Research Paper

Impact of *Mummy* Substance on the Proliferation and Migration of Human Adipose-derived Stem Cells and Fibroblasts in Separate or Co-culture Model

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Wound healing is critical to the regeneration of various organs and tissues. Traditional medicines, notably *mummy*, could serve as an alternative stimulator for the treatment of wounds. Till date, *mummy* has been used for the modulation of inflammation, articular injuries, and bone fractures, as well as wound healing. This study aims to evaluate the effect of *mummy* on the proliferation and migration of human adipose-derived stem cells and fibroblasts in single or co-culture *in vitro* conditions. The effective concentration of *mummy* substance was determined on fibroblasts and adipose-derived stem cells. The cells were treated with *mummy* separately or co-cultured over a period of 96 h. *In vitro* scratch assay was used to monitor cell migration rate. Effectiveness of *mummy* in inducing proliferation was evaluated by measuring the Ki-67 expression using flow cytometry analysis. Based on the data obtained, *mummy* was found to generate significant increase in the migration of fibroblasts as compared to untreated control in single culture system. On the other hand, an increased migration rate was recorded for adipose-derived stem cells, but it did not reach significant levels. Interestingly, the migration rate of co-cultured fibroblast and adipose-derived stem cells improved after exposure to *mummy* compared to matched controls. Incubation of adipose-derived stem cells, but not fibroblasts with *mummy* profoundly increased the expression of Ki-67 as compared to untreated cells. No significant effects were observed in the co-culture system. These data suggested that *mummy* altered the dynamics of stem cells and mature fibroblasts *in vitro*. Distinct cell responses could possibly be affected with regard to different cell types.

Key words: *Mummy* substance, wound healing, adipose-derived stem cell (ASCs), human fetal foreskin fibroblast (HFFF-2), co-culture, cell migration, cell proliferation

Skin, the external layer covering the body forms 8 % of the total body mass and is the largest body organ[1]. It consists of two main layers, epidermis and dermis. They are derived from different embryological origins and differ anatomically and functionally[2]. The overlying epidermis is mainly composed of ectoderm-derived keratinocytes that serve as a barrier against environmental insults[3]. The dermis provides support to the epidermis and is responsible for the strength and integration of the skin[4,5]. Any disruption in the normal anatomical structure, with a subsequent loss of functional integrity of the skin, is defined as a wound[6]. Fibroblasts play a key role in the production of dermis component and in wound healing[7].

Wound is a significant biomedical burden; millions of people around the world are affected by it. It is associated with morbidity and its annual cost is estimated to be $ 25 billion in the USA[8]. Cutaneous wound healing is to repair the injured skin, recover the tensile strength, integration, and immunological function of the skin[9]. Skin wound healing is an elaborate process that can be divided into four phases, haemostasis, inflammation, cell proliferation and remodelling[10,11]. During the third phase, fibroblast proliferation and migration into the extracellular matrix of the wound lead to the collagen deposition, thus steadily replacing the blood clots[9,12]. Finally, an intricate reorganization and maturation of

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Accepted 25 April 2018
Revised 07 October 2017
Received 10 January 2017
Indian J Pharm Sci 2018;80(3):516-524

newly formed collagen fibres occur in the remodelling phase[13]. In response to the cytokines secreted from neighbouring cells such as keratinocytes, fat cells, and migratory cells fibroblasts stimulate the production of extracellular matrix molecules and also growth factors, which in turn enhance keratinocyte proliferation in a paracrine manner[14].

Mesenchymal stem cells (MSCs) have been reported to play a role in wound healing[15]. Adult MSCs with their unique capacity for self-renewal and differentiation into other cells and tissues can orchestrate the main events occurring during the wound-healing process and emerge as a potential candidate for cell-based therapy in wound treatment[16,17]. Adipose-derived stem cells (ASCs) similar to bone marrow-derived multi-potent stem cells may be differentiated into adipogenic, chondrogenic, myogenic, and osteogenic lineages in response to specific stimuli[18]. ASCs can be obtained from liposuction aspirates with minimal patient discomfort and morbidity[19]. The application of ASCs to accelerate the wound-healing process and tissue regeneration has been investigated in different *in vitro* and *in vivo* experiments. Despite the considerable role of fibroblasts and stem cells in the promotion of wound healing, it is important to stimulate the ability of these cells using some enhancers[20-22].

For about 5000 y, nature has been considered as a potential source for the management of different diseases, such as wounds. In the past few years, the revival of interest in traditional medicine prompted investigations for a better understanding of the mechanisms underlying the mysterious effects of various traditional compounds; on the other hand, low-cost, minimal unwanted side effects, and better acceptance by patients make it important to further understand the role of these natural ingredients[23].

In ancient Persian and Egyptian medicine, *mummy* was used as a healer for inflammation, bone fracture, poisoning, and wounds[24]. *Mummy* is the substance used for mummifying famous Egyptian kings and is locally called *mummy* in most parts of Persia; it is a pitch-like substance found in some fractures of earth and also in rare caves. It is dark brown to black in colour and produced as a result of oil oxidation. It contains magnesium, sulphur, nitrogen, oxygen, and polysaccharide. *Mummy* is found in two types, fat-soluble and water-soluble. For topical application, it is dissolved in boiling water and massaged on to the affected site, such as a wound or an inflamed joint[24,25].

In recent years, various effects of *mummy* on fracture healing, gastric ulcer treatment, and animal model of wound healing have been investigated by some Persian researchers[25].

Considering the significant effects of *mummy* on the acceleration of wound healing, as claimed by local people and old Persian books such as Avicenna's Canon, as well as the role of fibroblasts and ASCs in the process of wound healing, the present study was designed to evaluate the effect of *mummy* on fibroblasts and ASC proliferation and migration in a two-dimensional culture condition.

MATERIALS AND METHODS

Adipose-derived mesenchymal stem cell isolation and expansion:

Human adipose tissues were obtained from patients undergoing laparotomy surgery. Exclusion criteria included any malignancy and the administration of hormones or chemotherapeutic agents. Written informed consent was obtained from all patients, and the proposal was approved by the medical ethics committee of Tabriz University of Medical Sciences. Adipose tissue samples were transferred to the cell culture lab and washed using phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 mg/ml streptomycin (1 % P/S; Gibco BRL). Samples were minced into small pieces using a sterile scalpel blade, weighed, and later digested with 0.2 % collagenase type I (Sigma, cat. no. C9891) per gram in shaking water bath for 60 min at 37°. Enzyme neutralizing was performed using fetal bovine serum (FBS, Gibco). After being passed through a 70 µm cell strainer (Fischer Scientific), cell suspension was centrifuged at 1500 rpm for five minutes. The cells were counted using 0.4 % Trypan-blue exclusion dye test on the haemocytometer slide and then seeded into T25 culture flask in Dulbecco's modified Eagle's medium (DMEM) low glucose (DMEM/LG, Gibco), supplemented with 10 % FBS and 1 % P/S at 37° till confluence. Medium exchanges were done every three days. Cells harvested at the third passage were used for the experiment. Previous studies from our laboratory provided evidence of the same characteristics as mesenchymal cells[26,27]. Human fetal foreskin fibroblast cell line (HFFF-2) was purchased from Pasteur Institute (Tehran, Iran). After thawing, cells were counted, plated at a density of 5×10^5 in the T75 culture flasks, and used for the experiment.

***Mummy* preparation:**

Fresh *mummy* was purchased from the local market in Kermanshah. Due to the absence of any *in vitro* study regarding the dosage of *mummy* in the first step, its effective concentration was determined using 3-[4,5-dimethylthiazoly-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay technique. Since *mummy* is water-soluble, it was solved in DMEM culture medium completely and filtered through 0.22 µm syringe filter for sterilization.

Cell viability assay by MTT:

The cell viability and proliferation were measured using MTT assay technique, in which the yellow tetrazolium MTT was reduced by mitochondrial enzyme namely succinate dehydrogenase to generate intracellular bluish purple formazan, which can be solubilized and quantified[28,29]. For this purpose, fibroblasts (HFFF-2 cell line)/ASCs (at third cell passage) were plated at a concentration of 1×10^4 cells/well in a 96-well plate for 24 h, and then cells in the control group were incubated in serum-free DMEM culture medium or treated with different concentrations of *mummy* (10-5000 µg/ml) for 24, 48, 72 and 96 h. In the next step, MTT reagent solution (5 mg/ml) was added to each well and the cultures were incubated at 37° for four hours while being protected from light. For solving the formazan crystals, dimethyl sulfoxide (Merck) was added to the wells; later, the absorbance was determined at 570 nm with a reference wavelength of 630 nm and measured using ELISA plate reader (Bio Tek).

Migration evaluation with *in vitro* scratch assay:

The effect of *mummy* on the migration of fibroblasts and ASCs was evaluated using *in vitro* scratch assay. Each well was plated with cells, including fibroblast (5×104/ml), ASCs (5×104/ml), co-culture of fibroblast and ASCs containing equal numbers of each (2.5×104), and their co-culture in a proportion of 30:70 (1.5×104 of ASCs and 3.5×104 of fibroblasts), and then incubated in DMEM supplemented with 10 % FBS for 24 h to allow cell adhesion and formation of a confluent monolayer. Afterwards, three linear scratches were performed in each well using a sterile pipette tip and the cellular debris was immediately removed by washing with PBS. In the next step, cells in control groups received fresh serum-free DMEM or were treated with DMEM containing 1000/2000 µg/ml of *mummy*. Photographs were taken with 4X magnification using an inverted microscope and digital camera (Olympus CK2, Japan) on days 0, 1, 2, 3, and 4. The acquired images were further analysed with the computing software Image J (ver. 1.49.) and the distances of each scratch closure were evaluated. All scratch assays were performed in triplicate, and the migration rate percent was calculated using the following formula[30], migration rate = average distance between scratch (day 0)–average distance between scratch (day 1 to 4)/average distance between scratch (day 0)×100.

Proliferation assay technique (Ki-67 detection):

Ki-67 protein as a marker for proliferation is expressed in all cell types during cell cycle. To understand the effect of *mummy* on cell proliferations, fibroblast cell line (HFFF-2) or ASCs alone or in co-culture condition in proportion of 50:50 or 30:70, as previously described, were plated. After adhesion to the culture flask, cells received culture medium as control or were treated with *mummy* at a concentration of 1000 µg/ml for 24 and 96 h. To preform Ki-67 proliferation assay, cells were trypsinised and neutralized with PBS containing 3 % FBS. Then, cell permeabilization was performed using 0.2 % Triton X-100 solution for 3 min and centrifuged at 800 rpm for 5 min. In the next step, cells were washed with PBS, supernatant was removed, and staining was performed using 5 µl Ki-67 antibody (RFF: 12-5699-41, San Diego, CA) in 100 µl of PBS for 30 min while protected from light and re-suspended using pipette tip. Later, 1000 µl of PBS was added and centrifuged at 800 rpm for 5 min, the obtained supernatant was removed, and the cells were suspended in 400 µl of PBS. In the final step, analysis was done using the flow cytometric method[31,32].

Stastical analysis:

Data are expressed as mean±SD. Statistical analysis was performed using two-way ANOVA and Tukey post hoc test and done with Graph Pad in Stat software version 2.02.

RESULTS AND DISCUSSION

Using the MTT assay, the effective concentration of *mummy* on fibroblasts and ASCs was determined. The *mummy* material was prepared at different concentrations (0.5, 10, 100, 500, 1000, 2000, and 7000 µg/ml) by dissolving in DMEM culture medium. Fig. 1A showed the viability of fibroblasts stimulated with different dosages. The results of ANOVA and Tukey post hoc test showed that *mummy* at a concentration of 1000 µg/ml generated the highest proliferation rate ($p<0.0001$). Stimulation of fibroblasts with higher dosages (2000 and 7000 µg/ml) resulted in

cell cytotoxicity and death ($p<0.0001$). Similarly, the most effective dosage of *mummy* on ASCs was found to be 1000 µg/ml and there was a sharp decrease in cell viability at 2000 and 7000 µg/ml (fig. 1B). This experiment was performed in triplicate.

To study the effect of *mummy* on fibroblasts and ASCs migration, *in vitro* scratch assay was used. As described earlier, fibroblasts and ASCs were seeded alone or co-cultured with 50:50 and 30:70 proportions. The length of scratch mark edges between days 0, 2 and 4 decreased. This reduction in length between the scratch was significant in the control group and treated groups ($p<0.0001$). The migration rate of fibroblasts in the group treated with 1000 µg/ml of *mummy* increased significantly ($p<0.0001$) through day 1-4 (Table 1). However, in 2000 µg/ml concentration of *mummy*, the migration rate of fibroblasts was increased on day 1 ($p<0.0001$) and decreased with passing time; by day 4, the cells were dead and detached from the bottom of the plate ($p<0.0001$; fig. 2A).

The effects of *mummy* on the migration rate and the distance between the edges of the scratch on ASCs are presented in Table 2. The length of scratch mark edges between days 0, 2 and 4 decreased. This reduction in length of the scratch was significant in the control group and treated groups at a concentration of 1000 µg/ml ($p<0.0001$), but not significant at 2000 µg/ml. The migration rate of ASCs in the group treated with 1000 µg/ml of *mummy* significantly increased on days 1 and 3. However, at 2000 µg/ml, the migration rate of ASCs decreased ($p<0.0001$; Table 2 and fig. 2B).

The migration rate of cells in 50:50 proportion of fibroblasts and ASCs at a concentration of 1000 µg/ml did not change in comparison to the control group (Table 3). However, in 2000 µg/ml concentration, the migration rate decreased significantly in comparison to the control group ($p<0.0001$; fig. 2C). Moreover, the length of scratch mark edges between days 0, 2 and 4 within all of the groups was not significant.

Table 4 showed the migration rate and distance between edges of scratch at 70-30 proportion of fibroblast and ASCs. At this proportion, the migration rate of cells significantly increased ($p<0.0001$) at 1000 µg/ml concentration. However, at 2000 µg/ml concentration, the migration rate decreased significantly on day 3 compared to the control group ($p<0.0001$), and on day 4, the cells died and detached from the bottom of plate (fig. 2D). The distance of scratch lines were significant

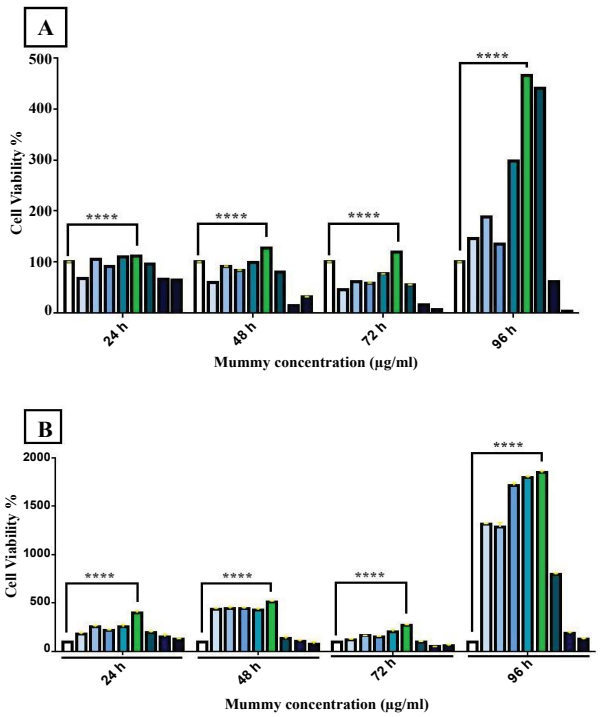

Fig. 1: MTT assay at *mummy* substance concentrations of 0.5-7000 µg/ml for 24, 48, 72 and 96 h
(A) Fibroblasts; (B) ASCs; □ control; ■ 0.5 µg/ml; ■ 10 µg/ml; ■ 100 µg/ml; ■ 500 µg/ml; ■ 1000 µg/ml; ■ 5000 µg/ml; ■ 7000 µg/ml; ****p<0.0001

in the control group and treated groups ($p<0.0001$). This experiment was performed in triplicate.

Ki-67 technique was used for the determination of cellular proliferation. The present investigation indicated that Ki-67 expression or proliferation decreased significantly in *mummy*-treated fibroblasts compared to the control group at 24 h but remained unchanged at 96 h (fig. 3A). Although the exposure of fibroblast to *mummy* for 96 h did not lead to a marked difference in Ki-67 expression compared to the control, the proliferation rate of the treated cells at 96 h significantly increased in comparison to that of the treated cells at 24 h, as is obvious in fig. 3A. In contrast, data obtained in this study revealed that the proliferation of ASCs, unlike that of fibroblast, increased significantly ($p<0.0001$) when treated with *mummy* substance both at 24 and 96 h (fig. 3B). Cellular proliferation of co-culture cells did not increase at any proportion at 24 and 96 h (fig. 3C and D). However, the proliferation rate of treated cells at 96 h was higher than that at 24 h. This experiment was performed in triplicate.

Skin wound healing is a harmonized process activated by injury, consisting of multicellular overlapping and the coordinated steps of inflammation,

TABLE 1: EFFECT OF THE *MUMMY* SUBSTANCE/ONLY DMEM ON *IN VITRO* SCRATCH ASSAY USING FIBROBLASTS (HFFF-2)

Treatment		Control	*Mummy*	*Mummy*
Dose (µg/ml)		DMEM alone	1000 µg/ml	2000 µg/ml
Distance between edges of scratch (µm)	Day 0	436.03±20.8	436.03±20.8	436.03±20.8
	Day 1	383.6±6.8	259.8±11.8****	278.5±10.8****
	Day 2	260.09±12.5	204.3±22.6****	235.8±9.20****
	Day 3	257±13.4	164.3±8.10****	233.5±11.6****
	Day 4	227.9±13.09	132.1±12.4****	X****
Test results for comparing days		****	****	****
% Migration rate of cells	Day 1	12±1.5	40.4±2.7****	36.1±2.4****
	Day 2	40.3±2.8	53.1±5.1****	44.7±1.2
	Day 3	40.8±3.08	62.3±1.8****	46.4±2.5
	Day 4	47.7±3	69.6±2.8****	X****

Values are presented as mean±SD; ****p<0.0001 vs. control group; X= scratch had disappeared

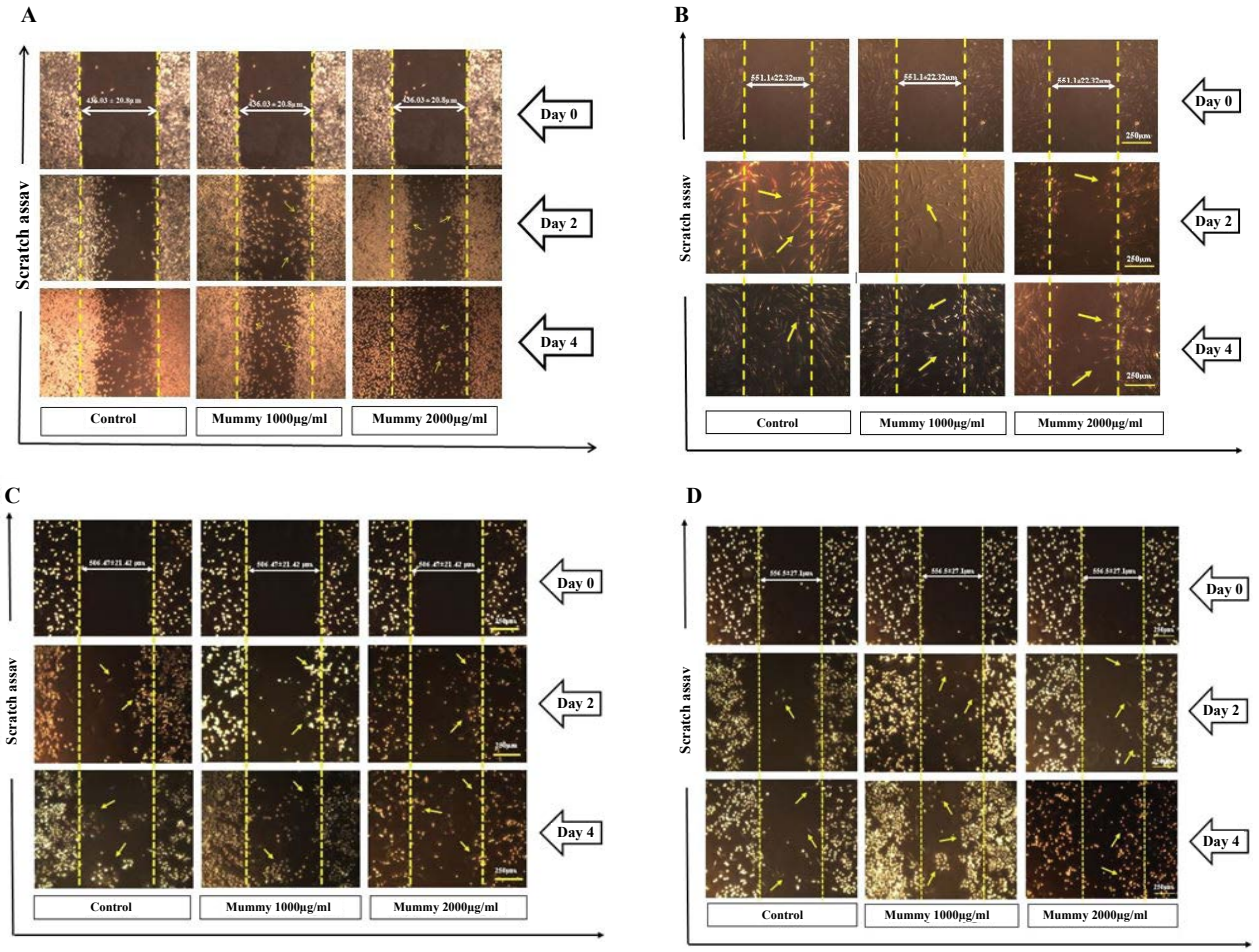

Fig. 2: Comparison of migration between control group and various cells after treatment with *mummy* substance at 24 and 96 h A-Fibroblast cells, B-ASCs, C- co-cultured fibroblasts and ASCs with 50/50 proportion, D- co-cultured fibroblast and ASCs with 70/30 proportion

angiogenesis, formation of granulation tissue, and re-epithelialization[33,34]. Wound healing is still a clinical challenge, especially in older patients, diabetic patients, heavy smokers, or burn patients, despite the current use of a wide array of wound dressings and ointments[35-39]. Novel strategies are required to enhance wound healing and repair.

Proliferation and migration are crucial factors in wound healing. Cell migration fibroblast migration in particular is one of the vital processes in wound healing[40]. Thus,

TABLE 2: EFFECT OF THE *MUMMY* SUBSTANCE/ONLY DMEM ON *IN VITRO* SCRATCH ASSAY USING ASCs

Treatment		Control	*Mummy*	*Mummy*
Dose µg/ml		**DMEM alone**	**1000 µg/ml**	**2000 µg/ml**
Distance between edges of scratch (µm)	Day 0	551.1±22.32	551.1±22.32	551.1±22.32
	Day 1	422.49±18.70	385.27±14.22	454.08±22.94
	Day 2	315.33±14.42	302.13±16.77	366.39±16.48
	Day 3	176.64±35.74	175.61±11.25	348.38±10.82****
	Day 4	54.52±6.84	46.65±21.46	323.91±25.25****
Test results for comparing days		****	****	
% Migration rate of cells	Day 1	23.33±3.3	30.09±2.75	17.60±4.16
	Day 2	42.66±2.51	45.17±3.03	32.19±0.59****
	Day 3	63.48±0.01	68.13±2.04	36.78±1.96****
	Day 4	88.18±2.62	91.53±1.24	35.16±2.90****

Values are presented as mean±SD; ****p<0.0001 vs. control group

TABLE 3: EFFECT OF THE *MUMMY* SUBSTANCE/ONLY DMEM ON *IN VITRO* SCRATCH ASSAY USING FIBROBLASTS+ASCs (50/50)

Treatment		Control	*Mummy*	*Mummy*
Dose (µg/ml)		**DMEM alone**	**1000 µg/ml**	**2000 µg/ml**
Distance between edges of scratch (µm)	Day 0	506.47±21.42	506.47±21.42	506.47±21.42
	Day 1	390.78±18.5	378.81±6.10	378.11±10.4
	Day 2	302.44±27.8	320.24±12.6	350.70±12.6****
	Day 3	280.54±15.8	283.32±20.0	324.7±9.77
	Day 4	266.94±12.9	237.92±13.3	417.37±16.7****
Test results for comparing days		**ns**	**ns**	**ns**
% Migration rate of cells	Day 1	22.8±3.66	25.20±1.20	25.34±2.06
	Day 2	38.24±4.51	36.77±2.50	30.75±2.49
	Day 3	44.60±3.13	44.05±3.95	35.88±1.93
	Day 4	47.29±2.54	53.02±2.64	17.59±3.30****

Values are presented as mean±SD; ****p<0.0001 vs. control group; 'ns' represent none significant

a study of the factors affecting fibroblast migration could improve wound healing[41]. Fibroblasts not only play a major role in epithelialization by providing extracellular matrix, but also contribute to wound contraction by transforming to myofibroblasts[42].

The potential impact of *mummy* on the migration and proliferation of dermal fibroblasts was studied. In the first step, the effective dosage of *mummy* on fibroblast survival was determined, showing that the 1000 µg/ml dosage is the optimal dose for fibroblast survival (fig. 1). Studying the effect of *mummy* on fibroblast proliferation and migration revealed that fibroblast proliferation does not increase under the effect of the 1000 µg/ml dosage of *mummy* (fig. 3A). However, with regard to the effect of *mummy* on fibroblast migration, the results showed that fibroblast migration was increased significantly (p<0.0001) in the presence of 1000 µg/ml of *mummy*; i.e. *mummy* might improve wound healing by promoting fibroblast migration to the wound site. The scratch assay involved the second phase of wound healing, characterized by the proliferation and migration of keratinocytes or fibroblasts[43-45]. It has been reported that *mummy* was effective in healing bone fracture[24]. In rats, it has been found that fibroblast migration was regulated by calendula extracts on Swiss 3T3 mouse fibroblast[45,46].

On the other hand, MSCs are more ideal for promoting wound healing[47]. The results regarding the effect of *mummy* on ASCs showed that ASC migration increased significantly in the presence of 1000 µg/ml of *mummy* (fig. 2B, Table 2). The current study demonstrated that *mummy* promoted the proliferation of ACSs at a dose of 1000 µg/ml (p<0.0001; fig. 3B). The MSCs of the skin populate the normal skin niche, remain quiescent, and become active after injury, helping in wound closure[48]. MSCs are applied to contribute to healing by releasing various cytokines and growth factors, by demonstrating therapeutic efficacy and the underlying

TABLE 4: EFFECT OF THE *MUMMY* SUBSTANCE/ONLY DMEM ON *IN VITRO* SCRATCH ASSAY USING FIBROBLAST+ASCs (30/70)

Treatment		Control	*Mummy*	*Mummy*
Dose (µg/ml)		DMEM alone	1000 µg/ml	2000 µg/ml
Distance between edges of scratch (µm)	Day 0	556.5±27.1	556.5±27.1	556.5±27.1
	Day 1	482.4±7.4	377.4±3.5****	480.2±13.9
	Day 2	399.8±9.5	281.7±10.8****	404.3±11.8
	Day 3	338.8±15.4	216.8±13.9****	400.4±7.4
	Day 4	311.1±13.7	158.6±11.04****	X****
Test results for comparing days		****	****	****
% Migration rate of cells	Day 1	13.2±1.3	32.1±0.6****	14.1±2.5
	Day 2	28.1±1.7	49.3±1.9****	27.3±2.1
	Day 3	39.09±2.7	61.02±2.5****	28.03±1.3****
	Day 4	44.09±2.4	71.4±1.9****	X ****

Values are presented as mean±SD; ****p<0.0001 vs. control group; X= scratch had disappeared

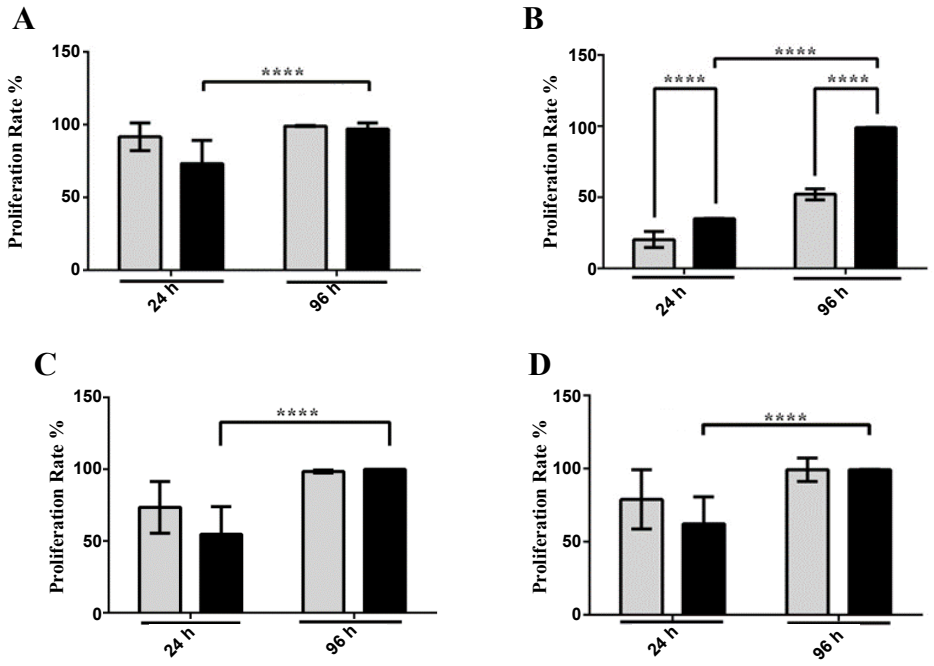

Fig. 3: Comparison of proliferation between control group and various cells after treatment with *mummy* substance at 24 and 96 h A- Fibroblast cells, B- ASCs, C-co-cultured fibroblasts and ASCs with 50/50 proportion, D-co-cultured fibroblasts and ASCs with 70/30 proportion. ■ Control ■ *mummy* 1000 µg/ml; ****p<0.0001

mechanism of MSCs on wound healing[49]. In fact, the antiinflammatory characteristics of MSCs increase their role in chronic wound treatment. In particular, vasculogenesis and angiogenesis crucial steps in wound healing are stimulated through paracrine factors released by MSCs[50]. At the same time, other studies showed that MSC-CM has little effect on fibroblast (L929) or keratinocyte HaCaT cell proliferation or survival[19,40,51].

The results regarding the effect of *mummy* on the co-culture of fibroblast and ASCs at proportions of 70:30 and 50:50 revealed that migration was enhanced under the effect of 1000 µg/ml *mummy* (Table 4, fig. 2). However, the effect of *mummy* on the co-culture of fibroblast and ASCs at both proportions (70:30 and 50:50) did not increase cellular proliferation. However, comparing the effect of *mummy* on proliferation at 24 and 96 h, the proliferation increase was significant ($p<0.0001$), while this was not the case in the untreated group (fig. 3). Thus, the stimulatory effect of *mummy* increased with passing time, confirming the healing effect of *mummy*[25]. In conclusion, *mummy* has a positive effect on the promotion of proliferation and migration of fibroblast and ASCs, suggesting that *mummy* might stimulate the proliferative and re-epithelialization phases of wound healing. The results

revealed that *mummy* accelerated wound healing, which was in accordance with public beliefs and ancient medicine.

Acknowledgements:

This work originated from a PhD thesis and has been financially supported by Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Conflicts of interest:

There are no conflicts of interest.

Financial support and sponsorship:

Nil.

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