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Research Article

Caffeic Acid Phenethyl Ester (CAPE) Effects on Dental Pulp Stem Cell (DPSC) Proliferation and Viability

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Received: May 14, 2022; **Published:** May 31, 2022s

Abstract

Introduction: Caffeic acid phenethyl ester or CAPE is an organic molecule produced by honeybees, which has been previously evaluated for its anti-inflammatory effects. Additional studies have demonstrated CAPE (also known as Propolis) may also exhibit many types of anti-tumor effects in various types of cancer – including oral cancers. However, to date no published studies to date have evaluated any potential effects of CAPE on other rapidly dividing oral cells and tissues, such as dental pulp stem cells (DPSC).

Methods: Cell cultures from an existing dental pulp stem cell (DPSC) repository were thawed and cultured. These included DPSC with rapid dividing time (rDT) dpsc - 3882, dpsc - 5653, dpsc - 7089 (~1 - 2 days), intermediate dividing time (iDT) dpsc - 9894, dpsc - 8694 (~4 - 6 days), and slow dividing time (sDT), dpsc-11750, dpsc-11418, and dpsc-11836. Cells were plated in 96-well assays to determine any effects of caffeic acid phenethyl-ester (CAPE) at three physiologically relevant concentrations (20 ug/mL, 50 ug/mL, and 100 ug/mL. Changes to cellular proliferation were measured using three-day proliferation assays.

Results: Growth of DPSCs increased significantly under CAPE administration compared with non-treated controls among rDT (14 - 16%), iDT (15 - 24%) and sDT (14 - 20%) isolates, $p < 0.05$. In addition, DPSC viability under CAPE administration also increased compared with non-treated controls among rDT (9 - 14%), iDT (8-9%), and sDT (8 - 12%) isolates, $p < 0.05$. Finally, these phenotypic changes were not associated with dysregulation of mesenchymal stem cell biomarker expression of NANOG, SOX2 or OCT4.

Conclusions: This study is among the first to evaluate different types of DPSC isolates (rapidly and slowly dividing) and the effects of CAPE. Although only DPSC explants from UNLV-SDM were tested, these results strongly suggest that additional studies should be conducted on other DPSC isolates to validate and confirm the potential positive effects of CAPE on DPSC phenotypes.

Keywords: *Caffeic Acid Phenethyl Ester (CAPE); Propolis; Dental Pulp Stem Cells (DPSC)*

Introduction

Caffeic acid phenethyl ester or CAPE is an organic molecule produced by honeybees, which has been previously evaluated for its anti-inflammatory effects [1,2]. CAPE has been demonstrated as a natural antioxidant with strong cytoprotective effects in various tissues and cell types [3,4]. Many studies have revealed that these properties may be strongly linked to the immunosuppressive activity of CAPE,

which has been shown to function through targeting of nuclear factor (NF) of activated T-cells and NFkB transcription factors [5,6]. Although the ability of CAPE to inhibit NFkB is important for anti-inflammatory and immunomodulatory functions, these properties have also been demonstrated as a functional mechanism to impact and protect against other conditions including osteoporosis and Parkinson's disease [7-9].

However, additional research has demonstrated CAPE (also known as Propolis) may also exhibit many important effects not related to immune or inflammatory modulation but as an antimicrobial agent [1,2,10]. For example, evidence has demonstrated that CAPE may be used to inhibit *Paenibacillus*, as well as clinically important *Vibrio* and *Candida* species [11-13]. This has led to further research to evaluate the potential role of CAPE to inhibit other important oral species, including the cariogenic bacteria *Streptococcus mutans* [14,15].

These studies regarding the antimicrobial properties of CAPE have led to the development of new research to evaluate the effects of CAPE on normal oral tissues and structures, which has demonstrated positive anti inflammatory effects on periodontal-associated macrophages as well as lipopolysaccharide-stimulated gingival fibroblasts [16,17]. Due to the nature of the oral administration of CAPE as an antimicrobial agent, the topical as well as systemic effects on normal as well as highly sensitive cells and tissues, such as stem cells should be considered [18].

However, only one study to date has evaluated the potential effects of CAPE on oral or dental stem cells from humans, which was focused exclusively on odontoblast precursors and differential potential [19]. Another previous study evaluated CAPE on dental pulp stem cells in Guinea Pigs - but to date, no published studies have evaluated the effects of CAPE on other rapidly dividing oral cells and tissues, such as dental pulp stem cells (DPSC) in humans [20]. Based upon the lack of evidence in this area, the primary objective of this study was to evaluate any potential effects of CAPE on the viability or proliferation of DPSC.

Materials and Methods

Study approval

This study involved a retrospective analysis of previously collected dental pulp stem cell (DPSC) isolates from an existing biomedical repository, as previously described [21,22]. The protocol and procedures for this study were reviewed and subsequently approved by the Institutional Review Board (IRB) from the University of Nevada, Las Vegas (UNLV) under Protocol #171612 - 1 "Retrospective Analysis of Dental Pulp Stem Cells (DPSC) from the UNLV School of Dental Medicine (SDM) Pediatric and Clinical Population" on February 21, 2021. The original DPSC isolation protocol was reviewed and approved by the IRB and UNLV Office for the Protection of Research Subjects (OPRS) under OPRS#0907 - 3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp" on 5 February 2010.

Each of the DPSC isolates was screened to determine the presence of specific stem cell markers, including CD73, CD90 and CD105 (and the absence of CD14, CD34, and CD45) according to the International Society for Cellular Therapy (ISCT) guidelines, as previously described [23]. Expression of additional stem cell markers, including NANOG, Oct4 and Sox2 was also confirmed using qPCR [24].

Cell culture

Cell cultures were maintained in an approved biosafety level 2 (BSL-2) humidified tissue culture cabinet from Fisher Scientific (Fair Lawn, NJ) at 37°C that was supplemented with 5% CO_2. Cells were maintained in alpha-MEM (minimal essential media) from Gibco (Waltham, MA) with the addition of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic solution - both obtained from Gibco (Waltham, MA). Each DPSC isolate had been previously cultured for a minimum of ten passages to determine viability using the Trypan Blue assay - as well as growth rates, which were characterized as having rapid doubling times or rDT (1 - 2 days), intermediate doubling times or iDT (5 - 6 days), or slow doubling times or sDT (10 - 12 days), as previously described [25,26].

RNA extraction and cDNA synthesis

RNA was obtained from each DPSC isolate using the phenol:chloroform extraction method, using TRIzol reagent, chloroform, isopropanol and ethanol from Fisher Scientific (Fair Lawn, NJ). In brief, equal volumes of cell suspension (400 uL) and TRIzol reagent (400 uL) were mixed in labeled microcentrifuge tubes with 200 uL of chloroform added. These suspensions were incubated on ice for five minutes prior to centrifugation at 10,000 x g for 15 minutes at 4°C. The upper aqueous phase (~350 uL) was transferred to a sterile microcentrifuge tube with an equal volume of isopropanol added to precipitate the nucleic acids prior to centrifugation at 10,000 x g for 10 minutes at 4°C. The isopropanol was removed and the pellet washed with ethanol prior to a final centrifugation at 10,000 g x five minutes. The ethanol was removed and the pellet was resuspended using 100 uL of RNAse-free molecular grade water from Fisher Scientific (Fair Lawn, NJ). Qualitative and quantitative analysis was performed using a NanoDrop Spectrophotometer and absorbance readings at A260 and A280 nm.

The One-Step Reverse Transcription Kit from ThermoFisher Scientific (Fair Lawn, NJ) was used to convert the total RNA derived from each DPSC isolate into cDNA. All samples were processed using a Mastercycler gradient thermal cycler from Eppendorf (Hamburg, Germany) for the reverse transcription performed at 47°C for 30 minutes.Qualitative and quantitative analysis of the resulting cDNA was done with the NanoDrop Spectrophotometer using absorbance readings at A260 and A280 nm.

qPCR screening

Screening of all DPSC cDNA was performed using the SYBR Green Master Mix from ThermoFisher Scientific (Fair Lawn, NJ). Each 25 uL reaction consisted of 12.5 uL of ABsolute SYBR green master mix, 1.5 uL of forward primer, 1.5 uL of reverse primer, 1.5 uL of sample and 8 uL of nuclease-free water. Cycling reactions were performed using an initial enzyme activation for 15 minutes at 95°C with 40 cycles of amplification that consisted of 15 seconds of denaturation at 95°C, followed by annealing at the primer-specific reaction temperature (listed below) for 30 seconds, and a final extension for 60 seconds at 72°C. Positive control primers were used, which consisted of beta actin - a cytoskeleton (structural) positive control and Glyceraldehyde 3-Phosphate Dehydrogenase or GAPDH - a glycolytic pathway or metabolic positive control.

Beta actin (structural) positive control primers

Beta actin forward; 5'-GTGGGGTCCTGTGGTGTG-3'; 18 nt, 67% GC, Tm: 69°C

Beta actin reverse, 5'-GAAGGGGACAGGCAGTGA-3';18 nt, 61% GC, Tm: 67°C

Optimal Tm: 62°C

GAPDH (metabolic) positive control primers

GAPDH forward: 5′ATCTTCCAGGAGCGAGATCC-3′; 20 nt, 55% GC, Tm 66°C

GAPDH reverse: 5′ACCACTGACACGTTGGCAGT-3′; 20 nt, 55% GC, Tm 70°C

Optimal Tm: 61°C

CD73 forward: 5′-AGTCCACTGGAGAGTTCCTGCA-3′; 22 nt, 55% GC, Tm: 70°C

CD73 reverse: 5′-TGAGAGGGTCATAACTGGGCAC-3′; 22 nt, 55% GC, Tm: 69 °C

Optimal Tm: 64°C

CD90 forward: 5′-ATGAACCTGGCCATCAGCA-3′; 19 nt, 53% GC, Tm: 67°C

CD90 reverse: 5′-GTGTGCTCAGGCACCCC-3′; 17 nt, 71% GC, Tm: 70°C

Optimal Tm: 68°C

CD105 forward: 5′-CCACTAGCCAGGTCTCGAAG-3′; 20 nt, 60% GC, Tm: 67 °C

CD105 reverse: 5′-GATGCAGGAAGACACTGCTG-3′; 20 nt, 55% GC, Tm: 66 °C

Optimal Tm: 67°C

CD14 forward: 5′-CTGGAACAGGTGCCTAAAGGAC-3′; 22 nt, 55% GC, Tm: 68°C

CD14 reverse: 5′-GTCCAGTGTCAGGTTATCCACC-3′; 22 nt, 55% GC, Tm: 67°C

Optimal Tm: 62°C

CD34 forward: 5′-CCTCAGTGTCTACTGCTGGTCT-3′; 22 nt, 55% GC, Tm: 68°C

CD34 reverse: 5′-GGAATAGCTCTGGTGGCTTGCA-3′; 22 nt, 55% GC, Tm: 70°C

Optimal Tm: 63°C

CD45 forward: 5′CATATTTATTTTGTCCTTCTCCCA-3′; 24 nt, 33% GC, Tm: 60 °C

CD45 reverse: 5′-GAAAGTTTCCACGAACGG-3′; 18 nt, 50% GC, Tm: 61 °C

Optimal Tm: 61 °C

NANOG forward: 5′-GCTGAGATGCCTCACACGGAG-3′; 21 nt; 62% GC; Tm 71°C

NANOG reverse: 5′-TCTGTTTCTTGACTGGGACCTTGTC-3′; 25 nt: 48%GC; Tm 69°C

Optimal Tm: 70°C

Oct-4 forward: 5′-TGGAGAAGGAGAAGCTGGAGCAAAA-3′; 25 nt: 48% GC; Tm 70°C

Oct4 reverse: 5′-GGCAGATGGTCGTTTGGCTGAATA-3′; 24 nt; 50% GC; Tm 70°C

Optimal Tm: 71°C

Sox2 forward: 5′-ATGGGCTCTGTGGTCAAGTC-3′; 20 nt: 55% GC; Tm 67°C

Sox2 reverse: 5′-CCCTCCCAATTCCCTTGTAT-5′; 20 nt; 50% GC; Tm 64°C

Optimal Tm: 65°C

Results

Several dental pulp stem cell (DPSC) isolates were successfully retrieved from cryopreservation storage, thawed and put into cell culture (Table 1). The growth rate or doubling time was verified, which was categorized as rapid doubling time or rDT, intermediate doubling time or iDT, and slow doubling time or sDT. Three DPSC isolates (dpsc - 3882, dpsc - 5653, dpsc - 7089) exhibited rapid doubling times between 1.9 and 2.6 days. Two DPSC isolates (dpsc - 9894, dpsc - 8694) exhibited intermediate doubling times between 5.1 and 5.5 days, with the remaining three isolates (dpsc - 11750, d-sc - 11418, dpsc - 11836) exhibiting slow doubling times between 10.2 and 13.1 days. Viability remained low averaging 29%, which ranged between 23% (dpsc - 7089) and 38% (dpsc - 11750).

Year	Cell line	Doubling Time	Growth rate	Viability
2012	dpsc-3882	2.6 days	rapid (rDT)	31%
2012	dpsc-5653	2.1 days	rapid (rDT)	31%
2012	dpsc-7089	1.9 days	rapid (rDT)	23%
2014	dpsc-9894	5.1 days	intermediate (iDT)	29%
2014	dpsc-8694	5.5 days	intermediate (iDT)	28%
2011	dpsc-11750	13.1 days	slow (sDT)	38%
2011	dpsc-11418	10.2 days	slow (sDT)	27%
2011	dpsc-11836	12.9 days	slow (sDT)	25%

Table 1: *Dental pulp stem cell (DPSC) isolates.*

RNA was subsequently extracted from all DPSC isolates placed into culture (Table 2). Analysis of the RNA concentrations from each DPSC isolate averaged 402.8 ng/uL and ranged between 377.1 and 422.1 ng/uL. Analysis of the A260:A280 ratio revealed an average of 1.72, which ranged between 1.70 and 1.74 and was within the acceptable range for cDNA synthesis reactions. All DPSC RNA extractions were converted to cDNA, which averaged 1079.2 ng/uL with a range between 1002.4 and 1211.3 ng/uL. Analysis of the A260:A280 ratio revealed an average of 1.82, which ranged between 1.80 and 1.84 and was within the acceptable range for qPCR screening.

Cell line	RNA concentration	A260:A280 ratio	cDNA concentration	A260:A280 ratio
dpsc-3882	377.1 ng/uL	1.71	1111.2 ng/uL	1.80
dpsc-5653	422.1 ng/uL	1.72	1002.4 ng/uL	1.82
dpsc-7089	411.3 ng/uL	1.70	1034.1 ng/uL	1.81
dpsc-9894	399.4 ng/uL	1.72	1211.3 ng/uL	1.84
dpsc-8694	400.8 ng/uL	1.71	1041.8 ng/uL	1.81
dpsc-11750	392.1 ng/uL	1.74	1122.5 ng/uL	1.80
dpsc-11418	404.2 ng/uL	1.72	1068.1 ng/uL	1.83
dpsc-11836	415.2 ng/uL	1.71	1042.2 ng/uL	1.82
	Average: 402.8 ng/uL Range: 377.1 - 422.1 ng/uL	**Average: 1.72 Range: 1.70 - 1.74**	**Average: 1079.2 ng/uL Range: 1002.4 - 1211.3 ng/uL**	**Average: 1.82 Range: 1.80 - 1.84**

Table 2: *RNA extraction, cDNA synthesis and analysis.*

Screening of all DPSC isolates was performed using validated qPCR primers (Figure 1). These data demonstrated that all DPSC isolates expressed the structural and metabolic positive controls, beta actin and GAPDH. This screening also confirmed all DPSC isolates expressed the ISCT stem cell markers CD73, CD90 and CD105 - although expression levels were variable among these isolates. None of the DPSC isolates expressed CD14, CD34 or CD45, also confirming the ISCT biomarker expression guidelines. In addition, the expression levels of NANOG, SOX2 and OCT4 were also detected in all DPSC isolates although expression appeared to be higher among the rDT isolates.

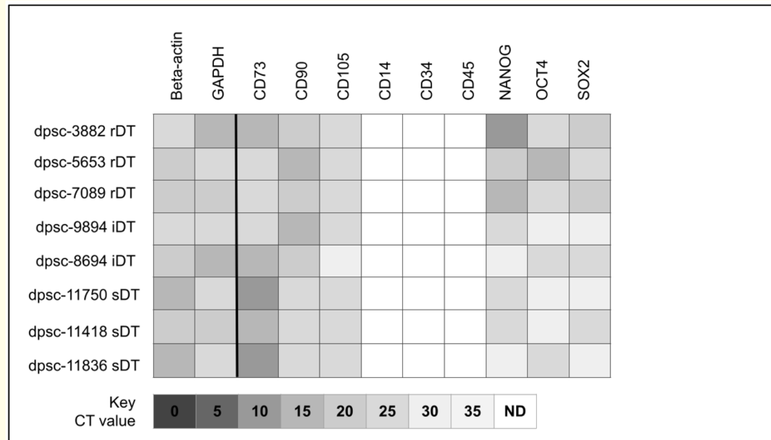

Figure 1: *qPCR screening of DPSC isolates. Confirmation of structural (beta actin) and metabolic (GAPDH) positive controls, as well as ISCT biomarker expression of CD73, CD90 and CD105 was observed. Negative results were confirmed for CD14, CD34 and CD45. MSC biomarkers NANOG, SOX2 and OCT4 exhibited variable expression among the DPSC isolates.*

To evaluate the effects of CAPE, DPSC were plated for three days in separate 96-well experiments with increasing concentrations ranging from 20 ug/mL to 100 ug/mL (Figure 2). These data demonstrated that all concentrations of CAPE increased DPSC growth compared to control (non-treated cells). More specifically, the growth of the rDT DPSC isolates increased by approximately 15% (dpsc - 3882: 15%, p = 0.041; dpsc - 5653: 14%, p = 0.044; dpsc - 7089: 16%, p = 0.039). In addition, the growth of the iDT DPSC isolates also increased significantly (dpsc - 9894: 24%, p = 0.028, dpsc - 8694: 15%, p = 0.040). Finally, significant increases in the growth of the sDT DPSC isolates was also observed (dpsc - 11750: 20%, p = 0.035; dpsc - 11418: 18%, p = 0.037; dpsc-11836: 14%, p = 0.043).

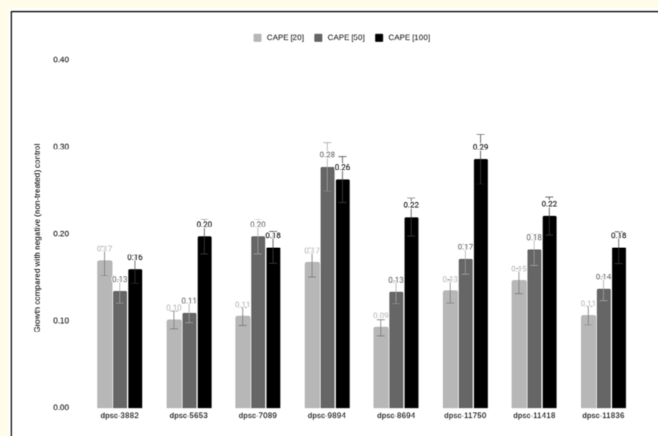

Figure 2: *Growth of DPSC isolates under CAPE administration. Growth of DPSC isolates under CAPE administration increased significantly compared with non-treated controls at 20, 50 and 100 ug/mL - but was not strictly dose-dependent. Growth of rDT isolates (range: 14 - 16%) was lower than iDT (range: 15 - 24%) or sDT (14 - 20%) isolates.*

To evaluate if the increase in DPSC growth was associated with other changes in DPSC phenotypes, viability under CAPE administration was also assessed (Figure 3). Analysis of these data revealed that viability increased among all DPSC isolates under CAPE administration. More specifically, viability among the rDT DPSC isolates increased between approximately 10% (dpsc - 3882: 9%, p = 0.048; dpsc-5653: 9%, p = 0.048; dpsc - 7089: 11%, p = 0.037). Viability among the iDT DPSC isolates also increased similarly (dpsc-9894: 8%, p = 0.031, dpsc-8694: 9%, p = 0.046). Furthermore, increases in the viability of the sDT DPSC isolates was also confirmed (dpsc-11750: 8%, p = 0.049; dpsc - 11418: 10%, p = 0.045; dpsc-11836: 12%, p = 0.04).

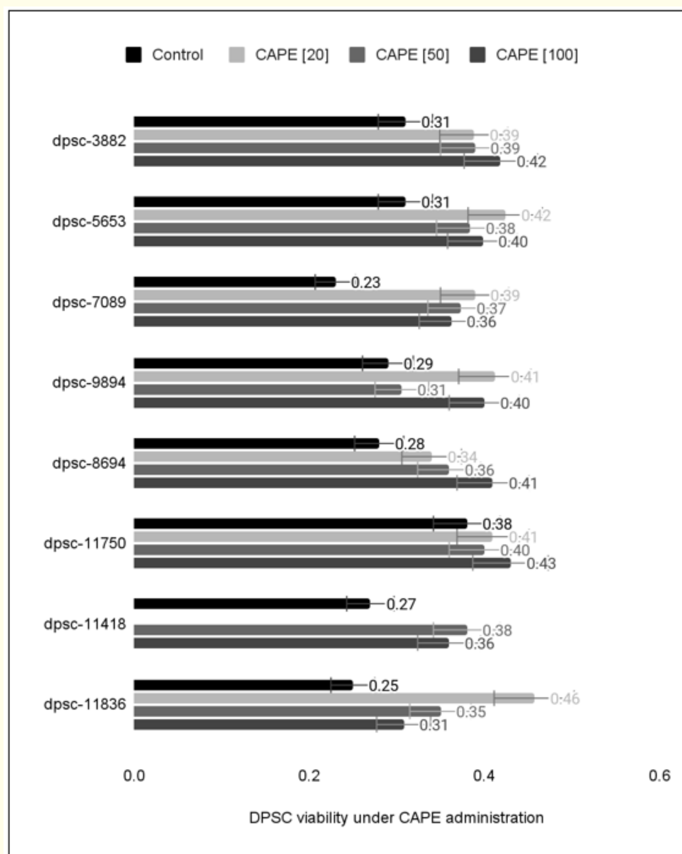

Figure 3: *Viability of DPSC isolates under CAPE administration. DPSC viability under CAPE administration increased compared with non-treated controls at 20, 50 and 100 ug/mL - but was not strictly dose-dependent. Viability of rDT isolates (range: 9-14%) was similar to iDT (range: 8-9%) and sDT (8-12%) isolates.*

To evaluate if CAPE induced any changes to DPSC biomarker expression, RNA extracted from each isolate was screened using qPCR (Figure 4). These data revealed that expression of MSC biomarkers NANOG, OCT4 and SOX2 were confirmed among all DPSC isolates under both control (non-treated) and experimental (CAPE) conditions. Although some variability in expression was noted among all DPSC isolates, reduced expression of NANOG was most notable among the sDT DPSc isolates (dpsc-11750, dpsc-11418, dpsc-11836).

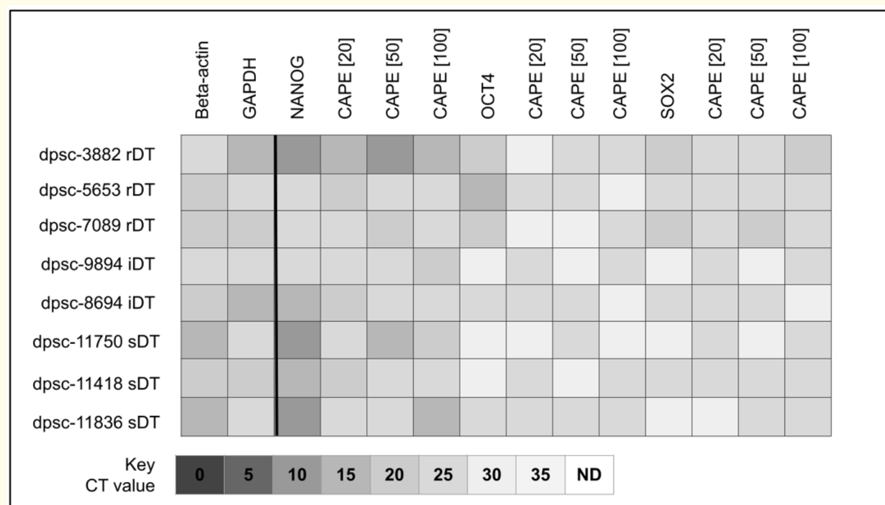

Figure 4: *DPSC biomarker expression under CAPE administration. RNA expression of MSC biomarkers NANOG, OCT4 and SOX2 were confirmed among all DPSC isolates under both control (non-treated) and experimental (CAPE) conditions with some variability noted with NANOG among the sDT DPSc isolates (dpsc-11750, dpsc-11418, dpsc-11836).*

Discussion

The primary goal of this study was to determine if there were any dental pulp-specific responses to CAPE administration. Although some non-human studies regarding CAPE and DPSC are available [19,20], this study may be the first to explore responses to CAPE in human DPSCs. These results demonstrated that CAPE administration is sufficient to increase growth as well as viability among DPSC, similar to other recent reports of increased viability induced by other natural compounds including quercetin (taxifolin) [27,28].

Although CAPE (or Propolis) is a bioactive molecule capable of inducing growth and modulating viability, it is notable that stem cell biomarkers and "stemness" were not adversely affected or significantly changed at any concentration evaluated - a possible side effect of changing culture conditions and long-term DPSC expansion [29,30]. The identification of biomolecules capable of modulating DPSC growth and viability without compromising multipotency is an important step for further development of this field towards translational applications [31], As more studies emerge to further the understanding of how to isolate and amplify DPSC for clinical use and regenerative medicine applications, any potential biomolecules that increase viability and growth may be particularly useful with DPSCs which exhibit heterogeneity in both proliferation rates and cell viability [32].

In addition to these findings, more evidence is accumulating that CAPE or Propolis supplementation is linked with a wide range of human health benefits [33,34]. These studies have revealed CAPE may function through direct or indirect modulation of specific transcription factors, including NFkB and JNK-associated pathways [35,36]. In fact, some evidence now suggests that NFkB-associated pathways may be modulated in DPSCs through administration of other bioactive natural supplements, such as Curcumin - although more research in this area will be needed as this has not been verified with CAPE or Propolis [37, 38]. As more clinical evidence accumulates that supports the use of CAPE or Propolis in root canal therapy, more research studies such as the current study will be needed to determine if these effects of modulating DPSC phenotypes, such as growth and viability are more broadly applicable [39-41].

Conclusions

This study is among the first to evaluate different types of DPSC isolates (rapidly and slowly dividing) and the effects of CAPE. Although only DPSC explants from UNLV-SDM were tested, these results strongly suggest that additional studies should be conducted on other DPSC isolates to validate and confirm the potential positive effects of CAPE on DPSC phenotypes.

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Volume 21 Issue 6 June 2022
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