EFFECT OF MORINGA OLEIFERA ON HUMAN DENTAL PULP STEM CELLS: AN IN-VITRO STUDY

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ABSTRACT

Objective: This research to evaluate and compare the effect of moringa oleifera (MO) extract on the stemness properties of human dental pulp stem cells (DPSCs). Subjects and methods: (DPSCs) were isolated from the pulp of three permanent teeth indicated for extraction for therapeutic purposes. DPSCs were cultured with moringa oleifera extract at concentration 100μg/ml, and with Ca(OH)2 and MTA extracts as positive controls. Cells cultured without any extract were used as negative controls. Stemness properties of DPSCs were assessed in terms of proliferation, migration, and the ability to enhance wound healing. Moreover, the osteogenic differentiation potential of DPSCs cultured with MO extract was evaluated.

Results: No significant statistical between groups was detected regarding cell proliferation rate. The number of migrated DPSCs towards the MO was significantly lower than that in the -ve control group (p<0.000005). Also, there was a significant difference between the number of migrated DPSCs in the MO group and that in the +ve control groups (p<0.000005). The ability to enhance the healing of the wound was higher in the MTA group rather than in the MO group. Finally, successful osteogenic differentiation of DPSCs manifested as calcified nodules occurred in all groups.

Conclusions: Despite the limitations of this study, MO proved to have no adverse effect on DPSCs proliferation rate and can induce osteogenic differentiation of DPSCs. On the other hand, MO showed little effect on cell migration and wound healing in DPSCs.

KEYWORDS: Dental stem cells, Cytotoxicity, Osteogenic differentiation, Calcium hydroxide, MTA, Moringa oleifera.

INTRODUCTION

Healing of the dental pulp is still a huge clinical obstacle as human teeth have little ability to regenerate (1). Dental pulp has largely proliferative stem/progenitor cells possessing self-regeneration and differentiation properties that commonly remain inactive when they are in the dental pulp and rapidly react after injury (2). Stem cells are defined as undifferentiated cells that can synthesize new stem cells (auto-renewal). These cells can differentiate into numerous special cell lineages that have further particular properties which ensure healing and regeneration of tissue all over life (3).

Vital pulp therapy comprises procedures such as direct pulp capping, indirect pulp capping, and partial or full pulpotomy (4). Direct pulp capping is

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a technique built to preserve exposed dental pulp with a covering agent, promoting hard tissue healing. The application of a material, on a thin layer of dentin where no vital pulp exposure occurs, is called indirect pulp capping. Pulpotomy varies from pulp-capping in that a part of the pulp is amputated before the capping material is applied. The materials applied in the vital pulp technique should have adequate bioactivity and biocompatibility to induce DPSCs activity and pulp repair in primary and permanent teeth (5).

Studying the properties of various capping materials is very important in the context of cell viability of regenerating cells, transcriptional profile, and their capability to affect the differentiation process. Calcium hydroxide \([\text{Ca(OH)}_2]\) is widely applied for direct pulp dressing with sufficient biological responses. Unfortunately, \([\text{Ca(OH)}_2]\) has a weak cohesive strength, marginal leakage, and insufficient antibacterial effect (6). Mineral trioxide aggregate (MTA) is a well-known pulp capping agent due to its high biocompatibility, antimicrobial properties, and sealing performance. In vitro, MTA can promote the proliferation and migration of mesenchymal stem cells derived from human bone marrow (7).

Moringa oleifera (MO) has been confirmed for antioxidant, anti-inflammatory, anticancer, and antimicrobial properties determining its potential application in the treatment and prevention of dental caries, periodontal disease, ulcers, and mucosal wounds. It has the potential to reduce pro-inflammatory mediators (prostaglandin synthesis and nitrous oxide production RNS in macrophages) (8). The osteoprotective properties of the MO plant have been demonstrated. MO leaves are known to treat many chronic diseases, especially diabetic patients as insulin deficiency has direct effects on bone. In other words, MO being antidiabetic stimulates bone density and strength (9).

The pulp reaction is thought to vary with the use of various available materials, depending on their biocompatibility, which may cause serious damage to this tissue (10). Cell culture methods are beneficial in evaluating the biocompatibility of materials used in dental practice. In fact, in vitro assays with cell cultures are frequently used to explain the methods involved in various biological reactions and to assess cell behavior in certain circumstances (11).

Therefore, this work was performed to evaluate the action of MO on the viability, migration, wound healing, and osteogenic differentiation of human DPSCs.

**SUBJECTS AND METHODS**

The study included four groups:
- Untreated cells as the negative control group.
- Moringa Oleifera.
- Mineral trioxide aggregate as a positive control group.
- Calcium hydroxide as a positive control group.

1- Isolation and culture of DPSCs

This study was approved by the Research Ethics Committee (REC) of the Faculty of Dental Medicine for Boys, Al-Azhar University (Ref No.78/82/03-19). Human first premolar teeth -indicated for extraction for orthodontic therapeutic purpose-were obtained from healthy individuals presented to orthodontic department – Al-Azhar university. The donor’s ages ranged from 12 to 18 years. Teeth were obtained from patients according to ethical guidelines of the Faculty of Dental Medicine for Boys, Al-Azhar University. Signed informed consent was obtained from the parents/guardians (n= 3 teeth).

Extripated pulp was cut into small fragments then digested using 0.2% collagenase type II (Serva Electrophores, Germany) for 1 hour at 37°C. Isolated cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Lonza Yerviers SPRL, Belgium) supplemented with penicillin/streptomycin (Invitrogen Co., USA), and 15% fetal bovine serum (FBS) (Life Science, United Kingdom), and then incubated at 37°C and 5% CO2. When the
adherent cells became 70%-80% confluent they were passaged. The cells at the fifth passage were used in the subsequent experiments.

2- Preparation of materials condition media:

Three gm of Moringa Oleifera (Imtenan, Egypt) were dissolved in 3ml distilled water at room temperature or 48 hours. The solution was then filtered through a 0.22 μm filter (Thermo Fisher Scientific, Nalgene, USA). The solution was then diluted to 100μg/ml (12).

According to the manufacturer’s instructions, the powder of MTA (ANGELUS, Brazil)/ Ca(OH)₂ (Prevest dentpro, India) was blended with distilled water. The mixture was sterilized by its exposure to ultraviolet light for 24 hours inside a biological safety cabinet. 5 ml of DMEM without FBS was applied to the prepared mixture after 24 hours and incubated for another 24 hours at 37°C. a 0.22 m filter was used to filter the solution (7,13).

3- Cell proliferation assay:

Cell counting kit 8 (CCK-8) (Sigma, Germany) was used to assess cell proliferation.—DPSCs (1x10⁴/well) were cultured for 24 hours in DMEM supplemented with 15% FBS in a 96-well plate. The media were then replaced with conditioned media of MO, MTA, and Ca(OH)₂ for 24 and 48 hours. 10μl/well of CCK-8 was added at each time interval and incubated for 4 hours. the absorbance was measured using a microplate reader (BMG Lab-Tech, Germany) at 450 nm. The experiments were carried out in triplicate (14,15).

4- Cell migration assay:

A. Transwell migration assay: A two-chamber Transwell system (Greiner bio-one, Switzerland) was used to test cell migration (8mm pore size and 6.5 mm diameter). In the lower wells of a 24-well plate, the materials extract was added and supplemented with 15% FBS. Migration Chambers were put in place and 0.5 x 10⁵ cells were suspended in 100 ul serum-free DMEM and seeded on the migration chambers. The upper chambers were moved to the lower wells and incubated for 24 hours at 37°C. The cells were then fixed for 2 minutes in 4 % formaldehyde and stained for 15 minutes with Giemsa stain (Biodiagnostic, Egypt). Cells that failed to migrate were gently wiped off with a cotton swab from inside the chamber. The numbers of migrating cells in each well were counted in random fields after the wells were examined under an inverted phase-contrast light microscope (DMi1-Leica, Germany). The tests were carried out in triplicate independently (16-18).

B. Wound healing assay: In a 6 well plate, DPSCs were cultured for 24 hours until they reached 70-80% confluency. With a sterile 1-ml pipette tip, a scratch was made. The materials extracts were added and supplemented with 15% FBS. Photographs were taken under the microscope to measure the wound width using image J software. all cells were incubated for 24 hours. The cells were then stained with Giemsa dye, and photographs were taken with Image J software to examine cell migration and wound healing. The tests were carried out in triplicate independently (5,19,20).

5- Osteogenic differentiation assay:

As a positive monitor, an osteogenic differentiation medium (Gibco®, StemPro®, USA) supplemented with 15% FBS was used. Different material extracts supplemented with 15% FBS were also used to culture DPSCs. All cells were incubated for 21 days and the media was changed every 3 days. the cells were fixed in 4% formaldehyde solution after 21 days and then stained with 2% alizarin red (Loba chemic, India). The experiments were carried out three times (6,21,22).

6- Statistical analysis:

SPSS 23 (Statistical Package for Scientific Studies) for Windows was used to conduct the data analysis. The description of variables was presented in the form of mean, and standard deviation (SD). The Shapiro-Wilk test was used to search for
normality in the results. The one-way analysis of variance (ANOVA) test was used to compare quantitative variables between classes, followed by Tukey’s Post hoc test. Results were expressed in the form of P-values. The significance level was set at P ≤ 0.05.

RESULTS

1- Cell proliferation:

Statistically, there was no significant difference between the proliferation rates of DPSCs in the MO group after 24 hours and 48 hours when compared to those in -ve Control group and MTA and Ca(OH)₂ as +ve Control groups (p>0.05) (Table 1 and Figure 1a).

2- Cell migration:

a) Transwell migration assay:

Generally, the number of migrated cells towards MO was the least in all groups. Image J analysis showed that the number of migrated DPSCs towards the Moringa oleifera (7.44±2.83) was significantly lower than that in the -ve control group (55.66±5.95) (p<0.000005). Also, there was a significant difference between the number of migrated DPSCs in the MO group and that in the +ve control groups; MTA (213.44±17.38), and Ca OH (83.11±4.62) (p<0.000005). (Table 2 and Fig. 1b).

b) Wound healing assay:

The mean area of wound in the untreated control cultures on day zero was 125.77±8.82 and on day one was 96.11±4.98 indicating the decrease of wound area by 24% while the mean area on day two was 31.33±5.93 indicating the decrease of wound area by 75%.

The mean area of wound in the CaOH group on day zero was 124.22±5.91 and on day one was 91±6.24 indicating the decrease of wound area by 27% while the mean area on day two was 39.55±3.90 indicating the decrease of wound area by 68%.

The mean area of wound in the MTA group on day zero was 122.77±6.13 and on day one was 88.77±6.62 indicating the decrease of wound area by 28% while the mean area on day two was 38.33±6.18 indicating the decrease of wound area by 69%.

The mean area of wound in the MO group on day zero was 124.44±7.40 and on day one was 121.22±8.84 indicating the decrease of wound area by 3% while the mean area on day two was 100.11±5.51 indicating the decrease of wound area by 20%. (Figure 1c).

TABLE (1): The mean absorbance rate of all groups after 24h & 48h

<table>
<thead>
<tr>
<th>Time</th>
<th>Control Mean ±SD</th>
<th>MTA Mean ±SD</th>
<th>Ca(OH)₂ Mean±SD</th>
<th>MO Mean±SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>10.49±6.66</td>
<td>10.29±8.18</td>
<td>11.62±11.56</td>
<td>14.87±10.05</td>
<td>0.984</td>
</tr>
<tr>
<td>48h</td>
<td>14.54±3.3</td>
<td>17.21±10.8</td>
<td>13.1±8.87</td>
<td>17.6±13.7</td>
<td>0.122</td>
</tr>
</tbody>
</table>

SD: Standard deviation

TABLE (2): The mean numbers of migrated cells in all groups

<table>
<thead>
<tr>
<th></th>
<th>Control Mean</th>
<th>MTA 213.44</th>
<th>Ca(OH)₂ 83.11</th>
<th>MO 7.44</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>55.66</td>
<td>213.44</td>
<td>83.11</td>
<td>7.44</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>SD±</td>
<td>±5.95</td>
<td>±17.38</td>
<td>±4.62</td>
<td>±2.83</td>
<td></td>
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SD: Standard deviation, *statistically significant p-value < 0.05
3- Osteogenic differentiation assay

Successful osteogenic differentiation of DPSCs manifested as calcified nodules occurred in all groups. However, the number and size of the calcified nodules were higher and bigger in the osteogenic media and MTA groups followed by the CaOH group and finally the MO (Figure 2).

FIG (1): Bar graph revealing the mean absorbance rate of all groups after 24h & 48h (a), Bar chart showing the mean numbers of migrated cells within all groups (b), and Bar chart showing the percentage of wound healing on day one and two in all groups (c).

FIG (2): Showing osteogenic differentiation of DPSCs on day 30 (Alizarine red stain). Arrows show the calcified nodules (original magnification x10).

DISCUSSION

The bioactivity of dental materials can be determined *in vitro* or *in vivo*. For the pilot evaluation of biological response to the test material, *in vitro* cell cultures provide a convenient, simple, repeatable, controllable, cost-effective method and more detailed analysis. Also, a small amount of test material is required, and the range of application is vast. Although *in vivo* testing of dental materials, on the other hand, stimulates real body conditions and clinically relevant but may be influenced by the dentist’s abilities, uncontrollable patient variables, and the material’s technological properties. Besides, it is expensive, time-consuming, and has more
complicated ethical and regulatory issues. As a consequence, the in vitro assay findings could be predictive of the effects observed in vivo (23).

The current study evaluated the effect of MO on cell proliferation. The time taken for the cells to proliferate when MO was used, was also determined, with the analyses of the cultures being reviewed at 24 hours and 48 hours exposure. Our results show that there was no significant difference between the proliferation rates of DPSCs in the MO group after 24 hours and 48 hours when compared to those in the -ve control group and +ve control groups (MTA and Ca(OH)\textsubscript{2}). In other words, neither MO possess proliferative nor cytotoxic effects on DPSCs.

Other studies investigated the effect of MO on cell proliferation and found that, when using concentrations lower than 5 mg/mL, MO promotes the proliferation of rat MSCs, fibroblast, and angiogenesis (12). Also, another study showed that lower concentrations of MO powder increased the proliferation of human primary fibroblasts (24). Furthermore, our findings agree with a study that investigated the impact of MO leaf extract on the viability of human fibroblast cells and discovered that the leaf extract was correlated with enhanced proliferation (25).

On the contrary, MO had a marked cytotoxic effect on both human breast adenocarcinoma and breast epithelial cell lines (12). These observations afford yet another evidence for the safety of MO for human consumption and consideration of their use in cell stimulatory drugs.

Upon examining the migration potential properties of MO on DPSCs, it was found that there were significantly fewer migrated DPSCs towards the MO than towards the -ve control group. Moreover, MTA and Ca(OH)\textsubscript{2} showed a better migration induction effect than that with MO test groups.

A decrease in the wound areas occurred in all groups and became more with time, but MO showed the least effect on the healing of the wound. According to the observed in vitro screening of wound scratch assays, MO may enhance faster wound healing because of its ability to stimulate fibroblast proliferation as reported in earlier studies that fibroblast cell migration and proliferation are essential events for tissue healing and are directly related to its success (25, 26).

Using In vitro Cellular Assays on rat fibroblasts and MSCs, a study confirmed that MO had the ability to accelerate wound healing (12). The results would be better if used material extract rather than crude material as stated by Muhammad et al. who studied the effects of MO leaf extract on human dermal fibroblast cells and confirmed that moringa leaf extract was correlated with enhanced excellent wound healing (25).

Another research discovered that MO aqueous extract has a definite prohealing effect in both steroid-suppressed and natural wound healing. MO aqueous extract showed a substantial improvement in percentage closing of excision wounds by improved epithelization in an excision wound healing model. This improved epithelization may be attributed to MO extract’s effect on increased collagen synthesis (27).

DPSCs differentiation into odontoblasts and osteoblasts is critical for early odontogenic differentiation and late dentine mineralization. Bioactive materials used for pulp tissue healing should enhance the dentinogenic potential of pulp stem cells (7).

That is to say, MO could enhance osteogenic differentiation of DPSCs, but failed to have the same osteoinductive effect as MTA and Ca(OH)\textsubscript{2}. Although no previous study has evaluated the effect of MO on the human stem cells osteogenic differentiation, some studies evaluated the effect of MO on stem cells isolated from rats’ bone marrow and osteointegration of bone in animals.

Corresponding results were obtained in an in-vitro study on differentiation of BM-MSCs derived from rats on MO leaf extracts and when it was combined with alkaline phosphatase, bone-like nodule formation was observed after 21 days in culture (28). Similar results were presented in a study that revealed that MO lam. leaves extract could induce osteogenic differentiation in porcine bone marrow.
derived MSCs (29). Additionally, the effect of the methanolic extracts of MO on osteoblasts was tested and resulted in significant osteoblast stimulating properties (30). Moreover, supplementing ovariectomized animals with MO proved that it has osteoinductive and positive effects on bone health (31).

Our results disagree with other studies which showed that MO can improve the proliferation and migration of MSCs. This could be attributed to the fact that only one concentration and one type of MO were used, which was available at the time of the study. Moreover, other extraction techniques should have been considered.

CONCLUSIONS

Under the limitations of that research, it was concluded that:

Moringa oleifera was neither cytotoxic nor can induce the proliferation of stem cells isolated from human premolar permanent teeth. Also, it was found that it has the lowest effect on migration of DPSCs and wound healing. Finally, MO can induce osteogenic differentiation of DPSCs but not the same osteoinductive effect as MTA and Ca(OH)₂.

Future implementation of MO in dental pulp capping materials may help to improve direct pulp capping, pulpotomy procedures, decrease its failure rate, and maybe a useful therapeutic agent for pulpal repair.

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