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## Research Article

### Effects of ethylenediaminetetraacetic acid on stem cells from the apical papilla: *in vitro* study

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**Running title** The effects of EDTA on SCAPs

1 **Original Article**

2 **Effects of ethylenediaminetetraacetic acid on stem cells from the apical papilla: *in vitro***  
3 **study**

4 **Abstract**

5 **Background/purpose** Ethylenediaminetetraacetic acid (EDTA) is used as an irrigant in  
6 regenerative endodontic treatment. The present study aimed to investigate the effects of EDTA  
7 on stem cells from apical papilla (SCAPs) *in vitro*.

8 **Materials and methods** Human SCAPs were isolated and characterised. The cells were treated  
9 with media supplemented with EDTA at concentrations ranging from 1.25% to 17%. Cell  
10 proliferation and apoptosis were examined using MTT assay and annexin V/propidium iodide  
11 staining. Cell migration was determined by a scratch assay. Gene expression was evaluated  
12 using a real-time polymerase chain reaction. Mineral deposition, a hallmark of osteogenesis *in*  
13 *vitro*, was determined using alizarin red s staining.

14 **Results** Overall, SCAPs exhibited mesenchymal stem cell characteristics. EDTA treatment at  
15 2.50% and 1.25% did not significantly exhibit cytotoxicity and alter cell morphology.  
16 However, EDTA attenuated cell proliferation and reduced *MKI67* mRNA expression in  
17 SCAPs. Further, EDTA significantly induced early cell apoptosis at 48 hours. Cell migration  
18 was delayed with EDTA treatment. After maintaining SCAPs in an osteogenic induction  
19 medium, EDTA diminished mineral deposition by SCAPs on day 14.

20 **Conclusion** EDTA treatment exhibits adverse effects on SCAPs *in vitro*. Hence, EDTA  
21 exposure to periapical tissues should be avoided to minimise the negative impacts on SCAPs  
22 cells in regenerative processes.

23 **Keywords:** EDTA; Stem cells from the apical papilla; Cell proliferation; Cell migration; Cell  
24 apoptosis; Osteogenic differentiation

25

## 26 **Introduction**

27           Regenerative endodontics is currently a valid treatment option for necrotic immature  
28 teeth.<sup>1</sup> Numerous publications indicate the benefit and success of regenerative endodontic  
29 procedures as determined by the continued root formation and increase in dentin thickness.<sup>1</sup>  
30 Various root canal irrigation regimens and final irrigants have been utilised.<sup>2</sup> EDTA at the  
31 concentration of 17% is recommended as the canal irrigant between each root canal treatment  
32 appointment before creating a blood clot inside the canal space.<sup>3</sup> The use of EDTA results in  
33 the release of bioactive molecules from dentin, influencing stem cell migration and  
34 differentiation in the root canal *in vitro*.<sup>4</sup> EDTA irrigation removes the smear layer and  
35 conditions dentin surfaces.<sup>5, 6</sup> The exposed collagen after EDTA treatment could further  
36 modulate cell functions, including odonto/osteogenic differentiation.<sup>6</sup>

37           Stem cells from apical papilla (SCAPs) reside in apical papilla tissues. These cells can  
38 differentiate into odontoblast-like cells and form dentin/pulp complex *in vivo*.<sup>7</sup> SCAPs can  
39 survive root canal infection even though total pulp degeneration usually occurs.<sup>8,9</sup> These cells  
40 have been speculated to be the primarily responsible cells in dentin/pulp regeneration following  
41 the regenerative endodontic procedure.<sup>9</sup> The proliferation, migration, and differentiation  
42 abilities of SCAPs play a crucial role in dental pulp tissue regeneration following regenerative  
43 endodontics. The effects of several irrigants, including 17% EDTA in SCAPs, are reported.  
44 17% EDTA exposure promotes more cell survival than other irrigants.<sup>5, 10</sup> Hence, the present  
45 study aimed to investigate further the direct effect of different EDTA concentrations on cell  
46 proliferation, apoptosis, migration, and differentiation of SCAPs *in vitro*.

47

## 48 **Materials and methods**

### 49 **Cell isolation and culture**

50 All experimental protocols were approved by the Human Research Ethical Committee,  
51 Faculty of Dentistry, Chulalongkorn University (approval No. 031/2020). All methods were  
52 carried out in accordance with the Declaration of Helsinki guidelines. Informed consent was  
53 obtained from all participants and/or legal guardian(s).

54 Apical papilla tissues from the immature impacted third molars scheduled for surgical  
55 removal according to the patients' treatment plan were collected for cell isolation using the cell  
56 explantation technique.<sup>11</sup> The isolated cells were maintained in Dulbecco modified Eagle  
57 medium (DMEM cat. no. 11960, Gibco, Grand Island, NY, USA) containing 10% fetal bovine  
58 serum (cat. no.10270, Gibco), 2 mM L-glutamine (GlutaMAX<sup>TM-1</sup>, cat. no. 35050, Gibco), 100  
59 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-  
60 Antimycotic, cat. no. 15240, Gibco) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. According  
61 to previous protocols, stem cell characteristics were analysed by cell surface marker expression  
62 and differentiation ability toward osteogenic and adipogenic lineages.<sup>11, 12</sup> For EDTA  
63 treatment, based on previous publications, cells were exposed to EDTA at different  
64 concentrations (17%, 12%, 10%, 5%, 2.5% and 1.25% v/v)<sup>13, 14</sup> for 30 and 60 seconds and  
65 subsequently rinsed with SCAPs growth medium.

#### 66 **Cell viability assays**

67 Cells ( $5 \times 10^4$  cells) were seeded in 24 well-plates and maintained in a growth medium.  
68 At designated time points, cells were incubated with MTT solution for 15 minutes, and formed  
69 formazan crystals were dissolved using DMSO/glycine buffer. The solubilised dye was  
70 measured at the absorbance of 570 nm using a microplate reader.

#### 71 **Cell morphology assay**

72 After EDTA exposure, cells were permeabilised with 0.1% Triton X-100 and stained  
73 with rhodamine-phalloidin (Invitrogen, Carlsbad, CA, USA) in 10% horse serum at 1:100

74 dilution. Nuclei were counterstained with DAPI at 1:1,000 dilution. F-actin and nucleus  
75 orientation were examined using a fluorescence microscope.

#### 76 **Cell apoptosis assay**

77 Cells ( $1 \times 10^5$  cells/well) were seeded in 6 well-plates and maintained in a growth  
78 medium. According to the manufacturer's protocol, cells were stained with Propidium  
79 Iodide/Annexin V staining (Roche Diagnostics, Mannheim, Germany) and further analysed  
80 using a FACSCalibur flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA).

#### 81 **Cell migration assay**

82 Cells ( $1 \times 10^5$  cells/well) were seeded in 6 well-plates and maintained in a growth  
83 medium. A scratch assay was created by scratching the monolayer of cells. Subsequently, cells  
84 were treated with EDTA. Images of the wound area were captured at 24 and 48 hours. The  
85 percentage of wound area was calculated using ImageJ software.

#### 86 **Gene expression assay**

87 Total RNA was extracted with RiboEx<sup>TM</sup> solution (cat. no. 301-001, GeneAll<sup>®</sup>, Seoul,  
88 South Korea) and converted to cDNA using reverse transcriptase Improm-II<sup>TM</sup> RT system kit  
89 (ImProm-II<sup>TM</sup>, cat. no. A3800, Promega, Madison, WI, USA). A real-time polymerase chain  
90 reaction was performed in a real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA)  
91 with SYBR<sup>®</sup> green detection system (FastStart<sup>TM</sup> Essential DNA Green Master; Roche  
92 Diagnostic). The primer sequences were *MKI67* forward: 5'-CGTTTGTTTCCCCAGTGTCT-  
93 3'; reverse: 5'-CTCCCTGCCCTTTCTATTC-3'; *GAPDH* forward: 5'-  
94 TCATGGGTGTGAACCATGAGAA-3'; reverse: 5'-GGCATGGACTGTGGTCATGAG-3'.

#### 95 **Mineral deposition assay**

96 Cells were fixed with cold methanol for 10 min and further incubated with the alizarin  
97 red s solution (2% w/v) for 3 min. The deposited dye was solubilised with a 10% cetylpyridium

98 chloride solution. The solubilised solution was measured at the absorbance of 570 nm using  
99 the same microplate reader as above.

## 100 **Statistical analysis**

101 Data were presented as mean  $\pm$  standard error of the mean. Statistical analysis was  
102 performed using Prism version 8 software (GraphPad Software, San Diego, CA, USA). Mann  
103 Whitney U test was employed for two-group comparisons. Kruskal Wallis test followed by  
104 Dunnett post hoc test was utilised for three or more group comparisons. All experiments were  
105 repeated with cells from at least four donors. A statistically significant difference was  
106 considered when the *P*-value  $< 0.05$ .

## 107 **Results**

### 108 **Stem cells characterisation**

109 Isolated human cells exhibited spindle shape morphology and expressed CD44, CD73,  
110 and CD105 but lacked CD45 expression (Fig. 1A-1E). After maintaining these cells in an  
111 induction medium, cells could differentiate into osteoblasts and adipocytes, as confirmed by  
112 the increase in mineral deposition (Fig. 1F and 1G) and intracellular lipid accumulation (Fig.  
113 1H and 1I).

### 114 **EDTA attenuated cell proliferation and promoted early apoptosis**

115 Cells were treated with EDTA at concentrations ranging from 1.25% to 17% for the  
116 30s or 60s and subsequently maintained in a growth medium for 24 h. Cells treated with EDTA  
117 exhibited a significant decrease in cell viability compared with the control. However, there was  
118 no statistically significant difference when cells were treated with 1.25% and 2.5% EDTA (Fig.  
119 2A). Cells treated with 1.25% and 2.5% EDTA appeared to have similar morphology to those  
120 of the control group while maintaining intact actin filaments at the cytoskeletal level (Fig. 2B).  
121 Hence, 1.25% and 2.5% EDTA were employed in further experiments from this point onwards.

122 The proliferation ability was attenuated when cells were treated with 1.25% or 2.5%  
123 EDTA for the 30s and 60s (Fig. 2C). In the control condition, significantly increased cell  
124 numbers were observed from baseline to day 7. However, in EDTA-treated cells, a slight  
125 increase of SCAP numbers on day seven was noted but not markedly different from baseline  
126 on day 1. Congruently, SCAPs treated with 1.25% or 2.5% EDTA also showed a decrease in  
127 *MKI67* mRNA expression compared to the control group (Fig. 2D).

128 Next, SCAPs were treated with 1.25% or 2.5% EDTA for the 30s and 60s and  
129 subsequently maintained in a growth medium for apoptosis assessment. Cells treated with 2.5%  
130 EDTA for the 60s had a higher percentage of early and late apoptotic cells than control cells.  
131 However, only a significant difference was observed in the percentage of early apoptotic cells  
132 at 48h (Fig. 3A-3C).

### 133 **EDTA attenuated cell migration and osteogenic differentiation**

134 Cell migration into the “scratched wound” area was observed in the control conditions  
135 at 24h. On the contrary, in all EDTA-treated SCAPs, delayed cell migration was noted (Fig.  
136 4A). Thus, the percentage of wound area was significantly higher with EDTA treatments  
137 compared with the control (Fig. 4B).

138 After maintaining SCAPs in an osteogenic induction medium for 14 days of culture,  
139 the mineral deposition was examined. The results demonstrated that EDTA treatment  
140 suppressed the mineralisation deposition process in SCAPs (Fig. 4C and 4D).

141

### 142 **Discussion**

143 Current clinical guidelines in regenerative endodontics are trending towards including  
144 cell-homing approaches. The laceration of periapical tissue lets progenitor cells from the  
145 periapical tissue migrate into the root canal system. SCAPs, which reside in the apical papilla,  
146 would migrate upward into the root canal and differentiate into dental pulp- and odontoblast-

147 like cells. These cells play a crucial role in dentin/pulp regeneration. The present study  
148 described the adverse effects of EDTA on SCAPs. In this regard, EDTA attenuated cell  
149 proliferation and induced early apoptosis. Further, cell migration and osteogenic differentiation  
150 were compromised with EDTA regardless of the concentration. These *in vitro* effects of EDTA  
151 on SCAPs should be considered for clinical implications.

152         17% EDTA root canal irrigation alone or combined with other irrigants enhanced  
153 SCAPs *in vitro* survival compared to other groups.<sup>5, 10</sup> Dentin conditioning with 17% EDTA  
154 allowed human dental pulp stem cell proliferation similar to those treated with distilled water.<sup>15</sup>  
155 However, the present study described the attenuative effects of EDTA on cell viability and  
156 proliferation of SCAPs. These distinctions might be ascribable to variations in methodology  
157 upon EDTA exposure to cells. For example, in previous studies, the root dentin was irrigated  
158 with EDTA and then allowed to dry before cell exposure.<sup>10</sup> In Martin et al., cells were seeded  
159 in hyaluronic acid-based constructs before EDTA treatment.<sup>10</sup> However, in our study, cells  
160 were directly exposed to EDTA in a monolayer culture system.

161         The present study showed that cell proliferation and *MKI67* mRNA expression were  
162 attenuated in EDTA-treated conditions. *MKI67* is detectable through the G1, S, G2, and M  
163 phases of the cell cycle.<sup>16</sup> The expression levels were low in G1 and S phases and peaked in  
164 the M phase.<sup>16</sup> Hence, the reduction of *MKI67* expression indicates that the cell proliferation  
165 rate is attenuated. EDTA not only negatively affects cell proliferation but also influences cell  
166 apoptosis. The present study revealed that the percentage of early cell apoptosis was  
167 significantly increased in 2.5% EDTA treatment for the 60s at 48 hours time point. Our study  
168 suggests that EDTA treatment attenuated SCAPs cell proliferation and promoted apoptosis *in*  
169 *vitro*.

170         SCAPs cell migration was also decreased when cells were exposed to EDTA. However,  
171 another study in human dental pulp cells demonstrated that 12% EDTA treatment significantly

172 enhanced stromal cell-derived factor-induced cell migration via transforming growth factor  $\beta$   
173 and chemokine receptor 4.<sup>17</sup> It should be noted that there were major differences between these  
174 two studies regarding cell types and migration assay techniques used, and the use of growth  
175 factor-induced migration.

176 EDTA-treated dentin discs promoted mineralisation by human dental pulp stem cells *in*  
177 *vitro*. The same irrigant drove bone/cartilage formation by murine-induced pluripotent stem  
178 cells adjacent to treated dentin *in vivo*.<sup>18</sup> Direct 12% EDTA treatment increased osteogenic  
179 differentiation of human dental pulp cells *in vitro* and promoted dentin bridge formation in  
180 direct pulp capping *in vivo* canine models.<sup>13</sup> However, this present study indicates a negative  
181 impact of EDTA on osteogenic differentiation in SCAPs after direct exposure, as shown by the  
182 decrease of mineral deposition ability. Despite the different cell types used in previous studies,  
183 comprehensive mechanisms related to cell signalling should be investigated in future *in vitro*  
184 research using EDTA treatment procedures.

185 When compared to clinical settings, it is essential to note the main differences in our  
186 findings, such as the absence of dentin and its components as well as the dimension of the cells  
187 exposed to root canal irrigants. The lack of dentinal components means that the effect of them  
188 being irrigated by EDTA generally attained from removing the smear layer exposes dentinal  
189 tubules leading to the release of growth factors such as transforming growth factor  $\beta$  (TGF- $\beta$ ),  
190 which were not involved in the present study in term of cell responses. Another notable  
191 distinction was that SCAPs in our study were treated in a monolayer culture system. In the  
192 clinical setting, SCAPs reside in the apical papilla tissues which have other components  
193 including the extracellular matrix. Hence, the way that SCAPs are actually exposed to the  
194 irrigant is much different from this *in vitro* study. Moreover, in standard clinical practice in  
195 endodontics, the root canal is usually treated with EDTA, dried with paper points and following

196 with blood clot creation in the root canal. Thus, this process could reduce the remaining of  
197 EDTA in the canal after irrigation and also dilute the final EDTA concentration at the apex.

198 From a clinical point of view, the present study supported the current clinical  
199 consideration for the regenerative procedure,<sup>3</sup> which encouraged the use of irrigation systems  
200 that minimise the chance of irrigant extrusion, for example, the use of the side-vents with a  
201 closed-ended needle, the proper position of irrigation needle, the use of gently irrigation force,  
202 the use of negative pressure irrigation system, and the appropriate use of irrigation activation  
203 with ultrasonic or nickel-titanium finishing rotary files. The root canal should be dried entirely  
204 after EDTA is used as a final rinse to avoid adverse effects on incoming SCAPs.

205 Despite the beneficial use of EDTA in clinical regenerative endodontic treatment  
206 procedures, EDTA herein exhibited a direct negative effect on cell proliferation, cell migration,  
207 and osteogenic differentiation in SCAPs *in vitro*. Hence, the clinical use of EDTA should be  
208 carried forward with caution. The flowing of EDTA liquid remnants into periapical tissue must  
209 be carefully avoided. The use of EDTA should be limited. Future *ex vivo* or *in vivo* effects of  
210 EDTA on SCAPs should be further clarified. Integrating a biological rationale into a clinical  
211 approach might accomplish the goals of regenerative endodontics.<sup>3</sup>

## 212 **Declaration of competing interest**

213 The authors have no competing interests relevant to this article.

## 214 **Acknowledgement**

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271 **Figure Legend**

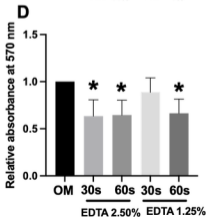
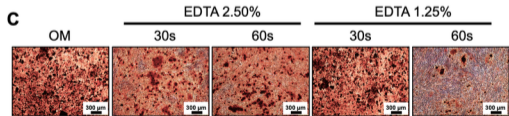
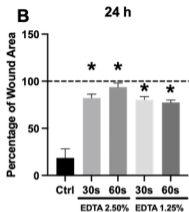
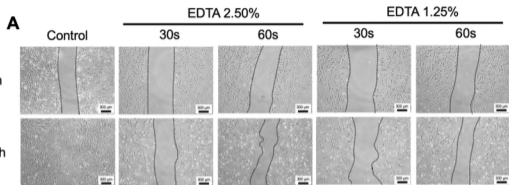
272 **Figure 1** Characterization of stem cells from apical papilla. (A) Morphology of the isolated  
273 cells. (B-E) Immunostaining of protein surface markers CD45, CD44, CD73, and CD105. (F-  
274 G) Mineralisation assay after osteogenic induction for 14 days. (H-I) Lipid accumulation assay  
275 after adipogenic induction for 16 days.

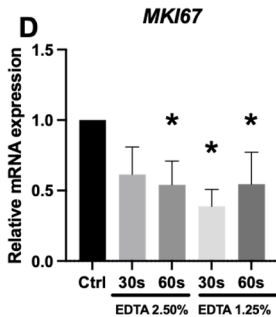
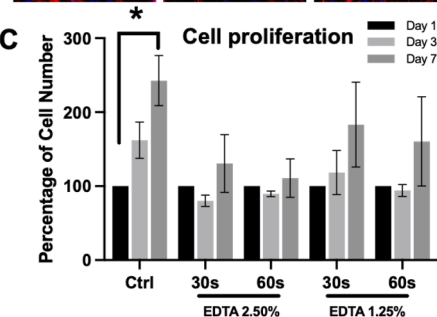
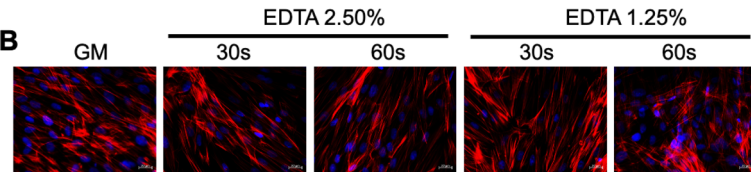
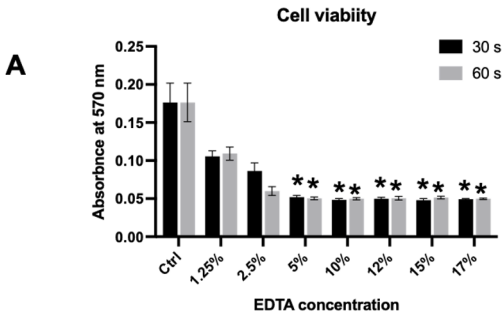
276 **Figure 2** SCAPs were exposed to 2.5% and 1.25% EDTA for the 30s and 60s. (A) Cell viability  
277 assay at 24h (B) Cell morphology evaluated by phalloidin staining at 24h. (C) Cell proliferation  
278 assay on day 1, 3, and 7. (D) *MKI67* mRNA expression was assessed by real-time polymerase  
279 chain reaction at 24h. Asterisks indicated a statistically significant difference compared with  
280 the control ( $P < 0.05$ ).

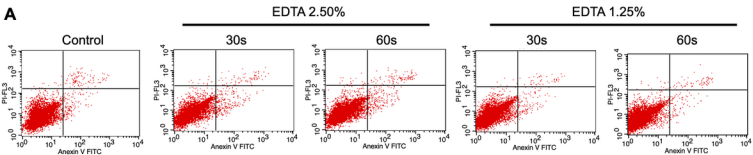
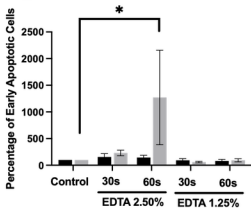
281 **Figure 3** SCAPs were exposed to 2.5% and 1.25% EDTA for the 30s and 60s. Apoptosis assay  
282 was performed using Annexin V/PI staining at 24h and 48h. (A) Representative cytograms. (B-  
283 C) The graphs demonstrated the percentage of early and late apoptotic cells. Asterisks indicated  
284 a statistically significant difference compared with the control ( $P < 0.05$ ).

285 **Figure 4** SCAPs were exposed to 2.5% and 1.25% EDTA for the 30s and 60s. Cell migration  
286 was examined using the scratch assay. (A) Representative images of cell migration at 0h and  
287 24h. (B) The graphs demonstrated the percentage of the wound area. (C) Representative images  
288 of mineral deposition determined by alizarin red s staining. (D) The graphs showed the  
289 quantitative analysis of solubilised dye. Asterisks indicated a statistically significant difference  
290 compared with the control ( $P < 0.05$ ). OM: osteogenic inductive medium condition; Ctrl:  
291 normal growth medium condition.

292





**A****B****C**