Impact of Mummy Substance on Proliferation and Migration of Human Wharton’s Jelly-Derived Stem Cells and Fibroblasts in an In Vitro Culture System

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Abstract

Objectives: Because of the high prevalence of chronic wounds, wound repair has become one of the health challenges. Numerous therapeutic strategies have been proposed for repairing wounds and recently, the use of herbal medicines has been considered because of lower costs and complications. The use of mummy is recommended in traditional medicine for treating bone fractures, bleeding control, poisoning treatment, headache relief and wound repair. Therefore, the purpose of the present study was to provide a scientific assessment of 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 Wharton’s jelly (WJSCs) were isolated by means of explant culture. MTT assay was used to determine the effective concentration of mummy. Scratch assay method was used to examine the effect of mummy on cell migration rate and flow cytometry was used to assess the rate of cell proliferation using Ki-67 antibody. WJSCs and HFFF-2, each under mono-culture and two-cell co-culture condition with 50-50 and 30/70 ratio respectively, were in an experimental group including culture medium and mummy and in a control group including culture medium only.

Results: Scratch assay results for HFFF-2 cell migration showed a significant increase ($P \leq 0.0001$), but the mummy had no significant impact on WJSCs and a significant increase was observed in 50/50 and 30/70 co-culture conditions with $P \leq 0.001$ and $P \leq 0.0001$, respectively. The proliferation rate of WJSCs increased significantly while no significant increase was found in fibroblast groups in mono-culture and co-culture conditions.

Conclusions: Results showed that by stimulating fibroblast cell migration both in mono-culture and co-culture conditions with stem cells and by increasing WJSCs proliferation, the mummy can be used as a treatment to accelerate wound repair procedure.

Keywords: Cell migration and proliferation, HFFF-2, Mummy Substance, WJSCs, Wound healing

Introduction

Skin, as the covering of the body, serves as a barrier against foreign pathogens, controls body temperature, provides sensation and prevents body water loss (1,2). The skin is composed of an outermost layer called the epidermis which overlies the underlying dermis (3). The dermis is an irregular, dense connective tissue predominantly consisting of extracellular matrix (ECM) with different origins and functions (4).

Fibroblasts are the main dermal cells responsible for the synthesis and maintenance of ECM components (3). A wound is an impairment of the normal structure of the skin that disrupts its function (5). Cutaneous wounds can occur as a result of surgical laceration, burns, pressure and diabetic ulcers (6). The prevalence of wounds is a financial burden. It is estimated that about $20 billion is spent annually on treatment of wounds and related complications (6,7).

Wounds can be classified as acute or chronic. Acute wounds occur as a result of surgical incisions, abrasions or lacerations and generally heal within three weeks (8). Chronic wounds involve all skin layers and normally occur as a result of diabetic foot ulcers or neurodegenerative disorders and tend to heal minimally within three months (7).

Wound healing is a complicated and dynamic process and proceeds through 4 distinct and overlapping phases: hemostasis, inflammation, proliferation and migration...
of cells and remodeling (9-11). Immediate coagulation at the wound site diminishes blood loss during hemostasis. During inflammation, neutrophils and other migratory cells invade through the diapedesis and remove debris and residual components of the matrix. This stage is followed by the proliferation phase in which fibroblasts proliferate and migrate into the wound in response to growth factors such as TGF-β and PDGF (3). Migrated fibroblasts begin to synthesize matrix macromolecules such as hyaluronan, collagen type I, elastin and proteoglycans (12, 13). During the final step, remodeling, collagen deposition increases the tensile strength of the skin that parallels fibronectin removal (13).

Fibroblasts are the most common connective tissue cells with a pivotal function in wound healing by migrating to the wound site through ECM cell deposition and remodeling to build a stromal environment (14). Other cells with active roles in wound healing are mesenchymal stromal or stem cells (MSCs). These are characterized by a prolonged self-sustaining capacity and ability to differentiate into various tissue types by asymmetric replication (15). The ability of MSCs in promoting cutaneous wound healing has well been understood (16). These cells can speed up wound closure (17), increase vascular endothelial growth factor (VEGF), enhance vasculogenesis (18) and modulate the inflammatory response (8), leading to successful wound healing.

Among available sources, the umbilical cord is a feasible and cost-effective source with no ethical considerations because of the fact that it is usually discarded as waste after birth (19). MSCs can be isolated from different regions of the umbilical cord, including the lining, subamniotic layer, perivascular region (zone) and Wharton’s jelly (19). It has been found that Wharton’s jelly-derived MSC (WJ-MSC) supernatant increases re-epithelialization, neovascularization and skin fibroblast proliferation and enhances wound healing in in vivo excisional skin murine models (8).

Fibroblast and stem cell proliferation and migration are involved in wound healing; hence, finding any factor to stimulate these cells is important (20). For thousands of years, in many ancient civilizations, traditional medicine has been applied to cure disorders such as cutaneous wounds (21). The inadequate efficacy and serious side effects of modern forms of treatment have prompted investigators to examine traditional medicine. It offers lower cost, ease of access, safety and acceptance by indigenous people, particularly in developing countries (22).

In ancient Persia, herbs and natural compounds were used for their potential therapeutic effects on wound healing. The great Persian physician, Bu-Ali Sina (Avicenna), wrote about the effectiveness of mummy in healing bone fractures, controlling bleeding, treating poisoning, relieving headaches, and healing wounds (23). Mummy, called mummayeh by local people in Iranian provinces such as Kerman, Chaharmahal and Bakhtiari, Kohgiluye and Kermanshah, is a semi-solid brown to black material formed as a result of oil oxidation from fractures (24). Chemical analysis indicates the presence of calcium, phosphate, carbonate, oxygen, nitrogen and polysaccharide in mummy (23).

The healing effect of mummy on bone fractures, in a rabbit model of cutaneous wounds and gastric mucosal damage, has been reported by several investigators (23-25). However, the effect of mummy on stem cells has not been studied. The aim of the present study is to investigate the effect of mummy on the proliferation and migration of human Wharton’s jelly-derived stem cells and fibroblasts separately or in co-culture models.

Materials and Methods

Isolation of Mesenchymal Stem Cells From Wharton’s Jelly

Women undergoing caesarean sections at Alzahra hospital in Tabriz, Iran, with no history of complications throughout their pregnancy were recruited for this study. All participants provided signed informed written consent forms. Umbilical cords were conveyed to the cell culture lab while immersed in the phosphate-buffered saline (PBS) containing 100 U/mL of penicillin and 100 mg/mL of streptomycin (P/S, GIBCO). After being washed three times, the samples were placed in 70% alcohol for 30 seconds and then were cut into 5 cm pieces using a sharp sterile blade. The samples were incised lengthwise and the vessels were removed. The WJ was gently separated from the amniotic cover and was further cut into 2×2 mm² pieces and explanted into T-25 culture flasks with low glucose Dulbecco’s Modified Eagle Medium (DMEM/LG; GIBCO) supplemented with 20% fetal bovine serum (FBS; GIBCO) and 1% P/S. The culture medium was exchanged two times per week. After about 2 weeks, MSC crawled steadily from WJ explants. After reaching confluence, the cells were detached with 0.25% trypsin-EDTA solution (GIBCO) and subcultured in new flasks until cell passage. HFF-2 was purchased from Pasteur Institute (Tehran, Iran). After thawing, the cells were counted and seeded at a density of 5×10⁵ in T75 culture flasks and used for experimentation. Such cells have been characterized as mesenchymal stem cells in previous studies (26, 27).

Determination of Mummy Concentration

Mummy was purchased from a local market in Kermanshah. No investigation has been reported regarding in vitro evaluation and dosage of mummy thus far. The effective dosage was determined using MTT assay. Since it is water soluble, it was dissolved completely in DMEM and filtered through 0.22 μm nylon mesh for sterilization.

MTT Assay

To understand the cell viability rate, MTT assay was used to determine the ability of the mitochondrial
enzyme (succinate dehydrogenase) in viable cells to convert soluble MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma), into a bluish product called formazan, which can be solubilized and quantitated (28,29). Fibroblast cell line/WJSCs (at the third cell passage) was seeded at a density of 5 x 10^3 cells/well in 96-well plate suspended in DMEM and 10% FBS and incubated for 24 hours. Afterwards, the cells were incubated in serum-free DMEM culture medium as a control or with different concentrations of mummy (0.5–7000 μg/mL) for 24, 48, 72, and 96 hours. Next, the cells from all groups were cultured with 5 mg/mL MTT reagent solution for 4 hours at 37°C in an incubator. After supernatant removal, dimethylsulfoxide (DMSO, Merck) was added to each well to preserve the formazan crystals and the optimal density was determined at 570 nm with a reference wavelength of 630 nm using an ELISA plate reader (Bio-Tek, USA).

Migration Evaluation: In Vitro Scratch Assay
The migration of cells, including fibroblasts (HFFF-2) and WJSCs (alone or in combination), in the presence of mummy was investigated using an in vitro scratch assay. Fibroblasts and WJSCs were seeded into 6-well culture plates at a density of 5 x 10^4 cells/mL. For the co-culture condition, equal amounts of cells (2.5 x 10^4 of each) or 70/30 proportion (3.5 x 10^4 fibroblasts and 1.5 x 10^4 WJSCs) were plated and incubated in DMEM containing 10% FBS + 1% P/S for 24 hours to reach monolayer confluence.

Next, three linear scratches were made in each well using a sterile pipette tip to form a scratch of about 0.5 mm in width. The supernatant containing cellular debris was removed, the cells were washed with PBS and free DMEM was added to the wells as control groups.

To evaluate the effect of mummy on cell migration and closure of scratches, cells from other groups were treated with DMEM containing mummy at concentrations of 1000 and 2000 μg/mL. Microphotographs (Olympus; CK2; Japan) were taken on days 0, 1, 2, 3, and 4 at ×4 magnification.

The images were analyzed quantitatively using Image J (version 1.49) and the distance between the edges of each scratch was determined. The percentage of migration was calculated using the following formula (20).

All the experiments were performed in triplicate.

Migration rate =
\[
\frac{\text{Average distance between scratch (Day 0)} - \text{Average distance between scratch (Day 1 to 4)}}{\text{Average distance between scratch (Day 0)}} \times 100
\]

Ki67 Proliferation Assay
Ki-67 protein was detected as a cellular marker in the proliferation assay. This protein is expressed in all cell types and can be detected during active phases of the cell cycle. To evaluate whether mummy can affect proliferation of fibroblasts, WJSCs alone or in 50/50 or 70/30 combinations, were seeded and treated with 1000 μg/mL of mummy or culture medium as the control group. After 24 and 96 hours, the cells in the different groups were trypsinized and then neutralized using PBS containing 3% FBS and centrifuged at 1500 rpm for 5 minutes. The cells were then permeabilized for 3 minutes using 0.2% Triton X-100 and then centrifuged at 800 rpm for 5 minutes.

After washing with PBS, the supernatant was removed and the cells were stained with 5 μl of Ki-67 antibody (REF: 12-5699-41, San Diego, CA) and dissolved in 100 μL of PBS for 30 minutes in a dark room while being agitated. Subsequently, 1 ml of PBS was added and the mixture was centrifuged for 5 minutes at 800 rpm. The supernatant was removed, 400 μL of PBS was added, the cells were analyzed by FACS Caliber and the data were analyzed using FlowJo software (version 7.6.1) (30,31).

Statistical Analysis
The data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using two-way ANOVA and Tukey post hoc test (for more than 2 groups).

**Results**

Determination of Mummy Dosage
To understand the effective dosage of mummy for the survival rate of fibroblasts and WJSCs, different concentrations (0.5, 10, 100, 500, 1000, 2000, 5000, and 7000 μg/mL) were prepared by dissolving the material in DMEM. Figure 1A shows that the highest effective dosage of mummy in the fibroblasts was observed at 1000 μg/mL. At higher concentrations (2000-7000 μg/mL), there was evidence of cytotoxicity as a result of decreased absorbance compared to the control. Similarly, stimulation of WJSCs with 1000 μg/mL of mummy resulted in the highest proliferation rate. Additionally, mummy at dosages of 2000-7000 μg/mL inhibited cell proliferation (Figure 1B). The experiment was performed in triplicate.

Effect of Mummy Substance on Cell Migration for In Vitro Scratch Assay
An in vitro scratch assay was used to evaluate the influence of mummy on the migration of fibroblasts and WJSCs. For this purpose, fibroblasts and WJSCs were cultivated separately and in a co-culture system (50/50 and 70/30).

As the data in Table 1 and Figure 2A show the migration rate of fibroblasts in the group treated with 1000 μg/mL of mummy increased significantly (P < 0.0001) from days 1 through day 4. However, at 2000 μg/mL concentration of mummy, the migration rate of fibroblasts was increased on day 1 (P < 0.0001) and decreased with passing time and on day 4 the cells were dead and detached from the bottom of the plate (P < 0.0001).

Table 2 shows the effect of mummy on the migration rate of WJSCs. Table 2 and Figure 2B show that the migration rate of WJSCs in the group treated with
1000 µg/mL mummy significantly decreased on day 1 \((P<0.001)\) and on days 2, 3, and 4 \((P<0.001)\) compared with the control group. The 2000 µg/mL concentration also showed a decrease in the migration rate of WJSCs over the experimental period \((P<0.0001)\).

Table 3 and Figure 2C show that the migration rate of cells in 50/50 co-culture of fibroblasts and WJSCs at 1000 µg/mL was higher on day 3 in comparison with the control group \((P<0.001)\). At 2000 µg/mL concentration, the migration rate decreased significantly on day 3 in comparison with the control group \((P<0.01)\) and on the day 4, the cells were dead and detached \((P<0.0001)\).

Table 4 and Figure 2D show the migration rate of cells in 70/30 proportion of fibroblasts and WJSCs, respectively. As shown, at this proportion, the migration rate of cells had increased significantly on day 4 at 1000 µg/mL concentration \((P<0.0001)\). At 2000 µg/mL concentration, the migration rate had decreased significantly on day 2.
The experiment was performed in triplicate.

**Effects of Mummy on Cell Proliferation Using Ki-67 Methods**

The findings indicate that Ki-67 expression and proliferation decreased significantly (P < 0.001) after 24 hours in the mummy–treated fibroblasts compared to the control group, but remained unchanged after 96 hours (Figure 3A). Significant up-regulation was observed between the treated cells after 24 and 96 hours (P < 0.0001; Figure 3A). The data also revealed that proliferation of WJSCs, unlike fibroblasts, increased significantly (P < 0.0001) when treated with mummy, after 24 and 96 hours (Figure 3B). Furthermore, mummy did not affect the percentage of Ki-67 in 70/30 and 50/50 co-cultured cells at any time (P > 0.05; Figures 3C and 3D). The experiment was performed in triplicate.

**Discussion**

Cutaneous wound healing is a complicated process, comprising the multicellular overlapping and coordinated steps of inflammation, angiogenesis, and formation of granulation tissue, re-epithelialization, proliferation, migration, matrix formation, and remodeling (32, 33). Although different ointments, bandages and devices have been developed and are commonly used, wound healing remains a challenge, especially in heavy smokers, patients with burns, the elderly and diabetics (34-37). Other remedies are required to upgrade or assist wound healing (32,33). An increasing amount of attention is being

![Figure 2. Comparison of Migration Between the Control Group and Various Cells After Treatment With Mummy at 24 and 96 Hours. A- Fibroblast cells, B- WJSCs, C- co-cultured fibroblasts and WJSCs with 50/50 proportion, D- co-cultured fibroblast and WJSCs with 70/30 proportion.](image)

<table>
<thead>
<tr>
<th>Treatment Dose µg/mL</th>
<th>Control</th>
<th>Mummy 1000 µg/mL</th>
<th>Mummy 2000 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance between edges of scratch (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>519.6 ± 9.8</td>
<td>519.6 ± 9.8</td>
<td>519.6 ± 9.8</td>
</tr>
<tr>
<td>Day 1</td>
<td>451.8 ± 16.5</td>
<td>430.1 ± 17</td>
<td>461.7 ± 12.3</td>
</tr>
<tr>
<td>Day 2</td>
<td>443 ± 22.2</td>
<td>423.1 ± 11.8</td>
<td>460.9 ± 17.5</td>
</tr>
<tr>
<td>Day 3</td>
<td>435 ± 26</td>
<td>381 ± 18.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>477.4 ± 15.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 4</td>
<td>402.8 ± 12.1</td>
<td>371.4 ± 39.5</td>
<td>X&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

| % Migration rate of cells |
| Day 1 | 13.06 ± 3.1 | 17.2 ± 3.3 | 11.1 ± 2.3 |
| Day 2 | 14.7 ± 4.5 | 18.5 ± 2.3 | 11.3 ± 2.8 |
| Day 3 | 16.2 ± 5 | 26.6 ± 3.6<sup>b</sup> | 8.1 ± 2.9<sup>c</sup> |
| Day 4 | 22.4 ± 2.3 | 28.5 ± 7.6 | X<sup>a</sup> |

Values represent mean ± SD.

<sup>a</sup>P < 0.0001 vs. control group; <sup>b</sup>P < 0.001 vs. control group; <sup>c</sup>P < 0.01 vs. control group; X = Scratch had disappeared.
focused on traditional medicines (21). Proliferation and migration are the major factors affecting wound healing. Cell migration and fibroblast migration, in particular, are vital processes influencing wound healing (38). The study of factors affecting fibroblast migration can help improve the process (20). Fibroblasts play a major role in matrix production, which is necessary for epithelialization, and affects wound contraction by transforming to myofibroblasts (39,40). In the present study, the influence of mummy on the migration and proliferation of dermal fibroblasts was investigated.

Since there is a lack of knowledge about the correct dosage of mummy in in vitro studies, the effective dosage for fibroblast survival was first determined. The findings showed that a dosage of 1000 μg/mL is optimal for fibroblasts in culture (Figure 1). The effect of this dosage of mummy showed that fibroblast proliferation did not increase under the effect of 1000 μg/mL of mummy (Figure 3A); however, fibroblast migration increased significantly ($P<0.0001$; Figure 2A and Table 1). This highlights the fact that promotion of healing is partly the result of increased migration and acceleration of wound closure in normal human skin fibroblast models. To the best of our knowledge, the stimulatory effect of mummy on fibroblast migration has not been reported. Mummy has been shown to be effective in healing bone fractures (23), gastric ulcers induced in rats (24), and skin wound healing in mice (24). Fibroblast migration was shown to be regulated by calendula extract in Swiss 3T3 mouse fibroblasts (41,42). These findings indicate that mummy, in general, could have a stimulatory impact on wound healing.

It has been reported that MSCs are ideally suited to prompt wound healing (18). The current results on the effect of mummy on WJSC proliferation and migration showed that the migration rate did not increase under all treatment conditions (Table 2). Figure 3B shows the proliferation rate of fibroblast-WJSC co-culture after treatment with mummy at different concentrations. The results indicate that mummy has a stimulatory effect on fibroblast migration, which is consistent with previous studies (23,24). These findings suggest that mummy could be a promising candidate for wound healing applications.

Table 4. Effect of Mummy at 1000 and 2000 μg/mL and DMEM Alone on In Vitro Scratch Assay Using Fibroblast-WJSCs (70/30)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Mummy 1000 μg/mL</th>
<th>Mummy 2000 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose μg/mL</td>
<td>DMEM Alone</td>
<td>1000 μg/mL</td>
<td>2000 μg/mL</td>
</tr>
<tr>
<td>Day 0</td>
<td>460.6 ± 30.1</td>
<td>460.6 ± 30.1</td>
<td>460.6 ± 30.1</td>
</tr>
<tr>
<td>Day 1</td>
<td>436.1 ± 16.9</td>
<td>411 ± 24.5</td>
<td>455.1 ± 27.2</td>
</tr>
<tr>
<td>Day 2</td>
<td>409.1 ± 27</td>
<td>399.2 ± 3.09</td>
<td>446.1 ± 22.8a</td>
</tr>
<tr>
<td>Day 3</td>
<td>404.5 ± 20.6</td>
<td>385.4 ± 22.1</td>
<td>410.4 ± 16.5</td>
</tr>
<tr>
<td>Day 4</td>
<td>396.5 ± 27.7</td>
<td>313.5 ± 13.7a</td>
<td>417.5 ± 29.2</td>
</tr>
</tbody>
</table>

Values represent mean ± SD.

$aP < 0.0001$ vs. control group; $bP < 0.05$ vs. control group.
the effect of 1000 μg/mL of mummy (Table 2 and Figure 2B); however, the results revealed that WJSC proliferation increased significantly (P<0.0001; Figure 3B). It is the first time that the effect of mummy on stem cells and its stimulatory effect on WJSCs proliferation in culture were investigated. It has been reported that mesenchymal stem cells of the skin populate the normal skin niche and remain quiescent, becoming active after injury and contributing to wound closure (43). In support of the present findings, it has been shown that MSCs can be applied to improve healing by releasing cytokines and growth factors, demonstrating the therapeutic efficacy and major underlying mechanism of MSC transplantation (44). The anti-inflammatory features of MSCs enhance their role in chronic wound treatment, especially vasculogenesis and angiogenesis, which are crucial steps in wound healing stimulated by paracrine factors released by MSCs (45,46).

The present study on the effect of mummy on proliferation and migration of fibroblasts and WJSCs in 50/50 and 70/30 co-cultures show that migration of co-cultured cells of both proportions increased under the effect of 1000 μg/mL of mummy (Tables 3 and 4 and Figures 2C and 2D). However, the results were not significant for the effect of mummy on co-culture proliferation (P>0.05). In support of the findings, earlier studies have demonstrated that human WJ-MSC-CM stimulated fibroblast proliferation and migration to coapt wound borders in vitro (19). This confirms that the stimulatory effect of mummy on wound healing is not a direct effect on mesenchymal stem cells or fibroblast proliferation and migration, but is an indirect effect modulated through increased angiogenesis, vasculogenesis and other types of cell proliferation.

Conclusions
In other words, mummy is mainly involved in ECM deposition that facilitates wound healing. In summary, mummy may accelerate wound healing by exerting a stimulatory effect on the proliferation and migration of fibroblasts and stem cells.

Conflict of Interests
None to be declared.

Ethical Issues
All phase of the current study were completed according to the guidelines of Local Medical Ethics Committee of Tabriz University of Medical Sciences (No: 9874/4/5) and the Declaration of Helsinki (1964).

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