

## REVIEW

# A critical review of *in vitro* research methodologies used to study mineralization in human dental pulp cell cultures

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## Abstract

**Background:** The pulp contains a resident population of stem cells which can be stimulated to differentiate in order to repair the tooth by generating a mineralized extracellular matrix. Over recent decades there has been considerable interest in utilizing *in vitro* cell culture models to study dentinogenesis, with the aim of developing regenerative endodontic procedures, particularly where some vital pulp tissue remains.

**Objectives:** The purpose of this review is to provide a structured oversight of *in vitro* research methodologies which have been used to study human pulp mineralization processes.

**Method:** The literature was screened in the PubMed database up to March 2021 to identify manuscripts reporting the use of human dental pulp cells to study mineralization. The dataset identified 343 publications initially which were further screened and consequently 166 studies were identified and it was methodologically mined for information on: i) study purpose, ii) source and characterization of cells, iii) mineralizing supplements and concentrations, and iv) assays and markers used to characterize mineralization and differentiation, and the data was used to write this narrative review.

**Results:** Most published studies aimed at characterizing new biological stimulants for mineralization as well as determining the effect of scaffolds and dental (bio) materials. In general, pulp cells were isolated by enzymatic digestion, although the pulp explant technique was also common. For enzymatic digestion, a range of enzymes and concentrations were utilized, although collagenase type I and dispase were the most frequent. Isolated cells were not routinely characterized using either fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) approaches and there was little consistency in terming cultures as dental pulp cells or dental pulp stem cells. A combination of media supplements, at a range of concentrations, of dexamethasone, ascorbic acid and beta-glycerophosphate, were frequently applied as the basis for the experimental conditions. Alizarin Red S

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(ARS) staining was the method of choice for assessment of mineralization at 21-days. Alkaline phosphatase assay was relatively frequently applied, solely or in combination with ARS staining. Further assessment of differentiation status was performed using transcript or protein markers, with dentine sialophosphoprotein (DSPP), osteocalcin and dentine matrix protein-1 (DMP -1), the most frequent.

**Discussion:** While this review highlights variability among experimental approaches, it does however identify a consensus experimental approach.

**Conclusion:** Standardization of experimental conditions and sustained research will significantly benefit endodontic patient outcomes in the future.

#### KEYWORDS

dental pulp cells, dental pulp stem cells, *in vitro* assays, mineralization, REPs

## INTRODUCTION

The dental pulp is a complex and specialized soft connective tissue surrounded by a hard tissue barrier of dentine. During the caries process, demineralization and destruction of dentine occurs as the biofilm develops and evolves due to the release of bacterial acids and their by-products. Subsequently, bacterial components stimulate cells of the dentine–pulp complex to invoke the hosts' defensive immune and inflammatory response (Cooper et al., 2011). With the progression of the infection unchecked, irreversible pulpitis develops. The pulp tissue becomes necrotic and is eventually destroyed due to a combination of virulence and degradative factors derived from the bacteria, along with molecules released during the host's inflammatory response (Cooper et al., 2017).

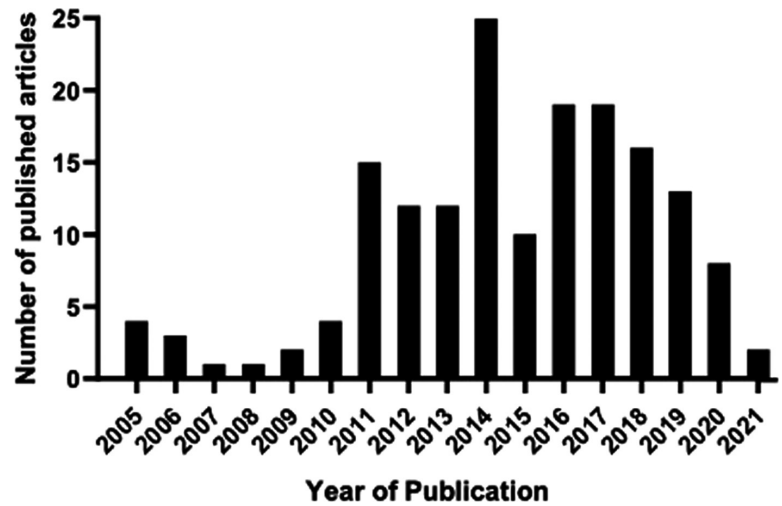
Traditionally, teeth with irreversible pulpitis and necrotic pulps require invasive root canal treatment (ESE, 2019). This approach is expensive and can result in the weakening of the remaining tooth structure, and this may ultimately cause tooth loss (Yang et al., 2016). Earlier intervention, before the pulp has become fully necrotic, offers opportunity for repair. Teeth with signs and symptoms indicative of reversible pulpitis still contain vital tissue and stem/progenitor cell populations and vital pulp therapies (VPTs), are therefore recommended (Arora et al., 2021; ESE, 2019). VPTs are included within regenerative endodontic procedures (REPs) targeted at salvaging the tissue and harnessing its natural capacity to repair hard and soft tissues. Clinical and animal studies have shown that following appropriate intervention and the generation of a conducive environment, vascularized pulp tissue can be revitalized, and a new hard tissue barrier can be formed (Nakashima et al., 2017; Zhu et al., 2018).

The pulp's natural repair and healing capacity are well documented with reactionary dentinogenesis occurring in response to milder tissue injury with surviving

odontoblasts secreting tertiary dentine. However, following more significant pulp injury from caries or trauma, reparative dentinogenesis is invoked if local environmental conditions are favourable. In this tissue healing scenario, the original developmentally derived odontoblasts are lost and a new population of odontoblast-like cells are stimulated to differentiate from stem/progenitor populations within the pulp (Simon et al., 2010). Reparative dentinogenesis is relatively more complex and involves several steps which include stem/progenitor cell recruitment, proliferation and subsequent differentiation to generate an odontoblast-like cell phenotype capable of synthesising and secreting a mineralised matrix. Notably, in reactionary dentinogenesis, the mineralized dentine formed has tubular continuity with the primary and secondary dentine, while in reparative dentinogenesis, osteodentine is formed which lacks this tubular integration. Both dentinogenic processes are shown to be regulated by archived growth factors released from the dentine either due to the disease or dental restorative process (Cooper et al., 2010).

Regenerative endodontic procedures represent an emerging clinical field and are defined as: '*biologically based procedures designed to replace damaged tooth structures, including dentine, root structures, as well as cells of the pulp–dentine complex*' (Murray et al., 2007). REPs focus on three major domains that form the basis of pulp regeneration/repair including: 1) a stem cell source which is capable of differentiating into odontoblast-like cells; 2) bioactive stimulants, such as growth factors, that can induce cell proliferation and differentiation and 3) a scaffold which supports cellular responses (AAE, 2013). For successful clinical induction of dentine–pulp complex healing, these components need to be integrated, along with the control of infection and inflammation (Cao et al., 2015). Consequently, to drive the field forward and develop optimal REPs there is a need to standardize and

**FIGURE 1** Frequency distribution of original research articles published between 2005 and 2021, retrieved from the PubMed database using the search term ‘human dental pulp cell culture mineralization’. The initial screened identified 343 publications which were then hand searched, as is described in the main text body, to identify relevant studies ( $n = 166$ )



adopt clinically relevant best research practices, including protocols which utilize a sound evidence-base.

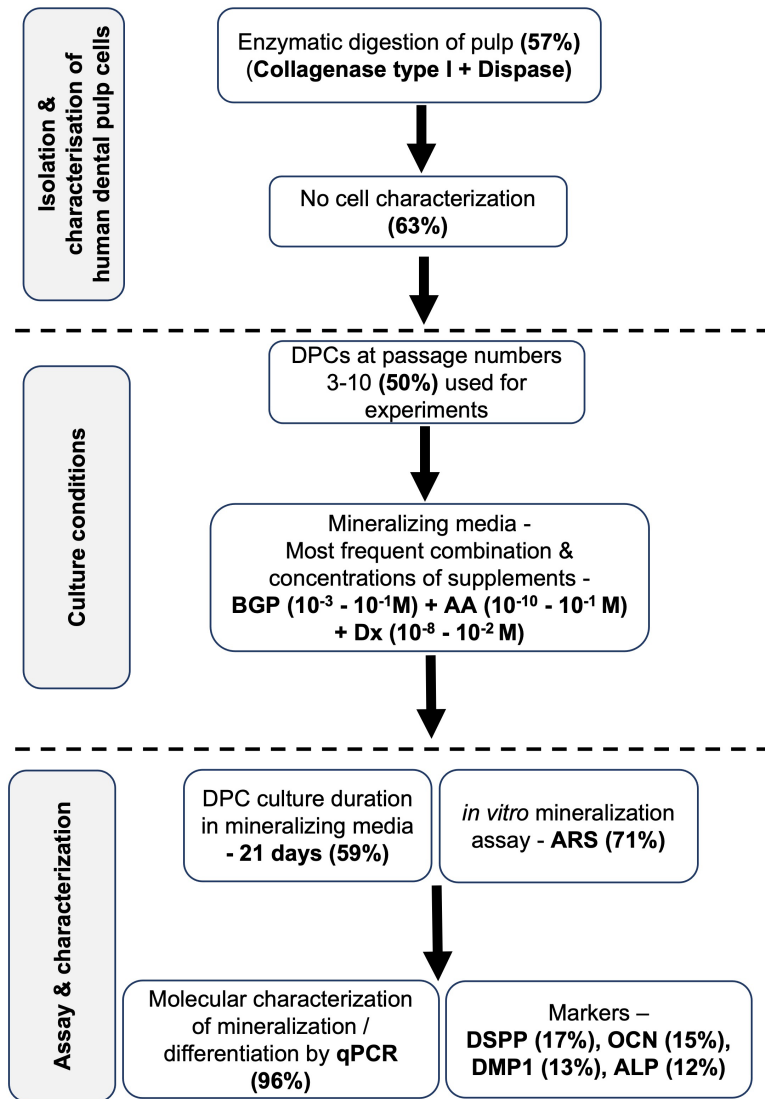
Preclinical studies utilizing *in vitro* and *in vivo* animal models for endodontic research are important for providing underpinning evidence for the development of new clinically effective techniques. It is therefore for the benefit of the field that laboratory studies are well planned and reported. A key component of contemporary basic scientific research utilizes relevant cell culture models. Two-dimensional (2D) cell culture systems utilize cell monolayers in culture dishes, while 3D culture models are more complex and generate more biomimetic cellular arrangements. This latter approach can be more technically challenging and incur increased expense. However, 3D culture systems reportedly offer advantages in being more representative of the *in vivo* environment allowing for more intricate interactions between multiple cell types as well as enabling more relevant tissue matrices to be replicated. Notably, the application of both approaches has contributed to the understanding of cellular and molecular processes which are relevant to oral health and disease. Within endodontics, these approaches have been used to characterize the modulation of mineralization processes occurring within the dental pulp (Diederichs et al., 2010; Pampaloni et al., 2007; Payr et al., 2021; Widbiller et al., 2016). Consequently, the purpose of this review is to provide a structured oversight of *in vitro* research methodologies which have been used to study human pulp mineralization processes.

To provide a framework for the review, a literature search was undertaken in the PubMed database (<https://pubmed.ncbi.nlm.nih.gov/>) up to March 2021 using the term ‘human dental pulp cell culture mineralization’. This approach initially identified 343 publications which were then further screened manually by reading abstracts and full texts to identify articles which were published in the English language only and involved laboratory studies of

human dental pulp cells (DPCs) cultured *in vitro* which assayed pulp mineralization. Articles that were reviews only, reports solely on animal studies, and/or that were published in languages other than English, were excluded. Consequently, 166 studies were identified, and their yearly distribution frequency over the time-period screened is shown in Figure 1. Interestingly, the data indicated an increasing trend in the number of yearly publications up to 2014, after which the number of articles published declined. Potentially, this initial upward trend may be linked to the first report on dental pulp stem cells (DPSCs) by Gronthos et al. (2000), as this may have stimulated an increase in the number of studies on mineralization *in vitro*. However, it is somewhat unclear as to why fewer studies appear to be reported in more recent years. Subsequently, due to the expansive nature of the field, to provide a more objective and representative overview the publication library was mined to identify the purposes of the *in vitro* mineralization studies, the approaches used to isolate and characterize the DPC populations, the mineralizing media and culture time-periods used, the types of mineralizing assay performed, and any other characterization techniques applied to identify the resultant cell phenotype. Based on this analysis and the evidence identified in the literature dataset, a flow chart of the most commonly used procedures (Figure 2) was generated. This methodology can now potentially serve as a consistent approach for future *in vitro* studies on human DPC mineralization assay. The literature review below reports on the outcomes identified from the analysis of the body of literature surveyed.

## PURPOSE OF PULP CELL *IN VITRO* MINERALIZATION STUDIES

The distribution of publications according to the purpose of mineralization research performed shows that the



**FIGURE 2** Flow chart as a guide for future *in vitro* studies on human dental pulp cells mineralization. The percentage values shown in the flow chart indicates the most frequently used procedures for human dental pulp cells isolation and assay of mineralization and differentiation. AA, ascorbic acid; ALP, alkaline phosphatase; BGP, beta glycerophosphate; DMP-1, dentine matrix protein-1; DPSC, dental pulp stem cells; DSPP, dentine sialo phosphoprotein; Dx, dexamethasone; OCN, osteocalcin.

proportion of articles identified are in line with the drive to identify new REPs which includes the determination of novel biological stimulants, including growth factors, transcription factors, plasma rich growth factors and enamel-derived proteins, in promoting mineralization, is a key area of research focus (Figure S1). In addition, a significant proportion of the studies have aimed at determining potential mineralizing effects of established or developmental dental biomaterials, particularly cements and tricalcium silicate-based materials. Ions and chemical compounds, including those containing calcium, zinc, strontium and magnesium, which can be released from different types of dental materials, were also investigated to determine concentrations at which they may have a beneficial mineralizing effect clinically. Drug repurposing, another clinically useful approach to identify compounds that may be effective at healing the dentine–pulp complex, was also explored. Examples of drugs investigated in the repurposing studies included simvastatin (Cassiano et al., 2020; Karanxha et al., 2013) and fluocinolone acetonide (Liu et al., 2013). Furthermore,

a relatively large number of studies explored the use of scaffolds such as hydrogels of chitosan and collagen, and compared different cell types, such as DPSCs, adipose tissue and bone marrow-derived stem cells, for their use in dentine–pulp complex tissue engineering (Jin et al., 2020; Kim et al., 2009; Vagropoulou et al., 2021; Wang et al., 2016). To enhance basic biological understanding of how the disease environment may influence mineralization, studies on culture density, 3D arrangements and disease relevant stimuli, for example, the role of the bacterial stimuli lipoteichoic acid and lipopolysaccharides, were also explored (Durand et al., 2006; Widbiller et al., 2018).

## ISOLATION AND CHARACTERIZATION OF DENTAL PULP CELL POPULATIONS

The application of DPCs is central to the study and development of pulp tissue engineering strategies for REPs

(Yen & Sharpe, 2008). It is well established that DPC populations contain multipotent mesenchymal stem cell (MSC) populations capable of differentiating down several lineages (Yasui et al., 2017). There remains a significant debate, however, as to what is the optimal approach for the isolation and characterization of DPSCs. The standardization of procedures would have considerable benefit for both the research and clinical communities and enable a more straightforward and robust comparison of studies (Ducret et al., 2015). Consequently, the manuscript dataset was screened to identify the laboratory procedures used prior to performing analyses of cellular *in vitro* mineralization. Data indicated that the most frequently used DPC isolation procedures were those of enzymatic digestion of dental pulp tissue and the dental pulp explant outgrowth approach (Figure S2A). Notably, while the enzymatic digestion approach ( $n = 94$ , 57%) was the most frequently used, compared with the explant approach ( $n = 44$ , 27%), the former approach was not well standardized and a variety of different enzymes, including collagenases type-I, -II, -IV, dispase, trypsin and accutase, as well as incubation time-periods were applied. It was however evident that a combination of collagenase type-1 and dispase was the most commonly used enzymatic cocktail (Figure S2B). Interestingly, it is likely that the use of different isolation procedures leads to variations in the phenotype and heterogeneity of the DPCs isolated (Bakopoulou et al., 2011; Huang et al., 2006). Indeed, some researchers have reported that DPCs isolated using the enzymatic approach exhibit higher proliferation rates compared with ones isolated using the explant method, while others have reported the opposite (Bakopoulou et al., 2011; Huang et al., 2006; Karamzadeh et al., 2012; Spath et al., 2010; Takeda-Kawaguchi et al., 2014).

Additional inconsistencies in this research field, in particular including the terminology used to describe the isolated cells or their origin, were also apparent. Seventy-seven articles (46%), which used either of the isolation approaches (enzymatic or outgrowth), referred to the cells obtained as DPSCs while 51 papers (31%), referred to them as DPCs. Furthermore, in eight publications (5%) the DPCs used in the mineralization assays were identified as being a 'kind gift' from another researcher/laboratory, nine publications (5%) reported using commercial cell lines and five publications (3%) used immortalized cell lines. No information was provided as to how pulp cells were obtained in five articles (3%). The passage number of the cells used also constitutes another key variable which is a significant factor in how primary cells behave in culture and therefore may influence experimental outcomes (Patel et al., 2009). Screening of the library revealed that studies used several different passage ranges for their studies. Within the 166 publications, 50% reported using

cells specifically at passages 3–10, 23% of studies used cells at passages 4–8, 13% used cells at passages 2–8, 11% of studies used cells at passages 5–10, 2% of studies used cells at passages 6–9 and in one study cells were solely used in passage 8. Notably, 30% studies did not report the passage number used. In manuscripts which reported the use of commercially sourced pulp cells the original isolation procedure and passage number were not identifiable.

Isolated DPC populations are generally considered heterogeneous, however, DPSCs reportedly express cell markers, such as CD44, CD73, CD90 and CD105, whilst not expressing CD14, CD34, CD45, CD79a and HLA-DR (Dominici et al., 2006; Huang et al., 2009). In recent decades, a range of techniques including immunocytochemistry/immunofluorescence (ICC/IF) and flow cytometry have been used to characterize DPC surface markers. Quantitative real-time polymerized chain reaction (qPCR) can also be used to characterize DPC populations, although this was not reported as a method of choice in the current dataset. Out of the 166 studies analysed, 28% papers were identified which characterized the isolated cells using fluorescence-activated cell sorting (FACS)/magnetic-activated cell sorting (MACS)/ICC, and reported them as DPSCs, while 7% of studies which used the same characterization techniques, referred to the isolated cells as DPCs (Table 1). Interestingly, three studies which undertook stem cell marker characterization termed their isolated population as either: dental pulp pluripotent-like stem cells (DPPSC), dental pulp mesenchymal stem cells (DPMSC) or dental pulp derived mesenchymal stem cells (DPMSC) respectively (Hasturk et al., 2019; Kawashima et al., 2017; Núñez-Toldrà et al., 2017). A relatively high proportion of studies (63%) did not report undertaking any characterization of the isolated cells although they still subsequently used a range of classification terms for their cell populations, including DPCs (39%), DPSCs (24%) or dental pulp stromal cells (1%) (Table 1).

Notably, to obtain homogenous populations of DPSCs, FACS and MACS have been used. These techniques provide a relatively small number of purified cells (<0.1%) which can make further downstream analyses challenging (Kawashima et al., 2017). The present data indicated that this approach was rarely performed as only 4% papers reported using FACS and MACS.

## CULTURE SUPPLEMENTATIONS AND ASSAYS FOR MINERALIZATION AND DIFFERENTIATION

To enable mineralization in cell culture, media such as Dulbecco's modified Eagle medium or minimum

Characterization and cell type reported as	n (%)
Cells characterised using FACS/MACS/ICC and reported as:	46 (28%)
DPSCs	12 (7%)
DPCs	
Cells characterized using FACS/MACS/ICC: and reported under different terminologies:	3 (2%)
i) Dental pulp pluripotent-like stem cells (DPPSC)	
ii) Dental pulp mesenchymal stem cells (DPMSC)	
iii) Dental pulp derived mesenchymal stem cells (DPMSC)	
No characterization: reported as DPCs	64 (39%)
No characterization: reported as DPSCs	40 (24%)
No characterization: reported under different terminology:	1(1%)
i) Dental pulp stromal cells	

Note: several publications applied multiple methods of characterization.

Abbreviations: FACS, fluorescence-activated cell sorting; ICC, immunocytochemistry, MACS, magnetic-activated cell sorting.

essential medium Eagle, alpha modification generally containing foetal calf serum or foetal bovine serum, are supplemented with  $\beta$ -glycerophosphate (BGP), ascorbic acid (AA) and dexamethasone (DX) (Digirolamo et al., 1999). The phosphate supplementation provided using BGP enables the chemical reaction for hydroxyapatite formation and DX steroid stimulates cell differentiation towards a mineralizing lineage via activation of Wnt/beta-catenin signalling pathway in this context. AA is a supplement necessary for facilitating the synthesis and secretion of collagen type I which subsequently provides the scaffold for the mineralized extracellular matrix (Hoemann et al., 2009; Langenbach & Handschel, 2013; Schäck et al., 2013). Different terminologies were identified within the dataset to refer to this supplemented media including: osteoinductive medium (Takizawa et al., 2019), osteogenic differentiation media (Okajcekova et al., 2020), osteo-odontoblastic culture medium (Mucuk et al., 2017), odontoblast differentiation medium (Itoh et al., 2018), odonto-induction medium (Kulan et al., 2018) and mineralization promoting media (Liu et al., 2007). Generally, it is within this basic background of BGP, AA and DX supplementation that experimental bioactives, stimulants and materials are assayed to determine their ability to further promote the mineralization process. For consistency, the relatively broad and encompassing term '*mineralizing media*' has been used.

The literature screen identified 10 different combinations for supplemented mineralizing media (Figure S3). The most commonly used combination included all supplements of BGP, AA and DX at a range of concentrations

**TABLE 1** The number (n) and % of total (166) of publications which undertook characterization of isolated dental pulp cells and how the cells were subsequently reported

**TABLE 2** Concentration ranges of supplements and reporting frequencies used in mineralizing media

Supplements	Concentration range (M)	Publications (n, %)
Beta-glycerophosphate	$10^{-3}$ – $10^{-1}$	101 (91%)
Ascorbic acid	$10^{-10}$ – $10^{-1}$	106 (97%)
Dexamethasone	$10^{-8}$ – $10^{-2}$	93 (85%)

Note: A range of units were reported within publications [number (n)], to enable comparison these have been converted to the same unit (M).

in 75% of papers (Table 2). The concentration ranges frequently added to the media for BGP, AA and DX were  $10^{-3}$ – $10^{-1}$  M (91%),  $10^{-10}$ – $10^{-1}$  M (97%) and  $10^{-8}$ – $10^{-2}$  M (85%) respectively. However, additional combinations and concentrations of these components were also reported (Figure S3). Interestingly, the commercially available mineralizing media, STEMPRO, was reportedly used in 5% of studies, however, no compositional details of this supplement were provided.

An additional variable for the study of mineralization in human DPCs is the duration of culture endpoints used for its assessment. Frequently cells were maintained in mineralizing media for 21 days (59%) prior to analyses. However, some studies performed analyses at earlier and later time-points with a high degree of variability reported (Figure S4). Selection of the analysis period is assay dependent and cellular mineralization processes can be determined using several experimental approaches, such as Von Kossa (VK) stain, Alizarin red S (ARS) stain and alkaline phosphatase (ALP) activity (Serguienko et al., 2018). Both

**TABLE 3** Number (*n*) and percentage of publications that used additional phenotypic characterization techniques to study pulp cell mineralization/differentiation

Technique	Gene expression analysis			Protein expression analysis			
	qPCR	Semi-quantitative PCR	Microarray	Western blot	IHC/ICC/IF	ELISA	FACS
<i>n</i> =	116	3	2	25	15	12	2
%=	96%	2%	2%	46%	28%	22%	4%

Note: several manuscripts applied more than one molecular characterization technique. Abbreviations: FACS, fluorescence-activated cell sorting; ICC, immuno-cytochemistry; IF, immune fluorescence; IHC, immune histochemistry; qPCR, quantitative polymerized chain reaction.

ARS and VK assays enable the visualisation and quantification of mineralized deposits. VK utilizes silver nitrate to stain mineralized deposits dark brown to black. This silver reduction reaction is precipitated by the binding of silver ions to anions, such as phosphates, sulphates, or carbonates, within the calcium salts. Conversely, ARS reacts with calcium cations to form a chelate and imparts red colouration to the calcium deposits (Wang et al., 2006). ALP activity is considered a marker of osteoblast differentiation and its assessment allows evaluation of the biological activity of cells towards a mineralizing phenotype (Macri-Pellizzeri et al., 2018). Since ALP is secreted in the early stages of differentiation ALP activity is generally assessed at relatively early phases *in vitro*. This is in agreement with the screened data which indicated that 41% studies have utilized ALP assay within 2 weeks of osteogenic induction in culture. The current observation also supports previous reports indicating that ARS staining (71% of studies) is the most frequently used assay, however, in 37% of the studies a combination of both ARS and ALP was applied (Figure S5). This finding most likely relates to the utility of the ARS assay as it is relatively straightforward to undertake, inexpensive, quantifiable and reproducible. Interestingly, the ALP assay was utilized relatively more frequently compared with any other assay, including that of VK staining (Figure S5).

The variability in the mineralization assays and experimental design makes comparison between studies difficult. Furthermore, it is speculated that different inductive culture condition requirements may be required due to the different initial progenitor populations used, that is, outgrowth or enzymatic digestion isolation. Consequently, this range of supplementations and associated concentrations may result in masking or enhancing the effects of the experimental mineralizing agents/conditions under investigation and this, may impact on overall clinical relevance. Indeed, it has been reported that if supplements are used in excess, then dystrophic mineralization can occur (Orriss et al., 2007). In addition, while it is apparent that ARS staining at 21 days of culture is the method of choice, this time-point also represents a significant variable. Future studies which enable determining

the longitudinal physiological relevance of *in vitro* culture conditions would be of benefit to the field and would facilitate the standardization of protocols to enable translational outcomes.

To complement the mineralization analyses and better understand the underpinning cellular and molecular differentiation processes, techniques such as PCR, ELISA, IHC/ICC/IF, and Western blotting can be performed to assess a range of different markers associated with osteogenic differentiation. In the literature sample, qPCR (96%) was the most frequently applied characterization technique used to assay transcript level changes associated with mineralization (Table 3). The high usage of qPCR is most likely due to well characterized assays being commercially available which can be used to robustly quantify multiple transcript levels in RNA derived from control and test samples. Consequently, this technique is a relatively cost-effective characterization approach. For quantitative protein expression analyses, Western blotting (46%), was the analytical method of choice, while ELISA was also relatively frequently applied (Table 3). Cytochemical approaches, such as IHC, ICC and IF, enable more qualitative assessment of protein marker expression and can be used in conjunction with quantitative approaches. Notably, there was good correlation between the relative frequency of the markers assayed at transcript and protein level (Table 4). Unsurprisingly, dentine sialophosphoprotein (DSPP) was the most frequently assayed marker as increased levels are associated with dentinogenesis and hence it is regarded as a robust marker of the mineralisation and differentiation process (Wan et al., 2016).

Similarly, increased dental matrix protein 1 (DMP1) expression is also regarded as being indicative of dentinal tissue differentiation however many of the other markers reported (Table 4) are associated with a more generalised mineralizing phenotype, that is, osteocalcin (OCN), ALP, collagen Type I Alpha 1 (COL1A1), osteopontin (OPN) and bone sialoprotein (BSP). Arguably, the application of a panel of markers and different complementary gene and protein analytical approaches will provide a more thorough and robust characterization of the mineralizing and

**TABLE 4** Frequency of gene/transcript and protein markers used as a complementary approach to assay mineralization and differentiation in pulp cell cultures

Marker	Gene n (%)	Protein
DSPP/DSP	77 (17%)	19 (27%)
OCN	68 (15%)	10 (14%)
DMP1	58 (13%)	11 (16%)
ALP	56 (13%)	6 (9%)
RUNX2	45 (10%)	4 (6%)
COL1A1	39 (9%)	6 (9%)
OPN	26 (6%)	6 (9%)
BSP	20 (5%)	3 (4%)
OSTERIX	14 (3%)	
BMP-1 & -2	14 (3%)	3 (4%)
ON	9 (2%)	2 (3%)
COL1A2	4 (1%)	
NESTIN	3 (1%)	
MEPE	3 (1%)	
IBSP	3 (1%)	
WNT-4 & -6	2 (0.5%)	
MMP-13 & -20	2 (0.5%)	
CBFA	1 (0.2%)	

Abbreviations: ALP, alkaline phosphatase; BMP-1 & -2, bone morphogenic protein-1 & -2; BSP, bone sialoprotein; CBFA, core-binding factor subunit alpha-1; COL1A1 & COL1A2, collagen type I alpha 1 and collagen type II alpha 2; DMP-1, dentine matrix protein-1; DSPP, dentine sialophosphoprotein; IBSP, Integrin binding sialoprotein; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinase; OCN, osteocalcin; ON, osteonectin; OPN, osteopontin; RUNX2, RUNX family transcription Factor 2; WNT, Wntless-related integration site.

differentiated cell phenotype. Interestingly, two studies were identified which utilized microarray analysis (Table 3). This high-throughput analytical process has the potential to provide added value compared with the more standard candidate marker approaches as it may identify novel pathways and markers involved in the mineralization and differentiation processes.

## CONCLUSIONS

To develop novel REPs, *in vitro* culture models provide an excellent experimental starting point from which new lead compounds, materials, hypotheses and clinical techniques can be tested and subsequently developed. This narrative review has not only highlighted the breadth of work which is being undertaken globally but also the variability in research approaches being used to study mineralization in human DPCs over recent decades. As

indicated previously, Figure 2 can potentially be used as a guide to study *in vitro* mineralization using DPCs by the researchers working in the field. It is appreciated, however, that other techniques and approaches could be used dependent on the expertise and resources available in individual research laboratories around the world. Ultimately, this guide may enable a more standardized approach and comparison between different future studies if core technologies and reporting terminologies are used.

## LIMITATIONS

To study future translation in REPs, the application of pre-clinical animal models to determine whether future human clinical trials are justifiable. Interestingly, within the literature dataset, 15% of the papers presented data using animal models, with 75% of those reporting positive correlation between *in vitro* and *in vivo* outcomes. These data are encouraging as they indicate that mineralization assays in culture can predict *in vivo* outcomes, albeit at the animal model stage only. The application of 3D cell arrangements and analyses which utilize hanging drop cultures, organoid, organotypic and 3D-printed cell culture techniques, may provide more relevance to predict mineralization processes within the dentine–pulp complex and facilitate translation. Interestingly, relatively few studies in the literature screen utilized these approaches and this may reflect the increased cost, time and technical challenges associated with them. Furthermore, it is notable that relatively few studies utilized high-throughput expression analyses, for example, microarrays, RNA sequencing methodologies, to characterize transcriptional changes which occur during mineralization in culture. It is expected that as these technologies become more accessible to researchers then temporal gene expression profiling will provide a greater insight into regulatory events which occur during the mineralization process, thereby identifying new opportunities for the development of future therapies.

It is apparent that more comprehensive analyses which enable determination of the most appropriate combinations of methodologies and analyses will enable better standardization of processes. Furthermore, it is acknowledged that it is not possible to exhaustively cover all areas of research related to this topic, and the application of many other variables could be explored, for example, the use of animal derived DPCs. However, the information provided indicate that better standardization of experimental approaches along with sustained research will ultimately lead to significant developments and benefits to the field of endodontics.



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## CONFLICT OF INTEREST

The authors have no conflicts of interests with this article.

## ETHICAL APPROVAL

Since it is a narrative review and it does not involve the use of human or animal subjects, ethical approval is not required.

## AUTHOR CONTRIBUTIONS

Shelly Arora and Paul R. Cooper performed literature screening, data extraction and wrote the manuscript. Shakila B. Rizwan and Haizal M. Hussaini prepared the figures, provided the expert comments and edited the manuscript. Lara T. Friedlander, Jithendra T. Ratnayake and Benedict Seo provided the expert comments and edited the manuscript. All the authors contribute to the manuscript revision and read and approved the submitted version, which was completed by Shelly Arora.

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