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Research Article

The Effect of *Morinda citrifolia* in Combination with Chelating Agent EDTA on Isolated and Differentiated Human Dental Pulp Stem Cells Attachment to Root Canal Dentine Walls

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Abstract

The present study compared the effect of different irrigation solutions including *Morinda citrifolia* (MC) as a natural irrigant on the Human Dental Pulp Stem cells (HDPSc) attachment to root canal dentine walls using Scanning Electron Microscope. Ten human third molars were collected for isolation of dental pulp stem cell from eight healthy medically free young patients of both sexes aging from 17 - 25 years and twenty five intact human first lower premolars were collected for addition of dental pulp stem cells. After the fourth passage of DPSCs, human bone morphogenic protein type II (rh-BMP 2) was added to Confluent cultures. Teeth were classified randomly into five equal groups according to the type of irrigant used. The cells were added to the cleaned and shaped root canals of the five groups. Group I irrigation was done using 5.25% NaOCl, Group II irrigation and final rinse was done using 5.25% NaOCl and 17%EDTA was used as a chelating agent, Group III irrigation was done using MC, Group IV irrigation and final rinse was done using MC and 17% EDTA was used as a chelating agent, Group V (control) Saline was used as irrigation. After fourteen days of cell culture, the teeth were removed and processed to be examined under scanning electron microscope. All data obtained were given as mean and standard deviation (SD) values. Cell numbers data were compared using non-parametric tests. Mann-Whitney U test was employed for pair-wise comparisons when Kruskal-Wallis test is significant. The significance level was set at $P \leq 0.05$. Results revealed that group IV had the statistically significantly highest mean number of cells. This was followed by Group II. Group III showed statistically significantly lower mean number of cells. Group V revealed statistically significant lower mean value. Group I showed the statistically significantly lowest mean number of cells. The results suggested that *Morinda citrifolia* is a biocompatible irrigant that promotes DPSC attachment to root canal dentin, together with EDTA which is essential to accomplish regenerative endodontic treatment.

Keywords: *Morinda citrifolia*; Regenerative Endodontics; Dental Pulp Stem Cells; Recombinant Bone Morphogenic Proteins; Odontoblastic Differentiation; EDTA; NaOCl

Abbreviations

MC: *Morinda citrifolia*; HDPSc: Human Dental Pulp Stem Cells; EDTA: Ethylene Diamine Tetra Acetic Acid; rh-BMP: Recombinant Human Bone Morphogenic Protein; FBS: Fetal Bovine Serum; DMEM: Dulbecco's Modified Eagle's Media; PBS: Phosphate Buffered Solution; SEM: Scanning Electron Microscope; HPDL: Human Periodontal Ligament

Introduction

Endodontic treatment of an immature permanent tooth is often a complicated procedure and protracted with an uncertain prognosis frequently resulting in premature tooth loss [1].

The ability of postnatal stem cells to produce pulp-dentine and cementum - periodontal ligament complexes *in vivo* suggest potential applications involving stem cells, growth factors and scaffolds for apexification or apexogenesis.

Regenerative endodontic procedures aim to replace damaged, diseased, or missing structures, including dentin, root structures and cells of the pulp-dentin complex, with live viable tissues preferably of the same origin that restore the normal physiologic functions of the pulp-dentin complex [2-5].

Little attention has been given to the possible cytotoxic effects of irrigating solutions when used as part of regenerative endodontic therapy, such as the addition of DPSC to the root canal, their effects on DPSc attachment to the root canal.

Morinda citrifolia juice has a wide range of therapeutic effects, including antibacterial, antifungal, antiviral, anti-inflammatory and immune enhancing effects [6] and has been known for its biocompatibility and low cytotoxicity.

In order to achieve suitable environment to facilitate the attachment of the DPSC to the root, the presence or absence of a smear

layer is of questionable effect. The cytotoxicity of the irrigating solution on the DPSc has to be investigated as well.

Material and Methods

Part I

Collection of Teeth for the addition of dental pulp stem cells

Twenty five intact human first lower premolars extracted for orthodontic reasons were used in this study. Inspection of teeth was performed to ensure that they were free of caries, previous restorations, and preexisting fractures. Radiographic images of the teeth were captured before inclusion to ensure that all the teeth had a single root canal. Steam autoclave was used to sterilize the teeth before experimentation at a temperature of 250°C for 15 minutes. The removal of the crowns of teeth at the cementoenamel junction was performed using a diamond rotary stone. Random division of the teeth to five equal groups according to the type of irrigating solution was done (Table 1).

Cleaning and shaping

All the teeth were handled using sterile gloves and sterile forceps in order to prevent contamination. Exploring of the canals was done using #15 K-file to ensure canal patency. During the process of cleaning and shaping, 1 mL of irrigating solution, dispensed by plastic syringes with 25-gauge needles, was used after each instrument size. Cleaning and shaping were performed using ProTaper universal system reaching F4. A total of 5 - 6 mL of irrigation solution was used in all groups during the biomechanical preparation using small plastic needles.

Collection and isolation of stem cells from human impacted third molar and removal of pulp

Immediately after extraction the impacted third molars were kept in Dulbecco's modified eagle's media (DMEM) to which Penicillin/Streptomycin and 10% Fetal bovine serum (FBS) were added. Phosphate buffered solution (PBS) was used to clean the third molar teeth to remove any debris. A sterile high-speed drill was used to make a longitudinal groove of 0.5 - 1.0 mm depth (occuluso-apically) of each tooth. Splitting of the teeth was performed by a chisel along the groove in order to expose the dental pulps. The exposed pulp tissue of each tooth was gently extracted using a sterile dental probe and a tweezer. To ensure removal of any exogenous debris or cells, pulp tissue was washed with PBS. The extracted pulp tissues were then minced into approximately 2 x 2 x 1 mm fragments using a sterile scalpel and kept in a tube containing DMEM to which Penicillin/Streptomycin and 10% FBS were added.

All cell culture procedures were performed under aseptic conditions, in a biological safety cabinet class II and the surfaces were continuously sanitized with 70% ethyl alcohol.

The dissected pulp tissue was then transferred to a polypropylene tube, washed again with PBS and centrifuged at 500 rpm for two minutes. The supernatant was then discarded. In a T25 flask, previously prepared 0.2% collagenase solution was added to the pulp tissue. The tube was put in a 37°C water bath for 30 minutes with gentle shaking in the water bath shaker. The tube then was centrifuged at 500 rpm for two minutes. The supernatant containing the digested pulp cells was then moved to another polypropylene tube and then centrifuged at 1500 rpm for 5 minutes. 10% FBS and DMEM were added to the digested pulp cells. Dispersed cells and the solution were then passed through 40 mm cell strainer to pass target cells only. The tube containing the primary total pulp cells was centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. The red blood cells were then lysed using 1% IO lysing solution. The tube was placed in vortex for 10 seconds, left at room temperature for 10 minutes and centrifuged again at 1500 rpm for 5 minutes.

The culture of the total primary pulp cells was done separately in T25 vented flasks in DMEM supplemented with 10% FBS and then incubated at 37°C and 5% CO_2 in incubator. Cells from each dish, at the time of passaging, were treated with sterile 0.5% Trypsin/EDTA for 5 - 10 minutes with gentle tapping. Once the detachment of cells from the bottom of the culture dish took place, the solution was neutralized with supplemented media. The cell suspension was then centrifuged at 1500 rpm for 5 minutes, supernatant was discarded and fresh supplemented media was added. All these cells were finally resuspended in four culture dishes.

In-vitro differentiation of HDPSc into odontoblast-like cells

In optimal media, the fourth passage culture of HDPSc were grown to 80 - 90% confluence in non-coated T25 vented flasks. The confluent monolayers was incubated with DMEM supplemented with 10% FBS and 50 µg/ml of L-ascorbic acid 2-phosphate for 7 days in order to induce odontogenic differentiation. The media was changed twice a week. Human bone morphogenic protein type II (rh-BMP 2) of concentration 10 ng/ml was added on the seventh day, to DMEM supplemented with 10% FBS.

Histochemical Staining

On the seventh day after addition of BMP-2, the dishes were stained using Alizarin red stain, as a histochemical marker, to identify mineralization as follows; after discarding the culture media, the cells were washed using PBS and fixed in 4% formalin for 15 minutes followed by the addition of Alizarin red stain for 5 minutes. The cells were finally washed with distilled water so as to remove any excess stain and monitored using inverted phase contrast light microscope and digital micrographs were taken (Figure 1). Proliferation and propagation of cells continued until 90% of the dish area was covered with cells by day 10, indicating a highly proliferative cell population in pulp (Figure 2).

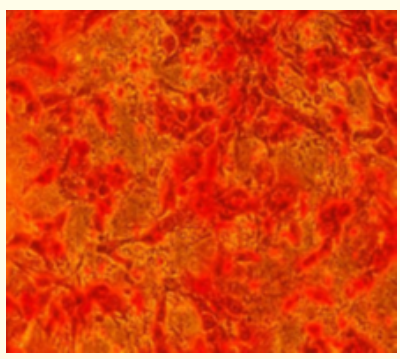

Figure 1: An inverted phase light micrograph showing Alizarin red staining. Experimental dish staining showing condensed calcification nodules original magnification X 40.

Part II

Addition of human dental pulp stem cells to prepared teeth.

Aseptic handling technique inside the laminar flow hood was adopted to deliver the cells into the root canals of the cleaned and shaped teeth of the five groups shown in (Table 1). The cells were transferred, using a microsyringe, to the root canals at a density of 1 X 10^6 cells per mL of DMEM culture media. In sterile 1.8-mL Eppendorf tubes, the teeth containing the cells were individually placed in an upright position (Figure 2) and maintained at 37°C in

a humidified atmosphere of 5% CO2 with the culture media being replenished every second day. After 14 days of cell culture, the teeth were removed and processed to be imaged under SEM.

Group	Irrigation solutions	Chelating agent	Final rinse
I	5.25 %NaOCl	None	None
II	5.25 %NaOCl	17% EDTA	5.25% NaOCl
III	*Morinda citrifolia*	None	*Morinda citrifolia*
IV	*Morinda citrifolia*	17% EDTA	*Morinda citrifolia*
V	Saline (control)	None	Saline

Table 1: Irrigation protocols used during biomechanical preparation of teeth.

Figure 2: The teeth containing the cells were individually placed upright in sterile 1.8-mL Eppendorf tubes.

Preparation of teeth for scanning electron microscope

Fixation of the teeth was carried out in 4% Glutaraldehyde on 0.2M Sodium cacodylate buffer (pH 7.3) for 4 hours, followed by post fixation in osmium tetraoxide OsO_4 for two hours. Teeth were rinsed three times by the same buffer solution and dehydrated through a graded ethanol series from 10 to 100%. Critical Point Dried instrument with liquid carbon dioxide was then used to dehydrate the teeth. The specimens were longitudinally cut and mounted on copper stubs with double sided adhesive tape and finally coated with gold using S150A sputter Coater.

Scanning Electron Microscope (SEM) Imaging

All specimens were examined and the micrograph images were stored as digital files on a Computer connected to the SEM. Each of the root canals was scanned individually in order to obtain an overview of the general surface topography. Micrographs of representative areas were captured, characteristic of the general surface topography of each specimen. A total of twenty five roots and images were analysed. The number of cells attached to the root canal walls per SEM micrograph field of view was assessed by two double blind independent reviewers using semi-quantitative criteria.

Statistical Analysis

Data were shown as mean and standard deviation (SD) values. Data of cells number were compared using non-parametric tests. Kruskal-Wallis test was employed to compare between the three irrigants as well as to compare between all groups. Mann-Whitney U test was employed for pair-wise comparisons when Kruskal-Wallis test is significant. Mann-Whitney U test was also used to compare between specimens without and with chelation. The significance level was set at $P \leq 0.05$. Statistical analysis was done with IBM® SPSS® Statistics Version 20 for Windows.

Results and Discussion

Isolation and Cell culture of human dental pulp stem cells

Cultured DPSCs showed scattered rounded and spindle shaped cells on day 1. No significant change was observed on day 2. On the third day, the cells showed increase in size with more spindle appearance and few scattered stellate cells (Figure 3a and 3b).

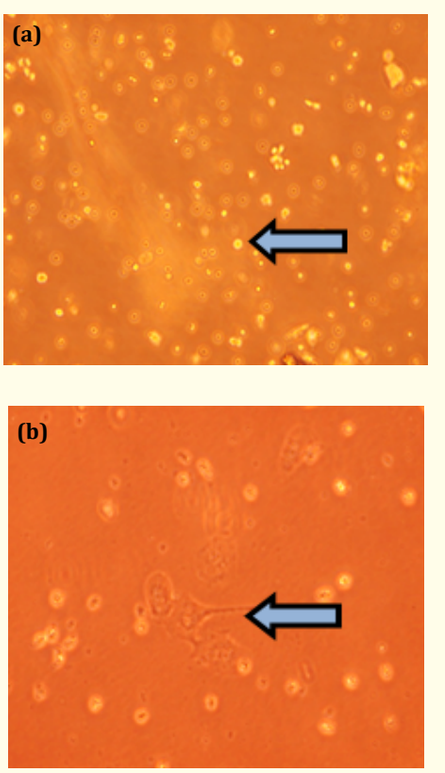

Figure 3: An inverted phase light micrograph showing cultured DPSCs (a) round and spindle shaped cells in culture days 1 and 2, (b) spindle shaped cells on day 3 and fibroblast-like cells showing growth and proliferation original magnification x 40.

Odontoblastic differentiation

After adding rh-BMP-2 and increasing the phosphate level in the experimental dishes, nodules-like structures scattered within

the cultured cells started to appear that were not seen in the control dishes (Figure 4).

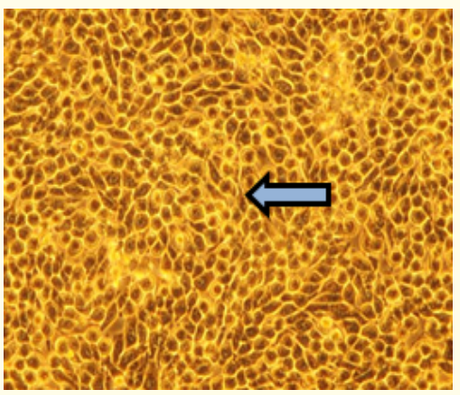

Figure 4: Odontoblastic differentiation of pulp stem cells on day 25 (original magnification x40): Experimental dish showing the calcification nodule.

Staining with Alizarin red

The experimental dish, were rh-BMP-2 was added to the culture exhibited a positive stain with Alizarin red with aggregates of intense staining revealing condensed calcification nodules (Figure 5).

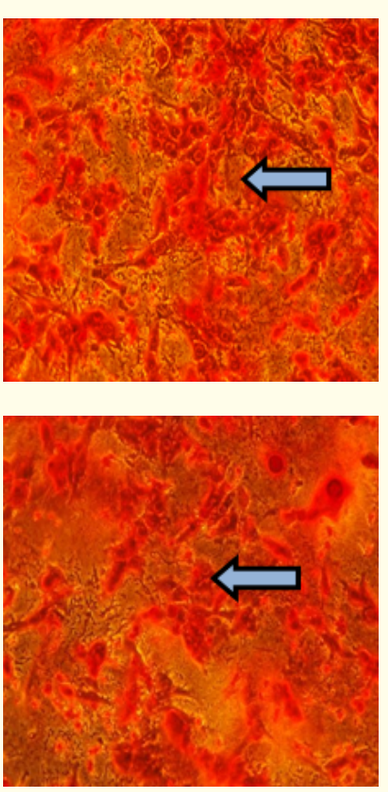

Figure 5: An inverted phase light micrograph showing Alizarin red staining. Experimental dish staining showing condensed calcification nodules original magnification X 40.

Scanning electron microscope results of attached cells

Effect of irrigation on attachment of DPSC to root canal dentine walls

Group I, SEM images revealed apparent smear layer on root dentine surface with rounded stem cells attached. Group II, SEM images revealed exposed dentinal tubules on root dentine surface with attached elongated stem cells with extensions appearing to resemble odontoblastic process extending in dentinal tubules. Group III, SEM images revealed smear layer on the root canal dentine with attached elongated stem cells with extensions appearing to resemble odontoblastic process extending in dentinal tubules. Group IV, *Morinda citrifolia* and 17% EDTA group SEM images showed exposed dentinal tubules on root dentine surface with attached elongated stem cells with extensions appearing to resemble odontoblastic process extending in dentinal tubules (Figure 6). Group V, SEM images revealed apparent smear layer on root dentine surface with rounded stem cells attached.

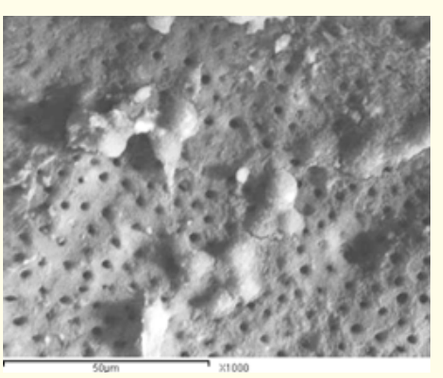

Figure 6: SEM image of group IV with magnification X1000 showing exposed dentinal tubules with elongated stem cells with extensions attached.

Overall comparison between the groups

Group IV showed the statistically significantly highest mean number of cells attached. This was followed by Group II. Group III revealed statistically significant lower mean number of cells attached. Saline showed statistically significantly lower mean value. Group I showed the statistically significant lowest mean number of cells attached (Table 2, Figure 7).

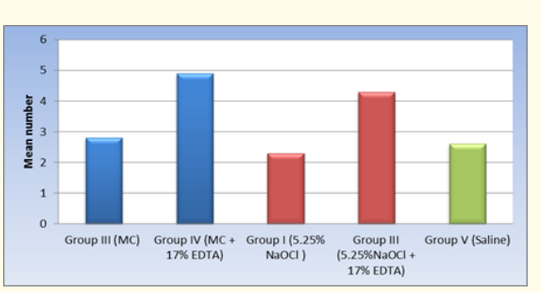

Figure 7: Bar chart representing comparison between all the groups.

Group	Mean	SD	P-value
Group I (5.25% NaOCl)	2.3[e]	0.2	0.001*
Group II (5.25% NaOCl + 17% EDTA)	4.3[b]	0.6	
Group III (MC)	2.8[c]	0.4	
Group IV (MC + 17% EDTA)	4.9[a]	0.2	
Group V (Saline)	2.6[d]	0.4	

Table 2: Descriptive statistics and test of significance for comparison between the number of cells attached in all groups.

*Significant at P ≤ 0.05

a, b, c, d and e: Different superscripts in the same column are statistically significantly different

In our study, stem cells were extracted from permanent wisdom teeth. Wisdom teeth were used to extract stem cells for several reasons. Firstly, they were easy to culture because of a lower chance of contamination with oral microflora so as to decrease probability of infection [7]. Secondly, wisdom teeth exhibit high proliferation and high frequency of colony formation that produce sporadic, but densely calcified nodules. Moreover, DPSCs are also capable of differentiating into other mesenchymal cell derivatives in vitro such as odontoblasts, adipocytes, chondrocytes and osteoblasts [8]. Human pulp cells can be induced *in vitro* to differentiate into cells of odontoblastic phenotype, characterized by polarized cell bodies and accumulation of mineralized nodules as described by Tsukamoto., *et al*. [9], About., *et al.* [10], Couble., *et al.* [11] and La Noce., *et al.* [12] and matches with our results.

In our study we used rh-BMP-2 to induce odontoblastic differentiation of DPSc. BMP-2 was found to induce a large amount of reparative dentin on amputated pulp *in vivo.* In addition BMP-2 regulates the differentiation of pulp cells into odontoblastic linage and stimulates reparative dentin formation [13].

Morinda citrifolia was investigated in this study as an example of a natural irrigant for its effect on the adhesion of DPSc to root canal dentin walls. MC is reported to have a broad range of therapeutic effects, including antibacterial, antifungal, antiviral, hypotensive, anti- inflammatory antitumor, antihelminthic and analgesic activity, Murray., *et al.* [6] suggested it as natural endodontic irrigating solution

The results indicated that the highest average numbers of DPSCs were attached to the root canals irrigated with MC and EDTA, MC + 17% EDTA showed statistically significant highest mean number of cells (4.9 ± 0.2). This was followed by NaOCl + 17% EDTA (4.3 ± 0.6). MC alone showed statistically significantly lower mean number of cells (2.8 ± 0.4). Saline showed statistically significantly lower mean value (2.6 ± 0.4). NaOCl alone showed the statistically significantly lowest mean number of cells attached (2.3 ± 0.2).

Although NaOCl is the most commonly used irrigating solution in endodontics which is attributed to a potent antimicrobial effect and effective dissolving of necrotic and organic tissue [14], it revealed the least attached count of stem cells. This may be due to its high pH (hydroxyl ion action), which interferes with cytoplasmic membrane integrity as Estrela., *et al.* [15] suggested. AlKahtani., *et al.* [16] stated that cell viability decreased significantly when the cells were exposed to NaOCl. This is also in agreement with the results of previous studies that reported on NaOCl toxicity [17,18]. NaOCl was reported to have a profound damaging effect on SCAP survival using an organotype human root model [19] which is concurrent with our results. Also, Trevino., *et al.* [20] concluded that NaOCl has a negative effect on odontoblastic differentiation of DPSCs *in vivo*.

On the other hand these are contradictory to Nosrat., *et al.* [21] who found NaOCl 5.25% passive irrigation for 20 minutes followed by triple antibiotic dressing can effectively disinfect necrotic immature root canals and leads to favorable clinical outcome, even in complex and immature root canal anatomy of the molar teeth, as root formation continued and revascularization process was considered a success. NaOCl with 17% EDTA showed statistically significantly higher mean number of cells (4.3 ± 0.6) than NaOCl alone (2.3 ±0.2). NaOCl group SEM images revealed apparent smear layer on root dentine surface with rounded stem cells attached. NaOCl + EDTA group SEM images showed exposed dentinal tubules on root dentine surface with attached elongated stem cells with extensions appearing to resemble odontoblastic process extending in dentinal tubules.

The cytotoxic effect of NaOCl appeared to be reversed with the application of NaOCl followed by 17% EDTA. That is in agreement with Martin., *et al*. [22] who concluded that dentin treatment with NaOCl resulted in reductions in dental stem cells but the addition of 17% EDTA reversed the effects of NaOCl and increased the survival of dental stem cells. MC SEM images revealed smear layer on the root canal dentine and attached elongated stem cells with extensions appearing to resemble odontoblastic process extending in dentinal tubules. MC + EDTA group SEM images showed exposed dentinal tubules on root dentine surface with attached elongated stem cells with extensions appearing to resemble Odontoblastic process extending in dentinal tubules. MC with 17% EDTA showed statistically significantly higher mean number of cells (4.9 ± 0.2) than MC alone (2.8 ± 0.4). That is in agreement with Ring., *et al.* [23] who stated that MC/EDTA is considered as one of the most optimal of the irrigating solutions to help maintain the survival and attachment of DPSCs to be used as part of regenerative endodontic treatment. Also Boonanantanasarn., *et al.* [24] found that MC promoted osteogenic differentiation as well as matrix mineralization in Human Periodontal Ligament cells and reported that noni leaf has therapeutic benefits in bone and periodontal tissue regeneration.

These results are similar to that of Pang., *et al.* [25] observing that EDTA induced cell attachment and odontoblastic/osteoblastic differentiation, which was observed exclusively in the group in which the DPSCs were placed in direct contact with the EDTA-treated dentin surfaces, concluding that EDTA is beneficial for achieving successful outcomes in regenerative endodontics. Also in agreement with Yamauchi., *et al.* [26] who reported that the exposure of the dentin matrix by EDTA appeared to enhance the adherence of the newly formed mineralized tissue to the root walls.

Conclusions

The use of *Morinda citrifolia* as a natural irrigant in combination with a chelating agent like EDTA favors adhesion and attachment of HDPSc to the walls of root canal. MC and EDTA might be recommended as an irrigation protocol of choice in future regenerative endodontic practice.

Conflict of Interest

The authors have no personal or financial conflicts of interests regarding this manuscript.

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