sciendo
10.2478/AMB-2018-0016

INFLUENCE OF CITRIC ACID ON THE VITALITY OF STEM CELLS FROM APICAL PAPILLA

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Abstract. *The endodontic treatment of immature permanent teeth with necrotic pulp is a serious clinical challenge. The chemical agents, used in regenerative procedures, should be selected not only based on their bactericidal/bacteriostatic properties, but also on their ability to ensure the survival of the patient's stem cells. The aim of this study was to evaluate the effect of citric acid on the vitality of SCAP in a model of an immature tooth root. Models of immature roots were created from 12 freshly extracted teeth. The models were gas sterilized with ethylene oxide and they were separated into three groups, based on the used combinations of irrigants: 1) 1.5% sodium hypochlorite / 17% EDTA; 2) 1.5% sodium hypochlorite / 10% citric acid; 3) saline. SCAPs in a hyaluronic acid–based scaffold were seeded into the canals and cultured for 7 days. Viable cells were quantified using a colorimetric assay. There was no statistically significant difference between the groups, irrigated with NaOCl/EDTA and NaOCl/ citric acid. The results from our experiment show that 10% citric acid can be used in combination with 1.5% NaOCl in a regenerative endodontic procedure.*

Key words: *apical papilla, stem cells, citric acid*

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INTRODUCTION

The apical papilla is a part of the dental papilla and is of ectomesenchymal origin [1, 2]. It is located on the tip of the developing root in the permanent teeth. This tissue is loosely attached to the apex and it can be removed easily. It is responsible for the development of the root together with Hertwig's epithelial root sheath [3, 4].

The apical papilla is histologically different from the dental pulp and it contains unique population of mesenchymal stem cells [5]. They are called stem cells from the apical papilla (SCAP). There is evidence to support the hypothesis that SCAP are sources of primary odontoblasts responsible for root dentin formation, whereas dental pulp stem cells are a probable source of substitution/replacement odontoblasts [3]. Apart from their mobility, SCAP have high proliferative potential and odontogenic capacity [4, 6]. Their preservation in the treatment of immature permanent teeth will allow the root development to continue throughout the apexogenesis [3, 5].

At the same time, a chronic infection and periapical inflammation can seriously damage the apical papilla or alter its properties. This is decisive for continuation of root development in immature teeth, undergoing regenerative endodontic treatment [3, 4].

The regenerative endodontic disinfection protocol of immature teeth with necrotic pulp includes minimal or no instrumentation, irrigation and intracanal medication with antimicrobic agents [7]. It relies on chemical cleaning for removal of necrotic residues [8-10]. The chemical agents in the regenerative endodontic procedures should possess bacteriocidic/bacteriostatic properties, but at the same time they should preserve the vitality and the proliferative capacity of the stem cells from the periapical region [11]. This approach is fundamentally different compared to the classical endodontic clinical protocol for root canal preparation [8].

The ideal endodontic irrigant should have broad antimicrobial spectrum of action and high efficacy against anaerobic and facultative microorganisms organized in a biofilm. The irrigant should be able to dissolve the necrotic residues from the dental pulp, inactivate the endotoxin and it should prevent the formation of a smear layer during the treatment or it should remove it after its formation [12].

None of the current irrigants combines all these abilities, which requires the use of more than one solution – sodium hypochlorite with antimicrobial action to remove the organic component of the smear layer, and EDTA or citric acid to remove the inorganic component of the latter.

The aim of this study was to evaluate the effect of citric acid on the vitality of SCAP in a model of an immature tooth root. The hypothesis we test is that citric acid has the same effect on the vitality of SCAP as EDTA. To achieve the aim of this study the following tasks have been set:

- Isolation of SCAP from freshly extracted immature teeth;
- Creation of a model of tooth with an immature root;
- Synthesis of hyaluronic hydrogel and incubation of SCAP in it;
- Evaluation of the effect of 10% citric acid on the vitality of SCAP, compared to 17% EDTA and saline.

MATERIAL AND METHODS

Our study includes 10 third molars of patients aged 14 to 17 years, extracted due to orthodontic reasons. Informed consent was signed before the use of the teeth. After the extraction, the teeth were placed in Dulbecco's modified Eagles Medium and they were transported to the laboratory within 60 minutes. The SCAPs were isolated by enzyme digestion method in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase. The cells were cultured with Dulbecco's modified Eagles Medium, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37 °C in 5% CO_2. When the cells reached 80% of confluence, they were subcultured, using 0.05% of trypsin. For the purposes of this study, cells from passages 3 to 5 were used. A flow cytometric identification of cell surface markers STRO-1, CD – 146 and CD – 34 was used to demonstrate the stem cell nature of these cells.

Additionally, models of immature teeth were created from 12 freshly extracted mature permanent teeth. The gingival and periodontal tissues were removed using surgical blade. Sterilized high-speed burs were used to section middle portion of the roots to obtain segment length of 5 mm. The segments were instrumented under constant irrigation with saline first with .02 hand K-files up to No. 40, then with Peeso – reamer to achieve 1.3 mm diameter of the root canal. The segments were sterilized with ethylene oxide and they were divided into three groups, based on the used combination of irrigants. The first group was irrigated for 5 minutes with 1.5% NaOCl (Chloraxid, Cerkemed), after that for 5 min with 17% EDTA (i-EDTA, i-Dental), and finally 5 minutes with saline, the second group – 5 min with 1.5% NaOCl (Chloraxid, Cercamed), 5 min with 10% citric acid (Citric acid, i-Dental) and 5 min with saline, the third group – 15 min with saline. Due to the high reactivity of NaOCl solutions, freshly unsealed newfactory packages were used for the purposes of this study.

Hyaluronic hydrogel was synthesised, containing hyaluronic acid (1.5 mg/ml), collagen (1.5 mg/ml) and laminin (1 mg/ml, Sigma – Aldrich) in a ratio of 1:1:1. Hyaluronic acid powder (HA sodium salt, Sigma – Aldrich) was diluted in 0.3 M NaH_2PO_4 with pH of 5.35. The desired concentration of the collagen was achieved after diluting it with 0.2% acetic acid. Buffer for fibrillogenesis, containing 162 mM NaH2PO4, adjusted to pH 11.2 with 10 N NaOH and filter sterilized, was added to the collagen. The collagen and 10xPBS were mixed in 4:1 ratio and the hyaluronic acid and the laminin were added.

Seven µl of hydrogel with 10 000 cells were pipetted into each segment and placed into a 24-well plate. Additionally, cells at dynamic concentrations (100 000, 50 000, 25 000, 12 000, 6000, 3000, 1500, 750 cells/7 µl) were added to the scaffold to serve as the control and to generate a quantification standard curve. DMEM was added and the samples were incubated at 37 °C and 5% CO_2 for a week.

The number of vital cells was determined using colorimetric assay Cell Counting Kit – 8 (CCK – 8, Sigma – Aldrich). The result was measured in microplate reader Varioskan (ThermoFisher Scientific). The experiment

was repeated three times. Data were analyzed with Parameter t-test and non-parametric test (Wilcoxon test) for difference in mean values at 95% significance level by statistical software SPSS – 17.

RESULTS

The vitality of SCAPs from the hyaluronic hydrogel, incubated in root segments, that were irrigated with different combinations of solutions, is shown in figure 1. The highest mean values are measured in the samples, that were irrigated only with saline (group 3, control).

However, this type of irrigation is not performed in vivo due to lack of antibacterial action of the saline [14, 15].

The mean values of the vitality of SCAP, incubated in the root segments with hyaluronic hydrogel, are shown in Table 1. Highest mean values were recorded in group 3 (m = 14614.1622, saline, Table 1). The mean values for the other two groups are almost similar – group 1 m = 4205.2267 and group 2 – m = 4206.011 (Table 1).

The results for the mean values of the three study groups were compared and presented in Table 2.

The statistical analysis of the results showed a statistically significant difference between group 3 and group 1 (Table 2, $p < 0.000$) and group 3 and group 2 (Table 2, $p < 0.000$). No statistically significant difference was found between the mean values of group 1 (NaOCl /EDTA) and group 2 (NaOCl /CA) (Table 2).

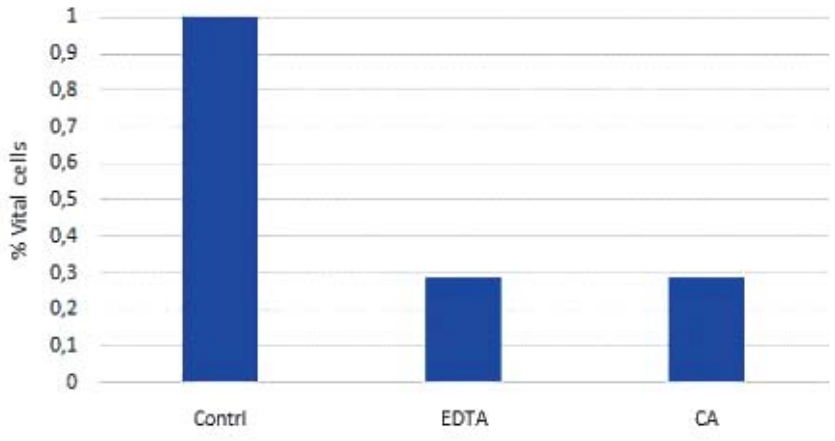

Fig. 1. Evaluation of the vitality of SCAP, incubated in root segments with hyaluronic hydrogel

Table 1. Mean values of the vitality of SCAP, incubated in the root segments with hyaluronic hydrogel, after irrigation with various combinations of solutions (group 1 – NaOCl/EDTA, group 2 – NaOCl/citric acid, group 3 – saline)

Group	Mean (m)	Standard deviation	Minimum	Maximum
Group 1	4205.23	866.47	3018.2	5683.33
Group 2	4206.01	801.14	3023.01	5550.76
Group 3	14614.16	1706.05	12311.30	17277.7

Table 2. Comparison of the mean values of the three study groups

Group	Compared with	Mean	Standard Deviation	Std, Error Mean	95% Confidence Interval of the Difference		T	P*
					Lower	Upper		
Group 1	Group 2	-0.7843	2949.71	851.51	-3879.61	-131.294	0.013	.990
Group 1	Group 3	-10408.9	2158.87	623.21	-11780.6	-9037.26	-16.702	.000
Group 2	Group 3	-10109.64	2238.18	646.11	-11831.72	-8987.57	-16.111	.000

DISCUSSION

Sodium hypochlorite remains the most used irrigant in the endodontics with bactericidal and dissolving the organic matter action [16, 17]. These two properties are crucial for the disinfection of the immature permanent teeth in the regenerative endodontics [18]. By examining the lower concentration of hypochlorite on SCAP, 1.5% of NaOCl has minimal effect on their survival and differentiation [19], whereas high concentrations have negative effect on the survival and differentiation of the stem cells from dental pulp into odontoblast-like cells both in vitro and in vivo. The use of 6% NaOCl has a detrimental effect on these cells and its application is not recommended [20]. The use of EDTA in the regenerative endodontics promotes the release of growth factors from the dentin [21, 22] and disrupts the adhesion of the biofilm to the root surface, which explains why EDTA decreases the amount of intracanal microorganisms compared to saline, despite its limited antiseptic properties [12, 23]. Dentin conditioning with 17% EDTA provides better survival of SCAP. Furthermore, a final irrigation of the canal with 17% EDTA after 6% NaOCl reduces the detrimental effect of the latter [24].

Important growth factors are found in the dentin matrix during the dentinogenesis. Some of them like transforming growth factor-beta stimulate the proliferation and differentiation of mesenchymal stem cells [25, 26]. EDTA dissolves these factors, thus increasing their bioavailability. The use of the inductive properties of dentin morphogens and growth factors is of great benefit to the clinician during regenerative procedures [22].

Citric acid has a similar action. The use of 10% citric acid in combination with 2.5% NaOCl removes the smear layer from the root canal. Its effectiveness does not differ from that of 17% EDTA, except in the apical part of the root canal in mature permanent teeth [27]. Various in vitro and in vivo studies have shown that 10% citric acid demineralizes the root canal dentin better [28] while having a lower cytotoxic effect than 17% EDTA [29-31] and exhibiting slight antimicrobial effect [32]. The low cytotoxicity of citric acid can be explained by its weaker proinflammatory effect compared to the EDTA-based solutions [33]. In addition, it plays an important role in the tricarboxylic acid cycle, which is crucial for regulating energy homeostasis and cell metabolism. EDTA is not a part of the cells and their biochemical processes [31].

The level of citrate in bones and teeth is about 20-80 μmol/g, which is 100 to 400 times higher than in plasma. According to new studies, it plays an important role in the bone anatomy and physiology as an integral part of bone nanocomposites [34, 35]. About 1/6 of the apatite nanocrystals‘ free surface is covered with citrate molecules that form bridges between the mineral plates, thus regulating bone crystallinity, which is directly associated with bone strength [34]. They provide more carboxylate groups to bind calcium ions than all non-collagen proteins together [35]. Exogenous citrate, added to the culture medium or released from biomaterial during its resorption, increases the expression of the alkaline phosphatase gene both in vitro and in vivo [36].

The results of our study show that there is no statistically significant difference in the survival of SCAP, treated with EDTA and 10% citric acid, which confirms the tested hypothesis (Tab. 2). The results are not related to the direct chemical effect on SCAP, since all segments were irrigated with saline before the cell incubation. It is important to notice the significant difference between the number of vital cells from the control group and the groups, irrigated with EDTA and citric acid (Tab. 2). This demonstrates the serious impact of the irrigants on the vitality of the SCAP, which, combined with the lack of validated clinical protocols, makes the result of a regenerative therapy still unpredictable.

CONCLUSION

Clinicians are often faced with the challenge of disinfecting large canals adequately. Microbial control is crucial in these procedures. Maintaining the vitality of mesenchymal stem cells is of great importance for the success of regenerative endodontics. It is necessary to use an irrigation protocol that ensures good disinfection and at the same time does not change the biological potential of stem cells. The results from our experiment show that 10% citric acid can be used in combination with 1.5% NaOCl in a regenerative endodontic procedure.

Funding

This work was supported by the Medical University of Sofia, Bulgaria [grant number 220/14.01.2015], [contract number 43/2015] and by the National Science Fund at the Ministry of Education and Science of the Republic of Bulgaria [grant number DFNI B02/15,12.12.2014].

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