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Survival and Adherence of Apical Stem Cells to Root Canal Dentin after Conditioning with Apple Vinegar

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ABSTRACT

Purpose: To evaluate the survival and adherence of apical stem cells to root canal dentin after conditioning with apple vinegar. **Materials and Methods:** Stem cells from apical papilla of immature third molars were isolated. Survival of cells was evaluated by MTT test. Adherence of cells was evaluated by level of fibronectin expression and scanning electron microscope (SEM) observation. Thirty dentin discs from premolar teeth, extracted for orthodontic purposes, were immersed in 1.5% NaOCl then washed by PBS and finally divided into five groups regarding the final irrigating solution and time of its application. Group I: EDTA for 5 minutes, Group II: Apple vinegar for 5 minutes, Group III: Distilled water for 5 minutes, Group IV: EDTA for 3 minutes and Group V: Apple vinegar for 3 minutes. Cellular morphology was evaluated by SEM. For that part of the study, twelve root segments were longitudinally sectioned into two halves, and then immersed in 1.5% NaOCl, then rinsed with PBS. Finally, they were divided into three groups regarding the final irrigation. Group I: EDTA, Group II: Apple vinegar and Group III: Distilled water. Samples were rinsed and prepared for examination by SEM. **Results:** The biocompatibility of tested solutions was arranged in descending manner as following: Apple vinegar > EDTA > cells in media, distilled water and saline > NaOCl. Apple vinegar for 5 minutes released highest amount of fibronectin with statistically significant difference from other groups. **Conclusion:** apple vinegar promoted survival and attachment of SCAPs better than EDTA.

KEYWORDS

Apical Papilla Stem Cells, Apple Vinegar, Fibronectin

- Paper extracted from Doctor Thesis titled "Survival and Adherence of Apical Stem Cells to Root Canal Dentin after Conditioning with Apple Vinegar".

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INTRODUCTION

Management of immature necrotic teeth is challenging as root formation is ceased following pulpal necrosis. Traditional management of such cases by apexification puts a tooth in a state called "arrested root development" [1].

Regenerative endodontics, a revolutionary treatment strategy, became an alternative procedure in the last two decades. It is considered a promising treatment trying to prevent the loss of the affected tooth. It is based on creating a conducive microenvironment that support stem cells survival and differentiation and aiming to complete root formation and apical maturation [2,3].

For regenerative endodontic procedures to be successful, it should follow the principles of the tissue engineering including, various stem cells, growth factors, scaffolds and a suitable microenvironment that induce regeneration of dental pulp and continuation of root development [4].

Steps of Regenerative Endodontic Procedures (REPs) have been mentioned before in several studies[5-7]. Success of such procedures depends mainly on chemical disinfection using adequate irrigation followed by placement of intracanal medication for 2 to 4 weeks. Mechanical instrumentation should be avoided to prevent tooth fracture, as dentinal walls are fragile and compromised. Moreover, complete instrumentation can unfavorably remove vital tissue from the apical area. After thorough disinfection, intracanal bleeding is induced to allow influx of stem cells. In addition, blood clot, with its scaffolding character, can support the stem cells [2].

Selection of irrigating solutions and intracanal medicaments should be done carefully as these chemicals represent the key factor for successful regeneration. It should balance between being efficient against bacteria and at the same time maintain the viability of the stem cells providing suitable microenvironment that help to obtain best possible outcome. Additionally, an ideal irrigant should promote attachment, proliferation and differentiation of stem cells. Unfortunately, no single chemical solution is enough to provide an appropriate disinfection and help to liberate the essential growth factors, thus, use of two or more irrigating solutions is necessary [2].

Stem cells are characterized by self-renewal and multiple cell lineages differentiation. According to tissue of origin, many populations of dental stem cells have been isolated [8,9]. Stem cells from the apical papilla (SCAPs) that reside in the apical papilla of the immature permanent teeth were first discovered in 2006.They are known by their high proliferative rate and reduced immunogenicity. They are also characterized by their ability to survive in spite the presence of infection. It has been found that these cells may be responsible or at least partially play a role in REPs. Since SCAPs can give rise to various lineages of cells, it can be considered a good source used in stem cell therapy [10].

For stem cell to lay down mineralized tissue like, it passes through multiple stages, migration, attachment, proliferation, and differentiation. Viability is affected by irrigants, medicaments[2] and material of the coronal barrier [10] . Attachment is affected by type of irrigant, chemical and physical properties of the surface [11].

Stem cells attachment to the surfaces can be evaluated by different techniques. Fibronectin is a glycoprotein with high molecular weight. It is associated with variable cell functions. It is considered one of the most common proteins responsible for cell adhesion [12].

Many studies evaluated the effect of irrigating solution on the viability and attachment of stem cells [2,4,11]. Sodium Hypochlorite (NaOCL) is used as main irrigant due to its antimicrobial ability and unique organic tissue dissolving action. However, it has been found that it decreases the viability of the stem cells in concentration and time dependent manner [13]. NaOCl to be effective, its application time should be about 5 minutes [4].

Final irrigation by 17% EDTA is recommended to release the growth factors from dentin that were sequestered previously during dentinogenesis. It has been found that 17%EDTA irrigation promoted survival, migration, attachment of stem cells to dentin and its differentiation into odontoblast-like cells. Moreover, 17% EDTA reverses the detrimental effect of NaOCl on SCAPs [2]. It was reported that 17% EDTA can be used as disinfectant [14]. It has been found that 17% EDTA affects the physicochemical properties of dentin, like changes in chemical composition, topography and wettability which in turn affects the attachment of stem cells to dentin [13].

Searching for more biocompatible solution than 17% EDTA, as EDTA cytotoxicity was reported, natural substituents have been tried to be used both in the conventional endodontics as well in REPs [15]. Examples for such natural alternates are, Morinda citrofila juice (MCJ) [16], chitosan [17] and apple vinegar. The use of apple vinegar as a final irrigant has been proposed and proved better results than 17%EDTA regarding the removal of smear layer especially in the apical third [18,19]. Apple vinegar use deserves special attention due to its antimicrobial effect [20], its biocompatibility and its cost effectiveness. It is formed of acetic, citric, formic, lactic, succinate and tartaric acids with small amounts of alcohol that results from the fermentation process. Alcohol is responsible for reducing the surface tension of the solution. However, the highest acid concentrations of the vinegar are achieved by the acetic (5%) and malic (0.35%) acids. Being one of the acids in the Krebs cycle, Malic acid boost the immune system [21].

Although apple vinegar showed promising results when used as an irrigant in endodontic procedures, the literature does not have data regarding its use in REPs. Therefore, this study aimed to evaluate the effect of apple vinegar on the survival and adherence of stem cells from apical papilla (SCAPs) to dentin. The null hypothesis was that both 17% EDTA and apple vinegar have same effect on the survival and attachment of SCAPs.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee (REC), Faculty of Dental Medicine for Girls, Al-Azhar University (code is REC-EN-21-02).

Collection of stem cells of apical papilla (SCAP) and cell culture [12]:

After obtaining a written informed consent, three human impacted third molars were extracted from one donor, aged 18 years old. The patient was free from any systemic or oral diseases. Teeth were extracted for orthodontic reasons at Oral and Maxillofacial Surgery Department, Al-Zahraa University Hospital. Extraction was done under general anesthesia. Teeth were put in a tube containing a-MEM supplemented with 100 U/mL penicillin/streptomycin and transferred on ice to the Molecular Biology laboratory at the Faculty of Medicine, Cairo University.

The apical papilla was separated from the tooth root using forceps and a size 11 surgical blade in a 10-cm tissue culture dish containing 10 mL wash buffer. Wash buffer containing the apical papilla tissue was transferred into a 14-mL round bottom tube and centrifuged at 400 × g for 10 min at 4°C. Apical papilla was resuspended and digested in a solution of 1 mL Type I collagenase (3 mg/mL final) and 1 mL dispase II (4 mg/mL final) for 1 h at 37 °C. An excess volume of wash buffer was added to the digested tissue to neutralize enzyme activity and then strained through a 70-μm cell strainer to remove undigested tissue from the liberated apical papilla cells. Apical papilla cells were pelleted by centrifugation at 400×g for 10min at 4 °C, resuspended in 2 mL wash buffer, and kept on ice. Nucleated cells were counted using a hemocytometer. Typically, the apical papilla cell count was ranged from 0.5 to 2.5 × 10^4 per one to three teeth processed. Isolated apical papilla-MSCs were grown using RPMI-160 culture media with 10% Foetal Bovine Serum (FBS), 2mM glutamine

and antibiotics (Penicillin with Streptomycin 1%) were added. Culture media and supplements were sterile. Aseptic conditions in a culture hood were followed to ensure sterility.

Cells were cultured in 75cm^2 flasks at 37°C/ CO_2 incubator. Cells were checked microscopically (Leica, USA) daily to ensure they were healthy and growing as expected. Cells were mainly attached to the bottom of the flask and elongated in shape. Culture medium was clear pinky orange in color that indicated absence of contamination. When the cells were approximately 80% confluent, sub-culturing was performed. SCAPs were morphologically characterized by inverted light microscope and their stemness was assured by flow cytometry. In this study cells at passage 3 were used.

Preparation of dentin discs:

As regarding the primary outcome, attachment of stem cells by fibronectin gene expression, and according to sample size calculation using the G power software, 6 teeth samples per group were appropriate for the study and the total sample size was 30 teeth (5 groups) with 80% power and α error probability = 0.05.

Premolar teeth were used, that were obtained from anonymous young (14-30 years old) healthy patients who have extracted their premolar teeth due to orthodontic purposes. Dentin from the middle third was transversely sectioned into a 1-mm thickness disc, under sterile phosphate buffer saline (PBS) irrigation. The dentin discs were sterilized using autoclave [12]. Thirty discs have been placed in two 24-well plates and then immersed in 1.5% NaOCl for 5 minutes in order to remove the organic debris.

Samples' grouping:

Dentin discs were divided into 5 groups, 4 experimental groups regarding to the final irrigating solution used and the time interval of immersion, and 1 control group as following; Group I, 1 ml of 17%EDTA for 5 minutes, Group II, 1 ml of apple vinegar for 5 minutes, Group III, 1 ml of distilled water for 5 minutes (control), Group IV: 1 ml of 17% EDTA for 3 minutes and Group V: 1 ml of apple vinegar for 3 minutes (Fig.1).

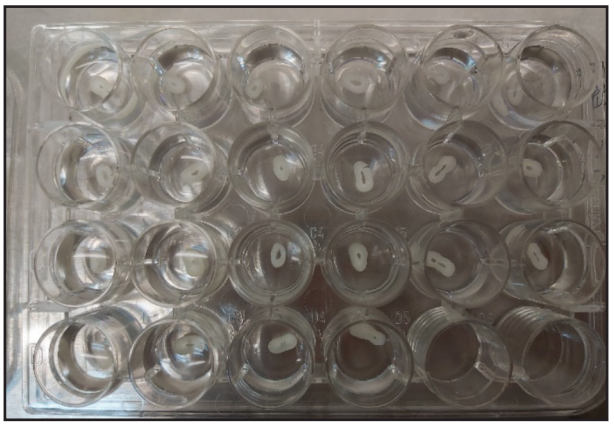

Figure (1): Dentin discs immersed in irrigating solutions in a well plate

SCAP seeding:

Dentin discs were rinsed with PBS to remove the conditioning agent residues [22]. Harvested SCAPs (1×10^6 cells /well) were seeded on the dentin discs and incubated for 4 hours to allow cell attachment. The medium was added to get final volume at 200 ml in each well, then the cells were cultured for 4 days.

Cell vitality and cell proliferation:

Evaluation of cell vitality was done using MTT assay [23]. MTT reagent supplied ready for use was obtained from (TACSTM TREVIGEN® 8405 Hegerman Ct. Gaithersburg). MTT assay is used for assessing cell metabolic activity through colorimetric analysis. Oxidoreductase enzymes are used under defined conditions to assess the number of viable cells present. These enzymes can reduce the tetrazolium dye. The number of living cells in culture is directly proportional to the intensity of the colour.

Cells (1×10^3) were placed in 96 well microplate 24hour before MTT assay to allow cell adherence to microplate, then 50 μL of serum-free media was added to cells followed by 10 μL of MTT solution into each well. The plate was incubated for 3 hours at 37°C. After incubation, 100 μL of detergent solution (MTT formazan) was added into each well. The plate was wrapped in foil, shacked on an orbital shaker for 15 minutes and incubated overnight at 37°C. Colour absorbance was read at OD=450 nm using an Enzyme-Linked Immuno-Sorbent Assay (ELISA) plate reader (Stat Fax 2200, Awareness Technologies, Florida, USA). Duplicate readings were taken and averaged for each sample.

Cell attachment:

Evaluation of cell attachment depends on the amount of fibronectin 1 (FN1) expression and SEM observation.

a. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) analysis:

The amount of fibronectin 1 (FN1) expression was assessed in 30 samples using quantitative real-time polymerase chain reaction (qRT-PCR) analysis which was performed by ABI 7500 software (Applied Biosystems, Foster City, CA).

b. SEM observation:

Samples for SEM observation were prepared by using 5 mm from the middle third of anonymous premolar teeth roots which were longitudinally cut into two halves. Samples were put in a 24-well plate and immersed in 1 ml of 1.5% NaOCl for 5 minutes then washed with PBS and finally divided into 3 groups regarding the final irrigating solution, 2 experimental and 1 control group as following; Group I: 17% EDTA for 5 minutes, Group II: Apple vinegar for 5 minutes and Group III: Distilled water for 5 minutes.

Samples were fixed in 4% paraformaldehyde for 30 minutes and dehydrated by using different concentrations of ethanol (30%, 50%,70%, 80%, 90%, and 100% for 10 minutes each). After that, all samples were coated with gold. Cellular observation was done under the SEM on basis of cellular morphology and density [12].

Statistical analysis:

One-way Analysis of Variance (ANOVA) test was used to compare between Fibronectin gene expression data in the different groups. Repeated measures Analysis of Variance (ANOVA) test was used to compare between MTT assay values in the different groups. Bonferroni's post-hoc test was used when ANOVA test is significant.

RESULTS

Flow cytometric analysis:

Results of analysis of flow cytometry confirmed the expression of stem cell surface markers, CD73 and CD29[24].

MTT assay:

Comparison between groups

After 6 ,24 and 48 hours, there was a statistically significant difference between mean MTT assay values of the six groups (P-value <0.001, Effect size = 0.973). Pair-wise comparisons between the groups revealed that Apple vinegar group showed the statistically significantly highest mean value. 17% EDTA group showed statistically significantly higher mean value than 1.5% NaOCl. There was no statistically significant difference between Sterile saline, Cells in media and Distilled water groups; all showed statistically significantly lower mean values. 1.5% NaOCl showed the statistically significantly lowest mean value (Table 1, Fig. 2).

Table (1): *Descriptive analysis of mean, standard deviation (SD) values and results of repeated measures comparison between MTT assay in different groups*

Group	6 hours		24 hours		48 hours	
	Mean	SD	Mean	SD	Mean	SD
1.5% NaOCl	0.39 ^{D}	0.08	0.19 ^{D}	0.06	0.75 ^{D}	**0.16**
17% EDTA	2.53 ^{B}	0.38	3.04 ^{B}	0.54	2.48 ^{B}	**0.43**
Apple vinegar	4.14 ^{A}	0.27	4.80 ^{A}	0.27	3.31 ^{A}	**0.52**
Sterile saline	1.57 ^{C}	0.19	1.75 ^{C}	0.21	1.21 ^{C}	**0.14**
Cells in media	1.40 ^{C}	0.10	1.87 ^{C}	0.09	1.22 ^{C}	**0.08**
Distilled water	1.46 ^{C}	0.10	1.79 ^{C}	0.13	1.19 ^{C}	**0.07**
P-value	<0.001*		<0.001*		**<0.001***	
Effect size (Partial Eta squared)	**0.973**		**0.970**		**0.919**	

**: Significant at P ≤ 0.05, Different superscripts in the same column indicate statistically significant difference between groups*

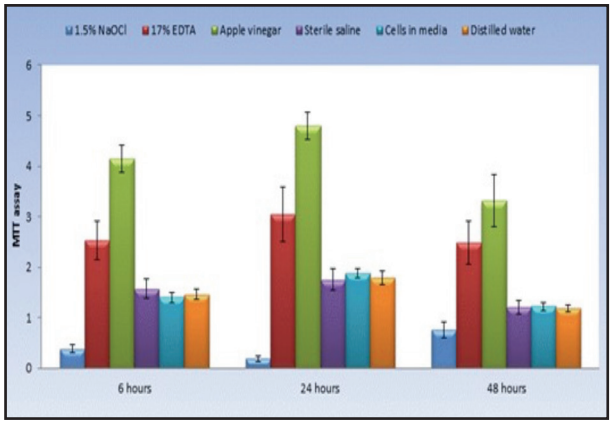

Figure (2) Bar chart comparing mean and standard deviation values for MTT assay results in the six groups

Attachment evaluation

I. Fibronectin gene expression (qRT-PCR)

Fibronectin gene expression values of the five groups showed a statistically significant difference. (P-value <0.001, Effect size = 0.888). Pair-wise comparisons between the groups revealed that Apple vinegar 5 minutes group showed the statistically significantly highest mean fold change. There was no statistically significant difference between Distilled water 5 minutes and Apple vinegar 3 minutes groups; both showed statistically significantly lower mean fold change values. There was no statistically significant difference between 17% EDTA 3 minutes and 17% EDTA 5 minutes groups; both showed the statistically significantly lowest mean fold change values (Table 2, Fig.3).

Table (2): *Descriptive analysis of mean and standard deviation (SD) values for comparison between Fibronectin gene expressions in different groups*

Group	Mean	SD	P-value	Effect size (Eta squared)
17% EDTA 5 minutes	1.92 ^{C}	0.27		
Apple vinegar 5 minutes	3.78 ^{A}	0.26		
Distilled water 5 minutes	2.57 ^{B}	0.19	<0.001*	**0.888**
17% EDTA 3 minutes	2.11 ^{C}	0.24		
Apple vinegar 3 minutes	**2.76** ^{B}	**0.29**		

**: Significant at P ≤ 0.05, Different superscripts are statistically significantly different*

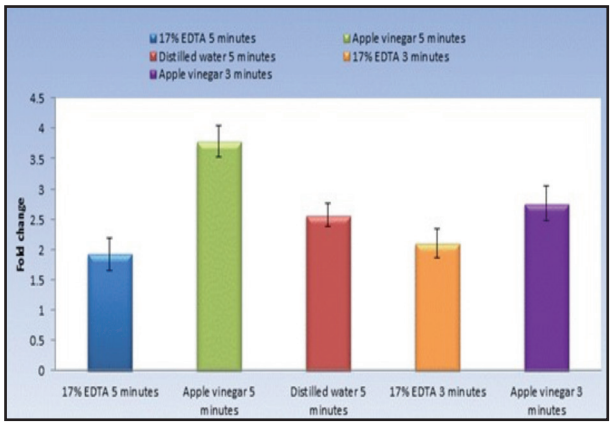

Figure (3) Bar chart showing mean and standard deviation values for Fibronectin gene expression in the five groups

II. SEM analysis:

SEM images showed the morphological differences of cells seeded on dentin discs that have been treated finally with different irrigating solutions; Group I; demonstrated clean dentin surface after smear layer removal, cells appeared flat shaped and stretched to dentin surface with low cell density. In Group II, cells also appeared flat shaped and stretched but with high cell density. In Group III, dentin surface demonstrated presence of smear layer patches. Cells were round to oblong with low density (Fig. 4).

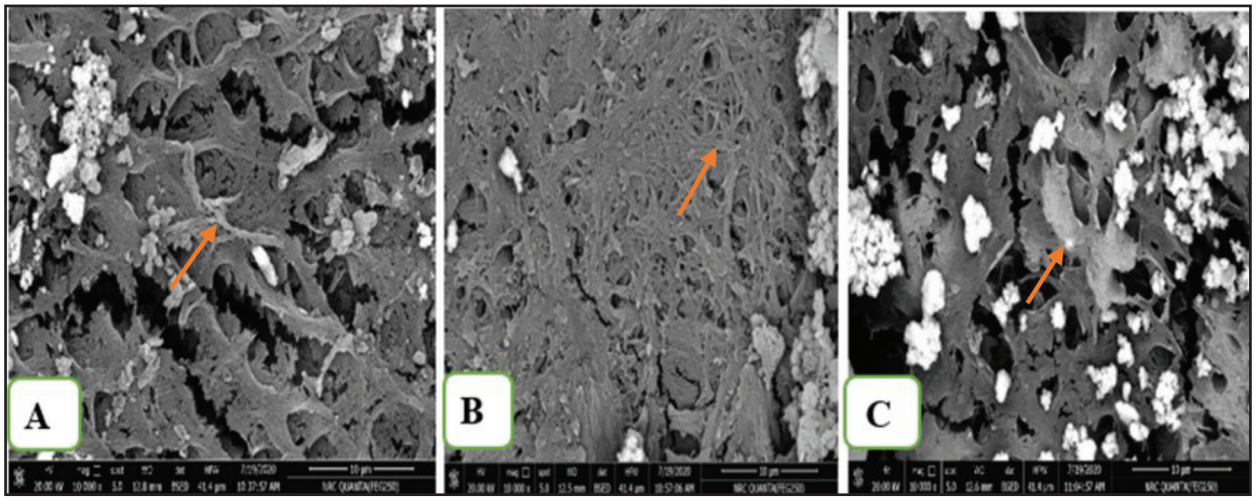

Figure (4) A scanning photomicrograph (X10000) of dentin discs with different final rinse: A) Group I: 17% EDTA (polygonal flattened cells with low cell density) B) Group II: Apple vinegar (polygonal flattened cells with high cell density) (C) Group III: distilled water (round to oblong cells with low cell density)

DISCUSSION

Regenerative endodontic procedures (REPs) have been widely applied clinically nowadays hoping to prevent the immature necrotic tooth from fracture and enhance the completion of the root development. Based on tissue engineering principles, a tetrad of elements forms the essential components in regenerative endodontics. These elements include, stem cells, scaffolds, growth factors and the microenvironment that allow the required outcome to occur [2-4].

Disinfection in regenerative endodontics is an essential step and is done using irrigating solutions and intracanal medication. Many irrigating protocols have been suggested. Besides being effective in disinfection, irrigating solution in REPs should maintain the viability of the stem cells, enhance attachment, proliferation and differentiation [2].

Sodium hypochlorite as a main irrigant has been widely used due to its antibacterial efficiency and its unique ability to dissolve the organic tissues. The use of 17%EDTA after NaOCl has been advised to reverse the detrimental effect of NaOCl on the cells [25-27]. Use of 17%EDTA is recommended in regenerative endodontics as a chelating agent that help the removal of the inorganic component

of the smear layer. Chelation helps in the dentin demineralization and the liberation of the essential growth factors present inside dentin. Acids also can be used for demineralization of dentin. Citric acid, phosphoric acid and phytic acid have been used previously and showed acceptable results regarding stem cell viability and growth factor liberation [25,28].

Apple vinegar, as a natural substitute to 17%EDTA, has been used in this study due to its antimicrobial effect, biocompatibility and cost effectiveness and its reported efficiency in smear layer removal [19].

Stem cells from the apical papilla are known by their high proliferative power and multilineage differentiation [9]. Therefore, SCAPs were used in the present study to assess the effect of apple vinegar on viability and adherence of stem cells to root canal dentin.

MTT assay has been conducted by exposing the irrigating solutions directly to the stem cells and the results have been taken after 6, 24 and 48 hours. Results were the same at the different time points where apple vinegar showed the highest mean absorbance value with statistically significant difference from other groups while 1.5% NaOCl showed the statistically significantly lowest mean value. Such result is in accordance with a previous study that reported higher biocompatibility of malic acid (main component of apple vinegar) than 17%EDTA [29]. Also, lowest mean value recorded by NaOCl is in an agreement with other studies that reported its cytotoxicity [2,4,6].

In the present study, attachment of apical papilla stem cells was evaluated quantitively by the amount of fibronectin release and qualitatively by scanning electron microscope imaging. Scanning electron microscope imaging holds many advantages. While it shows the morphological characters of the stem cells and cellular density, it also shows the effect of the conditioning agents on the dentin surface [30].

Results of fibronectin release showed highest mean value in apple vinegar group when it applied for 5 minutes with statistically significant difference from other groups. This may be attributed to the calcium ion released from dentin by the action of apple vinegar. It has been found that fibronectin synthesis increases in the presence of calcium ions [31]. The mechanism by which 17% EDTA demineralizes dentin is different from that occurs by apple vinegar. While 17% EDTA demineralizes dentin by chelation, apple vinegar demineralizes dentin by decalcification. Chelation is done when EDTA forms stable complex with calcium ions present in dentin and accordingly, EDTA carboxyl groups are ionized and the hydrogen ions that compete with calcium ions are released [19].

This finding was confirmed by comparing between apple vinegar conditioning for 5 minutes and 3 minutes. Fibronectin release was significantly higher when apple vinegar was applied for 5 minutes than 3 minutes. By time, decalcification increases and calcium ions in the environment also increase [19], accordingly, fibronectin synthesis increases.

Preparation and conditioning of the dentin surface affect the morphological characters and appearance of the stem cells [11]. Regarding results of SEM, Images were analyzed on basis of morphological characters of cells as well as the cell density [30]. Cells in 17% EDTA group and in apple vinegar group appeared flat and stretched in shape which means good attachment to dentin. However, cells in distilled water group appeared oblong to round shape which indicated less attachment of cells to dentin. This result is in an agreement with other studies which stated that 17% EDTA promotes stem cell attachment [2,25,30]. This result can be attributed to the ability of 17%EDTA and apple vinegar to remove the smear layer while in the control group, presence of patches hindered intimate contact and hence the attachment of the cells. It has been found that different degree of demineralization caused by different conditioning agents affected both cell morphology and attachment [11].

The difference in cell morphology may be due to different effect of the irrigating solutions on the physical and chemical properties. It is known that both 17% EDTA and apple vinegar lead to dentin demineralization, however the mechanism and the amount of demineralization is different. Even NaOCl has been found to produce to a little extent surface demineralization [33]. When the amount of calcium removal by 17% EDTA and apple vinegar was compared in a previous study, result showed that apple vinegar removed less amount of calcium leaving more calcium in dentin which means more stiffness of the substrate. With different degree of demineralization thus dentin stiffness varies accordingly. Such alteration in surface stiffness affects the attachment and cell morphology. Cells become more flattened when they are seeded on substrates with higher stiffness [34].

In the present study, cell density was higher in apple vinegar group than in 17% EDTA group. This result may be explained by the difference in the cytotoxicity between 17% EDTA and apple vinegar that was reported by the MTT assay. Cytotoxicity of an irrigant has an important role regarding the cell attachment. The initial attachment of the cell is deteriorated by cytotoxic irrigating solution [32]. Also, cell density is affected by the irrigant effect on the dentin wettability [30].

The effect of conditioning materials on dentin wettability has been studied. Wettability, an essential surface property for adhesion, has been found to be changed by irrigation. Wettability of a surface is mainly dependent on its chemical composition, roughness, hydration state, and can also be affected by the tubular density [35]. It was suggested that acidity increases the wettability. The increase in wettability may be explained by the enlargement of dentinal tubules which are filled with fluid, so increase surface hydration [36]. Hence apple vinegar (which has PH around 2) is thought to increase the dentin wettability. Wettability improvement may enhance the attachment of SCAPs, that would account for better outcome of regenerative endodontics [33].

CONCLUSION

Within limitation of this study, it has been concluded that:

1. Apple vinegar promoted the survival of SCAPs.
2. Apple vinegar improved the attachment of SCAPs.

RECOMMENDATION

1. More studies are needed to show the effect of the apple vinegar on migration and differentiation of stem cells.
2. Animal study should be done using apple vinegar to investigate its effect on the immature necrotic roots.

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AUTHOR DECLARATION

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