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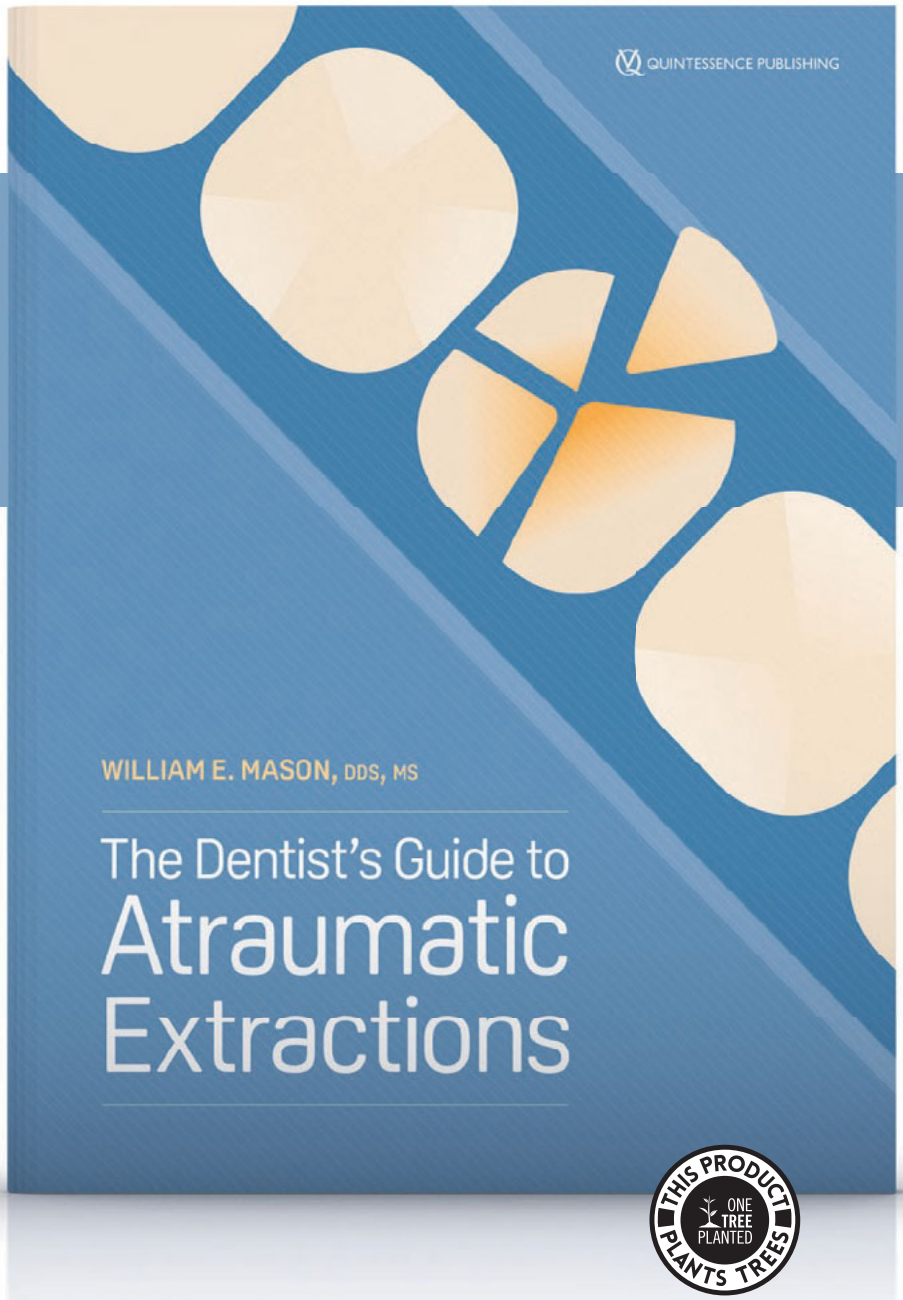
METHODS, TOOLS, AND TIPS



William E. Mason

The Dentist's Guide to Atraumatic Extractions

144 pages, 467 illus.
ISBN 978-1-64724-139-1
€88

Traditional methods of tooth extraction involve raising flaps and luxating the tooth to force it out of the bone. This often results in significant trauma and ridge resorption, which can compromise future implant placement. In this book, the author details his atraumatic extraction technique, which relies on systematic removal of root structure to protect the socket from deformation and thereby maintain the patient's original jaw anatomy and facial structure. With the root space creation extraction technique (RSCET), root structure is removed to allow space for the remaining tooth structure to be maneuvered and manipulated instead of pushed into the adjacent bony walls, thereby protecting the bone from trauma and resportion. Every tooth in the mouth can be removed atraumatically, and this book shows every step of the process and the tools required, including tips on atraumatic anesthesia.

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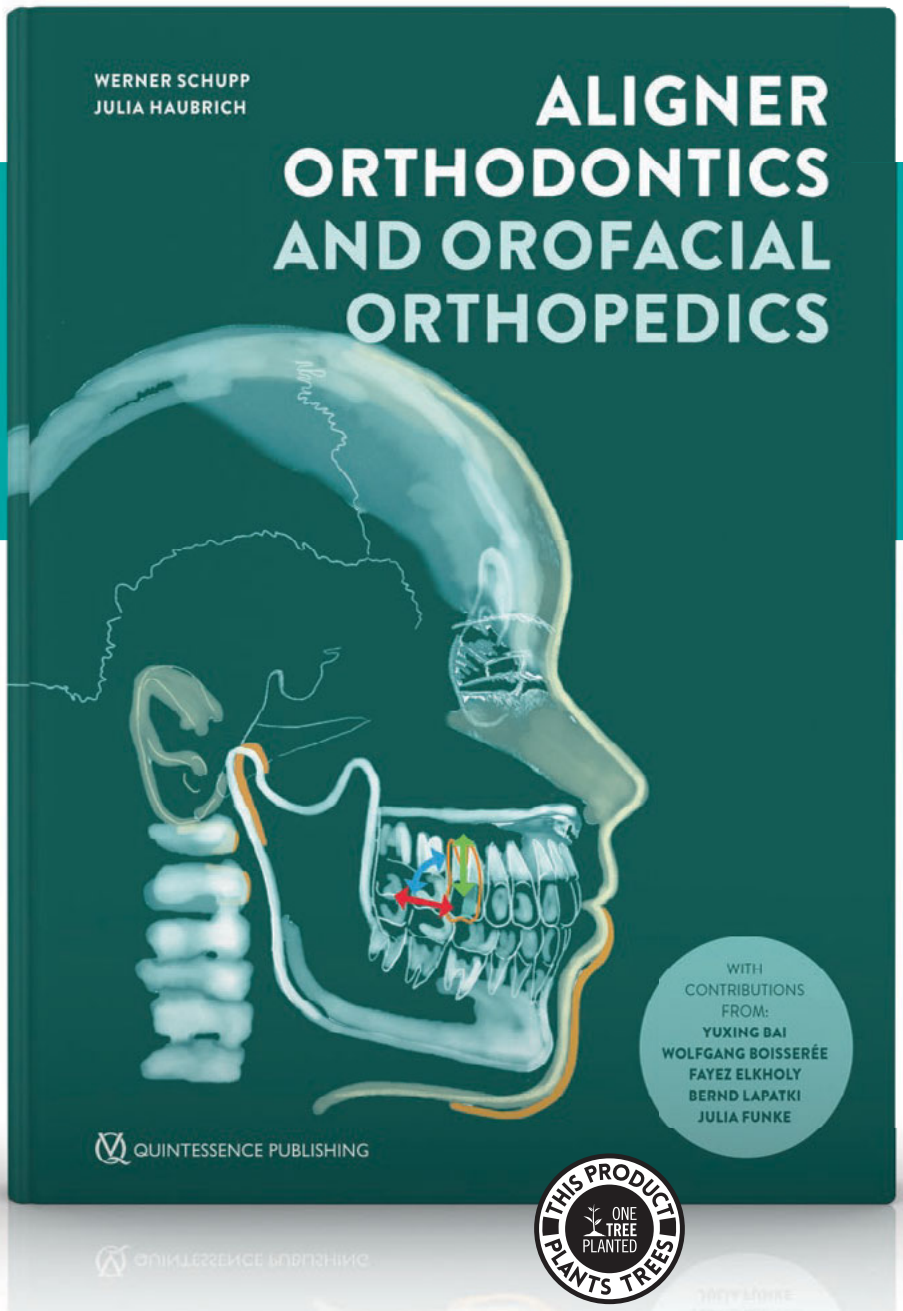

Werner Schupp | Julia Haubrich

Aligner Orthodontics and Orofacial Orthopedics

2nd Edition, 672 pages, 3,500 illus
ISBN 978-1-78698-106-6
€248

This book presents useful tips and strategies on how to integrate aligner orthodontics successfully into clinical practice, whether outsourced or with in-office aligner treatment. This second edition sees the authors review the diagnostic protocols and the biomechanics of aligners before presenting aligner orthodontics protocols. Supported by accompanying case documentation, the discussion of each malocclusion includes information on the associated symptoms, the rationale behind the selected treatment approaches, and the various outcomes achieved. The separation into sections on each malocclusion helps patients and clinicians in deciding whether this system can provide optimal treatment outcomes for a particular clinical situation. This is a practical manual for any clinician interested in the treatment modality of aligner orthodontics.

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Role of Dental Pulp Stem Cells to Promote Angiogenesis in Cell-based Regenerative Endodontics

Meng Qi TANG[1], Ling YE[1], Bo GAO[1]

Maintaining the viability and avoiding necrosis of dental pulp are crucial to preserving the structural integrity and functioning of teeth. In recent years, cell-based regenerative endodontics has emerged as a promising approach to achieve this goal and has gained increasing attention in scientific research; however, in the confined space of the root canal system, hypoxic conditions can be both beneficial and detrimental, as they may promote angiogenesis in the graft to some extent but also lead to tissue necrosis if prolonged. Dental pulp stem cells (DPSCs) have been verified as multipotent cells that can promote angiogenesis and are therefore ideal candidates for realising real dental pulp regeneration within root canals. Thus, we focus on the underlying mechanisms of DPSCs to promote angiogenesis and summarise some preclinical studies and clinical trials involving transplanting of DPSCs to achieve real dental pulp regeneration, in the hope that this intractable source of perplexity in regenerative endodontics may be resolved sooner.

Key words: *angiogenesis, dental pulp stem cell, pulp regeneration, pulp repair, regenerative endodontics*

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Regenerative endodontics refers to a collection of "biologically based procedures designed to replace damaged tooth structures, including dentine and root structures, as well as cells of the pulp–dentine complex"[1]. Its only officially approved protocol in the clinic as yet, revascularisation, also known as revitalisation, serves to restore the vitality of pulp and promote root maturation of necrotic immature permanent teeth[2-4]. Based on the principles of tissue engineering, this procedure requires high levels of irrigation rather than mechanical debridement to disinfect root canals and induce blood clot formation with sterile files, providing a favourable environment that contains various types of cells and growth factors for pulp regeneration[5,6].

The ultimate goal of regenerative endodontics is to achieve dental pulp regeneration, which aims to reconstruct complete vital pulp with both structural and functional tissue in situ[5,7]; however, a series of histological assessments of revascularisation in animal and human studies revealed that the new tissues regenerated in the root canals included cementum-, bone- and periodontal ligament–like tissues rather than the ideal formation of pulp–dentine complex[8-10]. This can be explained by the unpredictable composition or concentration of those trapped cells and growth factors in blood clots, leading to unforeseen tissue deposition[11] and even root canal obturation in long-term follow-ups[12]. Since the cells induced in the blood clots are unpredictable, revascularisation may result in uncontrollable outcomes in the process of pulp regeneration. Therefore, real pulp regeneration can be achieved if more controllable pulp stem cells are present in the root canals.

As the first dental-derived mesenchymal stem cells (MSCs) to be isolated and identified in 2000[13], dental pulp stem cells (DPSCs) possess the ability for self-renewal and multilineage differentiation as well as neurovascular properties, and can be seen as an excel-

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lent stem cell source for transplantation in cell-based regenerative endodontics[14]. Although only a limited number of animal studies and clinical trials have been conducted on this, this promising protocol shows great potential in real pulp regeneration and function restoration[15,16].

Angiogenesis refers to the formation of new blood vessels from the pre-existing ones and serves as the major mechanism whereby most tissues become vascularised during later embryonic development and adult life[17]. Timely angiogenesis for nutrient delivery and waste removal is believed to be crucial for cell survival in tissue engineering; otherwise, most tissue will undergo malnutrition under hypoxia[18]. Although hypoxia itself can promote angiogenesis of the graft, its speed is too limited to support the metabolism of the whole tissue[19]. Consequently, the inability to construct a sufficient blood supply promptly after cell transplantation can make it highly likely that pulp regeneration will fail.

Considering the neovascular property of DPSCs, we focus on the underlying mechanisms of DPSCs to promote angiogenesis and summarise some preclinical studies and clinical trials on transplanting DPSCs to achieve real dental pulp regeneration in the hope that this intractable source of perplexity in regenerative endodontics can be resolved sooner.

Angiogenesis: the essence of regenerative endodontics

Dental pulp is a highly vascularised tissue that supports all the other components within it and maintains the normal function of teeth[20]. If it is not promptly treated, contaminated pulp tissue can easily develop from reversible to irreversible pulpitis and even end in necrosis[21]. Consequently, pulpectomy must be performed as a last resort to preserve natural teeth but will lead to the loss of vital pulp and susceptibility to traumatic fracture and bacterial infection of teeth[22].

Cell-based regenerative endodontics can be an ideal alternative to pulpectomy that has great potential to overcome the aforementioned disadvantages[15]; however, due to being encased by rigid dentine and the lack of a collateral blood supply, transplanted stem cells are desperate for spontaneous angiogenesis to avoid unexpected cell distribution and differentiation in a nutrient and oxygen gradient environment[23]. It is universally accepted that low ambient oxygen concentration cannot maintain the normal metabolism of tissues as the oxygen diffusion distance is limited to 200 μm between the capillary wall and cell membrane[18]; however, hypoxia itself can be the environmental factor that conduces angiogenesis to some extent.

Hypoxia-inducible factor 1 (HIF-1), a transcription factor expressed under hypoxic conditions by almost all kinds of cells in mammals, consists of two subunits including HIF-1α and HIF-1β[24] and acts as a regulatory factor to oxygen homeostasis. The hypoxia response elements binding with HIF-1 can help identify target genes for HIF-1 to activate downstream[25]. Subsequently, HIF-1 enhances the transcription of multiple angiogenic growth factors such as vascular endothelial growth factor (VEGF), angiopoietin1 (Ang1), placental growth factor (PIGF), platelet-derived growth factor (PDGF) and angiogenic chemokines such as stromal cell–derived factor 1 (SDF-1), sphingosine-1-phosphate (S1P) and their receptors[26-28]. Those factors can either facilitate the differentiation of stem cells into endothelial cells (ECs) or recruit endothelial progenitor cells to promote angiogenesis[27]. Moreover, the migration speed of ECs subjected to hypoxia will be increased by reducing the expression of vascular endothelial-cadherin (VE-cadherin)[29]. As for DPSCs, hypoxia can enhance the expression of HIF-1[30,31] and activate the secretion of VEGF which is the primary transcriptional target for HIF-1 downstream[31]. Besides, increased expression of SDF-1 by overexpressing HIF-1α showed great proangiogenic characteristics of DPSCs in hypoxic ambience[32]. Han et al[33] demonstrated that preconditioned stem cells from human exfoliated primary teeth (SHED) by HIF-1 stabilisation showed positive results of a higher level of angiogenesis in the newly formed pulp-like tissue.

Despite the desirable effect of hypoxia on angiogenesis in the early stages, it cannot always exert a positive effect on it. An extremely low oxygen concentration below 1% and excessive accumulation of HIF-1 will both curb the collective migration velocity of ECs[34]. Thus, relying on hypoxia alone to promote angiogenesis may not be reliable, and the contribution of DPSCs to expedite this process needs to be illustrated in detail to offer a new perspective for desirable outcomes.

Proangiogenic property of DPSCs: residing in perivascular niches

DPSCs are derived from the cranial neural crest that forms the dental mesenchyme during early embryonic head development. This origin confers DPSCs with typical MSC properties, such as self-renewal, clonogenicity and multi-lineage differentiation[35,36], implying its proangiogenic potential that is worthy of investigation.

In addition, the proangiogenic property of DPSCs can be attributed to their local microenvironment in

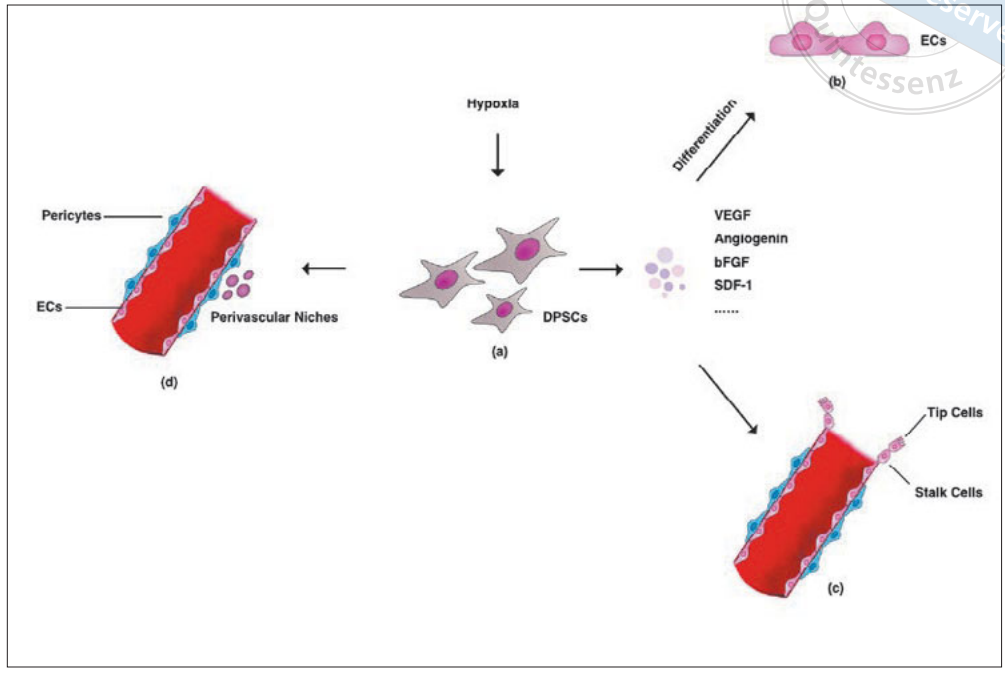

Fig 1 Summary of the underlying mechanisms for DPSCs to promote angiogenesis. **(a)** DPSCs secrete multiple proangiogenic factors in paracrine under physiological or hypoxic conditions. **(b)** These secreted proangiogenic factors contribute in turn to the differentiation of DPSCs into ECs. **(c)** The secreted proangiogenic factors can modulate the proliferation and migration of specialised ECs, such as tip and stalk cells, to promote angiogenesis in vivo. **(d)** DPSCs adapt to reside in perivascular niches, which can stabilise the neovasculature by acting like pericytes. Figure produced using Affinity Designer (Serif, West Bridgford, UK).

the pulp. Niches refer to the specific microenvironment in which stem cells are located. The concept of the perivascular niche was proposed initially for hematopoietic stem cells in bone marrow, where they reside near the vasculature[37]. This structure has been verified to exist in multiple human organs[38]. In a study by Shi et al[39], STRO-1, as an early marker present on different MSCs, was used to retrieve DPSCs from pulp tissue. This antigen was also found on dental pulp microvasculature, inferring the existence of perivascular niches for DPSCs in situ. Aldehyde dehydrogenase 1 (ALDH-1) is another marker associated with the stemness of cells. Both Machado et al[40] and Oh et al[41] demonstrated that DPSCs with high ALDH-1 were preferentially located proximally to vasculatures, indicating the presence of perivascular niches. Oh et al[41] also demonstrated that EC-derived interleukin-6 (IL-6) could induce the asymmetric division of DPSCs into either endothelial cell differentiation or an undifferentiated state and thus maintain both the perivascular niches and the multipotency of DPSCs. Consequently, the origin and locality of DPSCs reveal the close relationship between DPSCs and vasculatures in pulp and imply their proangiogenic property. Indeed, a study provided evidence to elucidate the mechanisms of DPSC-mediated angiogenesis, which mainly involve secreting proangiogenic factors or differentiating into ECs to form vasculature[7]. These mechanisms will be elaborated on in detail later in this article.

In addition, intriguingly, the neural origin of DPSCs from peripheral nerve–associated glia has been proven[42], which shows that DPSCs have a dual origin. The neurogenic potential[43] and the odontogenic and osteogenic ability[44] of DPSCs are generally accepted. Thus, given the easy availability of DPSCs from nonfunctioning third molars, all the factors above suggest this kind of cell is an optimal candidate for cell-based regenerative endodontics for the benefit of both angiogenesis and dental pulp regeneration.

Underlying mechanisms through which DPSCs promote angiogenesis

Angiogenesis is a complex process that can be divided into several phases: secretion of angiogenic factors as a response to physiological or pathological stimuli; degradation of the basement membrane and extracellular matrix with the purpose of allowing EC migration; migration and proliferation of ECs to form tubular structures; and maturation or stabilisation of vasculatures[45]. Correspondingly, there are four mechanisms through which DPSCs promote angiogenesis, as mentioned below (Fig 1).

Secretion of proangiogenic factors in paracrine

As previously aforementioned, DPSCs reside in perivascular niches and interact with ECs to regulate new blood

vessel formation. Hence, multiple signalling molecules secreted by DPSCs can be involved, among which the most detected factor is VEGF. Secretion of VEGF by DPSCs can be observed in almost every condition in both the cell lysates and the conditioned medium (CM)[46], and is even increased under hypoxia with the induction of HIF-1[47]. Using an angiogenic inhibitor to competitively bind with VEGF-A or knocking down the expression of VEGFR2 on DPSCs both lead to decreased blood vessel formation in vitro and in vivo, respectively, showing the impairment of the angiogenic capacity of DPSCs[48]. Conversely, transfecting DPSCs with high-level overexpression of VEGF showed a great ability for vascular tube formation[49]. In addition to VEGF, other proangiogenic factors secreted by DPSCs can be detected, including angiogenin, Ang1, monocyte chemoattractant protein-1 (MCP-1), hepatocyte growth factor (HGF), IL-8, basic-fibroblast growth factors (bFGF), SDF and PDGF[46,50-52]. Once the vessels are formed, it is imperative that they become stabilised and avoid regression. Compared with cultures of EC alone, the administration of DPSCs in a previous study did stabilise the pre-existing vessel-like structures formed by ECs[53]. The recruitment of mural cells, including pericytes and smooth muscle cells (SMC), plays a pivotal role in stabilising neovasculature and maturation of vessels[54]. In the process of angiogenesis, PDGF-BB can be secreted by DPSCs and as a chemoattractant for SMCs to be recruited and expanded around neovasculature to promote its maturation[55]. Meanwhile, Ang1 secreted by DPSCs activated Ang1/Tie2 signalling to maintain the quiescent of ECs and strengthen the interaction between mural cells and ECs, leading to the stabilisation and maturation of newly formed blood vessels[56].

Moreover, with the exception of those functioning growth factors, an enzyme named urokinase plasminogen activator was found to be expressed by DPSCs, which can stimulate the degradation of extracellular matrix and may facilitate the proliferation, migration and invasion of ECs[46]. All the proangiogenic factors mentioned above synergistically endow DPSCs with proangiogenic properties to a certain extent.

Intriguingly, however, some other antiangiogenic factors or enzymes have been detected. Insulin-like growth factor binding protein-3 (IGFBP-3) was found to be expressed by DPSCs[46,50] and acted to inactivate the ERK1/2 signalling pathway and Elk-1, leading to the reduced transcription of their downstream proangiogenic target genes, such as PDGF and bFGF[57]. Enzymes like tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1expressed by DPSCs can inhibit the degradation of extracellular matrix[46]. Thus, the balance between these proangiogenic and antiangiogenic factors may influence the interaction between DPSCs and ECs and eventually the formation of new blood vessels.

Promoting proliferation and migration of ECs

One of the most important processes for angiogenesis is the proliferation and migration of ECs[45]. Endothelial tip cells, as specialized ECs, serve as the navigators to guide endothelial sprouts with their long filopodial protrusions. Endothelial stalk cells, another specialised type of EC, follow behind the tip cells and elongate the sprouts to form vascular lumens[58,59]. This process has been proven to be modulated by miscellaneous proangiogenic factors[60]. VEGF is known as the primary regulator for angiogenesis in either a physiological or pathological state[61]. Binding with its main signalling receptor VEGFR2, which is predominantly expressed on ECs, will sequentially activate several kinases and guide EC proliferation and migration[62].

Recent studies have shown that human umbilical vein endothelial cells (HUVECs) incubated in DPSC-CM can proliferate significantly faster and enhance the proliferation rate of HUVECs compared with controls[52,63]; however, Bronckaers et al[46] demonstrated that DPSC-CM did not facilitate the growth and proliferation of ECs compared with the control groups containing 10% foetal bovine serum. It is assumed that this consequence may be attributed to the existence of antiangiogenic factors and insufficient proangiogenic factors to induce the proliferation under malnutrition conditions. The addition of fetal bovine serum which can increase the amount of angiogenic factors can solve the problem[64]. This may suggest that angiogenesis in cell-based regenerative endodontics can be promoted by adding extra proangiogenic factors.

With regard to EC migration, Dissanayaka et al[65] indirectly co-cultured DPSCs and HUVECs using a transwell migration assay to identify the impact of DPSCs on HUVEC migration. It emerged that HUVECs incubated over a DPSC monolayer had formed round spindle-shaped morphologies and were organised in vascular lumen networks[65]. This result can be attributed to the secretion of VEGF by DPSCs which was detected in the DPSC monocultures. In addition, the application of anti-VEGF antibodies inhibited the migration of ECs significantly, which verified the chemotaxis of VEGF further[46]; however, insufficient inhibition of EC migration implied that there may be other factors involved in this process. SDF-1α and CXCR4 axis has been known to regulate hematopoiesis[66]. In the study by Nam et al[51],

incubating HUVECs in DPSC-CM in vitro or encapsulating DPSCs and HUVECs in vivo while both were treated with CXCR4 antagonist would inhibit the migration of HUVECs and decrease the formation of microvessel structures. DPSCs express high levels of SDF-1α and low levels of CXCR4, but the opposite is true for ECs[51,67]. Thus, SDF-1α serves as another significant mediator in angiogenesis that strongly demonstrates the angiogenic capacity of DPSCs.

Differentiated into endothelial-like cells

DPSC has been verified to possess the ability to differentiate into diverse tissues, such as osteoblasts, adipocytes, neurons and endothelial cells, which makes it an optimal choice for cell-based regenerative endodontics[14]. It is well established that VEGF can induce stem cells into ECs. Through VEGF induction, DPSCs can display some features of ECs by upregulating the expression of von Willebrand Factor, CD31, CD34, CD54, CD105, CD106, VEGFR-1 and VEGFR-2 and VE-cadherin, and by forming capillary-like structures in vitro[68-70]. Use of an angiogenic inhibitor that can complete with VEGF revealed that the endothelial differentiation of DPSCs is dependent on VEGFR-2 activation and its downstream ERK signalling pathway[48]. Although VEGFR-1 shows a higher affinity for VEGF, VEGFR-2 is the main receptor for VEGF to activate downstream signalling pathways. VEGFR-1 displays weak autophosphorylation and is often regarded as a decoy receptor to prevent VEGF from binding to VEGFR-2[62]; however, VEGFR-1 also participates in endothelial differentiation of DPSCs. Zhang et al[71] demonstrated that it was VEGFR-1 that activated the downstream canonical Wnt/β-catenin signalling pathway and mediated the process of DPSC endothelial differentiation in response to VEGF. A more recent study even showed that only 10% to 15% DPSCs can express constitutive VEGFR-1 and that this unique sub-population of DPSCs was primed for angiogenic differentiation[72]. Since DPSCs have various subpopulations with significant heterogeneities[73], this may imply that sifting and purifying DPSC subpopulations with high angiogenic properties may enhance these properties[74,75].

After differentiation into ECs and formation of vascular-like structures, these newly formed blood vessels still need to anastomose with host vasculatures to provide nutrients and oxygen to the graft. VE-cadherin is a calcium-dependent protein that mediates endothelial cell-to-cell adhesion and is essential for vascular integrity[76]. Sasaki et al[77] recently revealed the underpinning mechanisms of how DPSC-derived neovasculature anastomoses with host vasculature to form a functional vascular system. They demonstrated that the induction of VEGF can sequentially activate the ERK signalling pathway and its downstream ERG transcription, which bound to the VE-cadherin promoter and enhanced its expression on DPSCs. Silencing VE-cadherin in DPSCs resulted in either disorganised formation of blood vessels in vitro or a decreased number of functional microvessels anastomosed with host vasculature in vivo, which further verified the role of VE-cadherin in sprouting and anastomosis of DPSC-derived vasculature.

Stabilising neovasculatures by acting like pericytes

Besides ECs, which shape the profile of vascular lumens, mural cells are also essential for vascular formation in angiogenesis[54]. Mural cells surround the ECs and display great abilities to stabilise the neovasculature by inhibiting EC proliferation and migration, regulating endothelium permeability, forming the basement membrane and preventing its regression[54,78].

It has been recognised that a subpopulation of pericytes could express MSC markers, which indicated another derivation of MSCs[79,80]. Similarly, DPSCs have been shown to possess pericyte-like topography and express different levels of perivascular markers, such as alpha-smooth muscle actin, 3G5, NG2 and CD146[51,81-83]. Considering the close relationship between pericytes and DPSCs, Caplan[79] proposed that pericytes were sources of precursors of MSCs, but not all pericytes were MSCs. Dentine sialophosphoprotein (Dspp), along with other odontogenic genes, were found to be in a transcriptionally permissive state that gave pericytes their multipotent differentiation ability in the dental pulp[80,84]. Feng et al[85] demonstrated that pericytes were able to possess MSC-like properties and could be differentiated into specialised mesenchymal-origin cells such as odontoblasts; however, pericyte-derived odontoblasts accounted for part of the total newly differentiated cells, indicating that not all MSCs were pericyte-derived and that MSCs in dental pulp may have distinct origins.

On the other hand, DPSCs can be induced to differentiate into pericyte-like cells with high positive expression of pericyte markers[86], acting like pericytes to stabilise vascular-like structures formed by ECs and prevent their degeneration[87]. Moreover, DPSCs treated by transforming growth factor-β (TGF-β) can be induced into SMCs with high expression of specific markers and improvement of contractile function[56,88]. Zhang et al[56] demonstrated that DPSCs treated by TGF-β can function as pericyte-like cells and express high levels of angi-

opoietin 1, a pro-maturation factor for blood vessels, to significantly inhibit EC migration and endothelial sprouting. Besides, angiopoietin 1/Tie2 signalling can act as a key regulator to maintain the stability of blood vessels by increasing vascular endothelial-cadherin expression and enhancing endothelial cell-cell adhesion[89,90].

Applying DPSCs in cell-based regenerative endodontics

Since the first case report of regenerative endodontics was published in 2001[91], there has been a growing interest in this field among scientists worldwide. With the advancement of tissue engineering concepts[92], including the use of stem cells, scaffolds and signalling molecules, novel approaches have emerged with the aim of achieving true pulp regeneration, such as cell-based and cell-homing approaches[93]. DPSCs can be obtained inviolately from extra third molars or orthodontic teeth and are easy to isolate and cultivate from pulp tissue. They can also differentiate into multilineage tissues in regenerative endodontics[13,94]. Moreover, DPSCs have been shown to promote angiogenesis, which can overcome the challenge of insufficient blood supply after transplantation[95].

The application of DPSCs in cell-based regenerative endodontics can be divided into mainly four different approaches to be mentioned below, namely applying DPSCs alone or with scaffolds; applying DPSCs with growth factors; co-transplanting DPSCs with ECs; and prevascularisation in vitro beforehand. The application of the above methods in preclinical studies using animal models will be discussed below (Table 1), and the clinical trials will be outlined in detail later.

Applying DPSCs alone or with scaffolds

Currently, regenerative endodontics used clinically is revascularisation [do you mean 'use of regenerative endodontics in clinical settings involves revascularisation'?], where an induced blood clot functions as a scaffold to trap cell and growth factors, initiating pulp regeneration[96]. Likewise, cell-based regenerative endodontics also entails the involvement of natural or synthetic scaffolds[92]. Scaffolds are porous biomaterials with good biocompatibility that allow seeded cells to adhere, aggregate into 3D structures and be positioned correctly[97]. Moreover, the porosity of scaffolds facilitates the permeation of growth factors and nutrients, supporting cell proliferation, migration and differentiation[98]. Most importantly, scaffolds should be mimetic to the physical and biochemical microenvironment of the root canal for optimal cell survival and viability[98].

Collagen, as the most abundant component in the extracellular matrix, plays a pivotal role in angiogenesis. The remodelling of the extracellular matrix, particularly the degradation and assembly of collagen, is attuned with the migration of ECs to modulate the formation of vasculatures[99]. This has made collagen the most widely investigated natural scaffold applied with DPSCs. Combining DPSCs or their CM with collagen scaffolds and subcutaneously transplanting them into immunodeficient mice can increasingly generate pulp-like tissue with well-organised vasculature[100]. Compared with bone marrow and adipose tissue–derived MSCs, DPSCs demonstrated a greater ability to stimulate EC differentiation and promoted endogenous cell migration, both essential steps in angiogenesis[100]; however, in a study by Ravindran et al[101], DPSCs co-transplanted with collagen scaffolds can be differentiated into ECs only at the periphery. The addition of decellularised extracellular matrix, which contains a rich source of growth factors and phosphorylated proteins, has been shown to significantly improve the deposition of collagen fibres and hydroxyapatite, as well as the formation of pulp-like tissue and organised neovasculature[101]. This may indicate that the presence of additional bioactive molecules can enhance pulp regeneration[102]. As well as natural scaffolds, synthetic ones with excellent properties have also been studied. PuraMatrix (3-D Matrix, Tokyo, Japan) is a liquid synthetic peptide hydrogel that self-assembles and polymerises when exposed to physiological conditions[103]. This makes it a promising candidate for regenerative endodontics, as it can adapt to the variable and complicated shape of the root canal system[103]. Most importantly, compared with other traditional scaffolds such as collagen and fibrin, PuraMatrix can initiate a rapid onset of vascularisation which is essential for transplanted cells[104]. Encapsulating PuraMatrix with DPSCs into full-length human root canals and implanting them subcutaneously into immunodeficient mice can generate functional pulp-like tissue with a microvessel density similar to that of normal human dental pulp[65,105]. This further strengthens the significance of providing an appropriate microenvironment for dental pulp regeneration. With the development of materials and technology, a growing number of novel scaffolds such as self-assembled peptide RAD/Dentonin hydrogel and coagulated platelet poor plasma have been investigated and shown great potential in promoting angiogenesis and tissue regeneration[106,107].

Table 1 Summary of the different strategies for applying DPSCs in cell-based regenerative endodontics

Strategies	Examples	Method	Conclusion
DPSC alone	3D DPSC constructs[16]	In vivo (animal)	Transplanting DPSCs can regenerate blood vessel–rich pulp-like tissue without any scaffolds and exogenous growth factors
DPSC with scaffolds	DPSCs with collagen[100]	In vivo (animal)	DPSCs demonstrated greater abilities of promoting EC differentiation and cell migration compared with MSCs from bone marrow and adipose tissue
	DPSCs with extracellular matrix embedded collagen/chitosan scaffold[101]	In vitro and in vivo (animal)	Presence of additional bioactive molecules can enhance the regenerative potential of DPSCs
	DPSCs with PuraMatrix[65,105]	In vivo (animal)	Strengthening the significance of providing an appropriate microenvironment for dental pulp regeneration
DPSCs with growth factors	DPSCs with VEGF loaded microspheres[110]	In vivo (animal)	Regenerated pulp-like tissue extending from apex to middle third of the root
	DPSCs with hierarchical VEGF-loaded nanofibrous microsphere scaffolding system[111]	In vivo (animal)	Sustained and prolonged releasing of VEGF can help to reach successful pulp-like tissue regeneration to full-length of human root canals
	DPSCs with CGF[116]	In vitro	Concentrated growth factor enhanced the proliferation, migration, and odonto-/osteogenic differentiation of DPSCs under inflammatory conditions
DPSCs with ECs	DPSCs with HUVECs in GelMA hydrogel[116]	In vivo (animal)	Formation of highly cellularised and vascularised pulp-like tissue
	DPSCs with HUVECs in PuraMatrix[65]	In vitro and in vivo (animal)	HUVECs were induced to form endothelial vascular tubes by the VEGF secretion from DPSCs
	DPSCs with adipose tissue-derived microvascular fragments[123]	In vivo (animal)	Adipose tissue-derived microvascular fragments contained ECs and pericytes, facilitating the formation of vascularised pulp-like tissue
	DPSCs with induced DPSCs[125]	In vivo	Formation of vascular-like networks and host blood vessels invading from apical foramen
Prevascularisation	Prevascularised construction of DPSCs and HUVECs[128]	In vivo (animal)	Prevascularised microtissues enhanced the neovascularisation in regenerated pulp-like tissue, resulting in anastomose with host vasculatures
	Prevascularised construction of DPSCs and induced DPSCs[129]	In vivo (animal)	Prevascularised constructs possessed greater ability to promote neovascularised pulp-like tissue regeneration compared with DPSC constructs without prevascularisation beforehand

Despite those positive aspects, there are still concerns about the application of scaffolds. Scaffolds should biodegrade synchronously at a controllable rate with pulp regeneration, providing enough room for pulp regeneration and averting the surgical removal of scaffolds[97]; however, this process can be hard to control, especially in the complex and variable internal environment that varies from person to person[108]. Thus, the solution of using scaffold-free tissue engineering technology emerges. In a study by Itoh et al[16], a rod-shaped 3D DPSC construct was fabricated with a thermoresponsive hydrogel mould. After inserting the DPSC constructs into human tooth root segments and subcutaneously transplanting them into immunodeficient mice for weeks of incubation, regenerated pulp-like tissue was observed[16]. These tissues contained multiple neovasculature perfused with host blood cells, indicating that transplanted DPSC can be induced to form blood vessels and anastomose with host blood vessels to establish vascular access and ensure blood supply to regenerated tissues[16]. Moreover, DPSCs adjacent to dentine were induced into odontoblast-like cells by the rich source of growth factors in dentine, resulting in mineral deposition[16]. Since instantaneous angiogenesis is essential to the survival of graft material, this experiment demonstrated the great potential of using only DPSCs to regenerate blood vessel–rich pulp-like tissue without the need for scaffolds and exogenous growth factors.

Applying DPSCs with growth factors

Due to the narrow apical foremen and the single blood supply of the pulp, barely relying on DPSCs may not be

sufficient to achieve desirable angiogenesis. The growth factors secreted by DPSCs activate multiple signalling pathways for promoting cell differentiation, proliferation, migration and neovasculature stabilization, which emphasises the significance of the involvement of growth factors.

It is known that VEGF plays a pivotal role in both angiogenesis and dentine regeneration[61,109] and is the most widely investigated growth factor in regenerative endodontics. Many studies have been conducted to explore the effectiveness of VEGF on pulp regeneration and achieved desirable results. Injecting DPSCs with VEGF-loaded microspheres into human tooth root lumens and transplanting them subcutaneously into nude mice led to significant formation of pulp-like tissue extending from the apex to the middle third of the root, with abundant neovasculature and odontoblast-like cells lining the tubular dentine[110]. To better simulate physiological conditions for full-length human root pulp regeneration, a hierarchical nanofibrous microsphere scaffolding system was fabricated to function as both a cell carrier and a controllable VEGF delivery vehicle[111]. With sustained and prolonged release of VEGF, vascularised pulp-like tissue was regenerated, reaching the coronal third of the canals. Compared to groups that transplanted DPSCs alone, the application of controllable release of VEGF yielded promising results not only in pulp regeneration but also in promoting angiogenesis. Strong immunohistochemical staining of von Willebrand factor and CD31 was observed, with neovasculature distributed throughout the full length of the root canal. Intriguingly, the coronal third of the root canal, which is farthest from the apical foramen, generated the highest number of vasculatures, further proving the angiogenic effect of hypoxia. Apart from VEGF, other growth factors such as SDF-1 and bFGF combined with different scaffolds can also yield satisfactory outcomes in pulp regeneration[112-114]. These cytokines can be used respectively or matched with each other to further promote pulp regeneration and angiogenesis. Derived from platelet concentrate, concentrated growth factor is a reservoir rich in multiple growth factors, such as TGF-β1, PDGF-BB, insulin-like growth factor-1, bFGF and VEGF115. This makes it an ideal bioscaffold for supporting cells and provides ample cytokines at the same time. In a study by Xu et al[116], concentrated growth factor significantly enhanced the proliferation, migration and odonto-/osteogenic differentiation of DPSCs under inflammatory conditions stimulated by lipopolysaccharides. Merely transplanting concentrated growth factor into beagle dogs' immature teeth revealed the continued development of teeth and normal pulp-like tissue regeneration with neovasculature and palisading-arranged odontoblasts. Beyond concentrated growth factor, co-transplanting DPSCs with decellularised extracellular matrix[117], platelet-rich fibrin[118] and platelet-poor plasma[107], all serving as cytokine-rich bioscaffolds, provided a variety of promising strategies to promote angiogenesis and dental pulp regeneration.

Co-transplanting DPSCs with ECs

It is well acknowledged that both DPSCs and ECs are significant in the angiogenesis process, in which ECs constitute the wall of vascular lumens whereas DPSCs act as pericytes to stabilise the neovasculature[59,87]. Reports have revealed that HUVECs cultured with CM of DPSCs could markedly promote proliferation, migration and tube formation of HUVECs in vitro[119]. Thus, a novel method, namely co-transplanting DPSCs with ECs into root canals, was used to achieve vascularised dental pulp regeneration. In an in vivo study, it was reported that DPSCs can be encapsulated in GelMA hydrogel scaffolds with HUVECs and subcutaneously transplanted them with human teeth segments into nude mice. This resulted in highly cellularised and vascularised pulp-like tissue with with patent blood vessels and identifiable host red blood cells[120]. Compared to transplanting DPSCs alone, co-transplanting DPSCs and HUVECs can enhance HUVECs migration significantly to form vascular tubes through the interaction between the two kinds of cells, which was clearly orchestrated by VEGF secretion from DPSCs[65].

Apart from HUVECs, adipose tissue–derived microvascular fragments are another source of functional vessel fragments that can be easily harvested from fat tissue[121]. This kind of material contains several significant cell types, such as ECs, pericytes and MSCs. In vivo transplantation has shown that these fragments can rapidly interconnect with each other and even with host blood vessels to form perfused microvascular networks at an early stage[122]. Recently, a study conducted by Xu et al[123] subcutaneously implanted human tooth root segments containing DPSC aggregates and adipose tissue–derived microvascular fragments into immunodeficient mice. This resulted in more positive pulp-like tissue with more obvious and abundant neovasculature, both in quantity and diameter, compared to application of DPSCs only. Adipose tissue–derived microvascular fragments not only supply ECs but also provide ample proangiogenic factors that promote both angiogenesis and odontogenesis, providing perivascular niches for DPSCs and preventing their senescence[121,123]. Apart

from harvesting ECs from adipose tissue–derived microvascular fragments, the multi-lineage differentiation property of DPSCs allows them to be angiogenically induced into endothelial-like cells, providing another rich source of ECs[68]. Induced DPSCs even displayed better angiogenic properties than microvascular ECs in vitro, as the latter were more sensitive to the right cell density seeded on scaffolds. Co-culturing induced DPSCs with non-induced DPSCs demonstrated an analogous capacity to form vascular networks in vitro just as observed between microvascular ECs and DPSCs in vivo[124]. Moreover, in vivo transplantation using ectopic neovascularisation mouse models showed the formation of vascular-like networks and host blood vessels invading from the apical foramen[125], which was an essential step in promoting pulp regeneration within the root canals.

Prevascularisation in vitro beforehand

Establishing a sufficient blood supply as soon as possible by promoting angiogenesis and anastomosis with host vasculatures is unquestionably critical for graft survival; however, due to the limitation of host vasculature invasion[19] and the distance of oxygen diffusion[18], this process may be time-consuming and lead to necrosis of the graft. Constructing prevascularised tissue in vitro beforehand can be a promising solution to the hindrance posed by rapid angiogenesis in tissue engineering and can be perfused apace after transplantation in vivo[126]. Applying this concept in regenerative endodontics with DPSCs also shows giant potential in promoting vascularised pulp regeneration.

When ECs were co-cultured with DPSCs in vitro, PDGF-BB secreted by ECs was revealed to be responsible for the recruitment of DPSCs as perivascular cells, promoting basement membrane deposition and neovasculature maturation formed by self-assembled ECs. After transplantation in vivo, blood perfusion was enhanced significantly, demonstrating the perspectives of prevascularised tissue constructs in regenerative endodontics[127]. For pulp regeneration, in a study by Dissanayaka et al1[28], self-assembly of DPSCs and HUVECs were constructed into microtissue spheroids, and organised vascular networks were induced by these cells apparently in vitro culture beforehand. Tooth root fragments loaded with those prevascularised microtissues were transplanted subcutaneously into the immunodeficient mice. Compared with the neovascularisation achieved through applying DPSCs alone, prevascularized microtissues enhanced neovascularisation in regenerated pulp-like tissue with higher numbers of perfused vessels, resulting in anastomosis with the host vasculature. Considering the restricted and impractical selection of HUVECs in the clinic, inducing endothelial differentiation of DPSCs and fabricating prevascularised DPSC constructs in vitro can be another option. Partially due to the limited blood supply from the apical foramen, applying DPSCs alone may result in incomplete pulp regeneration with cavities formed within the root canals[16]. Inducing DPSCs into ECs and prevascularising the cell construct may facilitate full-length root canal pulp regeneration. Thus, the same group of scientists conducted this study and demonstrated that prevascularised DPSC constructs possessed a greater ability to promote higher density neovascularised pulp-like tissue regeneration compared with DPSC constructs without prior prevascularisation[129]. Besides, differentiated DPSCs expressing VE-cadherin can contribute to neovasculature derived from DPSCs anatomosing with the host vasculature[77].

Clinical trials for pulp regeneration using a cell-based approach

Clinical trials can only be conducted based on miscellaneous preclinical studies involving multiple animal models. Compared to a large number of preclinical studies conducted over the past decades, the number of clinical trials is limited and have not made significant progress until recently.

The safety and efficacy of transplanting DPSCs with granulocyte-colony stimulating factor have been certified using immunodeficient mice and pulpectomised dog teeth, respectively. No toxicity or adverse events were observed, and desirable vascularised and innervated pulp regeneration was achieved[130]. Subsequently, the first clinical trial applying DPSCs for pulp regeneration was conducted by the same group of researchers[74]. Five patients aged 22-40 years with irreversible pulpitis were included in the study. Autogenous mobilised DPSCs by granulocyte-colony stimulating factor were transplanted into pulpectomised teeth and followed up for 24 weeks. Four of them showed a positive response to electric pulp testing after 4 weeks, indicating reinnervated pulp regeneration within the root canals. Complete pulp regeneration was detected by MRI and three patients demonstrated lateral dentine formation by CBCT; however, a histological analysis to verify the regenerated tissues in detail could not be performed in this study[74]. Another clinical trial transplanted DPSCs from primary teeth into pulpectomised immature permanent teeth in patients suffering from pulp necrosis and periapical periodontitis

after trauma[15]. Compared with those teeth accepting apexification as controls, experimental groups showed significant root development with a closed apical foramen and thickened dentine walls after 12 months of follow-up. Furthermore, histological analysis was conducted in one case due to accidental retraumatisation of the implanted tooth[15]. This revealed the regeneration of vascularised and innervated 3D dental pulp tissue with odontoblastic differentiation and neuronal marker expression. Moreover, a clinical case used autogenous DPSCs and leukocyte platelet-rich fibrin to design a personalised cell-based trial and yielded desirable outcomes in both radiographic and clinical evaluation for pulp regeneration[131].

Although current clinical trials have shown positive achievements to acquire dental pulp regeneration by transplanting DPSCs without toxicity and adverse events, there amount of published experimental data remains limited. Thus, more high-quality clinical trials are needed to support the safety and efficacy of this protocol. Apart from this, there are existing challenges in cell-based regenerative endodontics that needed to be resolved, such as the isolation, culture, expansion, and storage of stem cells, as well as facilities and technical problems[93]. In clinical trials, contamination should be tightly controlled; this can be more difficult in the oral cavity compared to animal studies conducted in sterile environments. Otherwise, the presence of micro-leakage can lead to persistent apical periodontitis, which eventually affects pulp regeneration[74].

Conclusions and perspectives

Maintaining the viability and avoiding the necrosis of dental pulp are crucial for preserving the structural integrity and function of teeth. Cell-based regenerative endodontics provides a promising way to achieving real dental pulp regeneration, compared with the unforeseen and undesirable results that revascularisation achieves in the clinic currently[132]. DPSCs promote angiogenesis through multiple mechanisms and are easily available, making them an ideal candidate for transplantation; however, they can do much more than this. They reside in the niches of neurovascular bundle and deriving from peripheral nerve–associated glia endow DPSCs with the ability to reinnervate regenerated dental pulp. Moreover, odontoblast-like cell differentiation and dentine-like structure deposition have been observed, resembling the process of dentinogenesis[133]. These properties make it possible to achieve real pulp–dentine complex regeneration with cell-based regenerative endodontics.

Nevertheless, technical, ethical and economic issues must be addressed before this protocol can be adopted officially in clinical practice. Additional bigger animal models applying orthotopic rather than ectopic sites to mimic the physical and clinical conditions in humans are needed to provide more accurate data. More high-quality and standardised clinical trials are necessary for practical transfer. The ultimate goal of regenerating both functional and structural natural pulp can only be achieved through generations of endeavors.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Meng Qi TANG contributed to the literature collection and draft of the manuscript; Drs Ling YE and Bo GAO supervised the study design and revised the manuscript.

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Unique Features of Nanomaterials and their Combination Support Applications in Orthodontics

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Nanotechnology is a rapidly evolving field with numerous biological applications and is becoming increasingly significant due to its immense potential to enhance the properties of orthodontic and biomaterials. It is employed in various emerging areas of orthodontics, focusing on improving the performance of diverse orthodontic appliances and accessories, as well as nanoelectromechanical systems (NEMS) and nanorobots. Nevertheless, the biocompatibility and cytotoxicity of nanomaterials in orthodontic applications require further investigation. This paper reviews the latest applications of nanomaterials in orthodontics, elucidates their unique features and synergistic applications in orthodontics, and outlines prospective developments in the field.

Key words: *clinical application, nanomaterials, orthodontics*
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Nanotechnology is an interdisciplinary field that encompasses the manipulation, precise positioning, modelling and fabrication of materials at molecular and atomic scales. The concept of nanotechnology was first introduced by American physicist and Nobel Prize laureate Richard Phillips Feynman, while the term "nanotechnology" itself was coined in 1974 by Norio Taniguchi[1,2].

Nanomaterials are defined as materials with components smaller than 100 nm in at least one dimension and may include grains, fibres, atomic clusters, films, nanoholes or combinations of these forms. One-dimensional nanomaterials are known as sheets, two-dimensional nanomaterials are referred to as nanowires and nanotubes, and 3D nanomaterials are called quantum dots[3]. As material size decreases to the nanoscale, the smaller particle size enables enhanced permeation into deeper lesions. The surface–volume ratio rises dramatically with the reduction in material size, which in turn improves catalytic activity and alters physical and chemical properties. Both the internal tiny diameter of nanowires or nanorods and the perfection of facets of the nanostructure contribute to the enhancement of mechanical strength. Mechanical strength increases with decreasing size, but only when the diameter is less than 10 microns[4]. Nanofabrication can be achieved through two approaches: the top-down approach, which employs miniaturisation techniques to create micro-/nanoscale structures from macroscopic materials, and the bottom-up approach, which involves constructing macroscopic structures from atoms or molecules that possess self-organising or self-assembling capabilities[5].

Nanomaterials, with their smaller particle size, can be readily incorporated into orthodontic materials for the purposes of modification. For instance, they can serve as material coatings to reduce friction[6,7], alter hydrophilic and hydrophobic properties[8] or impart antibacterial properties as functional coatings[9].

Over the past decade, nanotechnology has shown promising applications in enhancing dental treatment and care and prevention of oral diseases[10]. The superior inherent characteristics of nanomaterials compared

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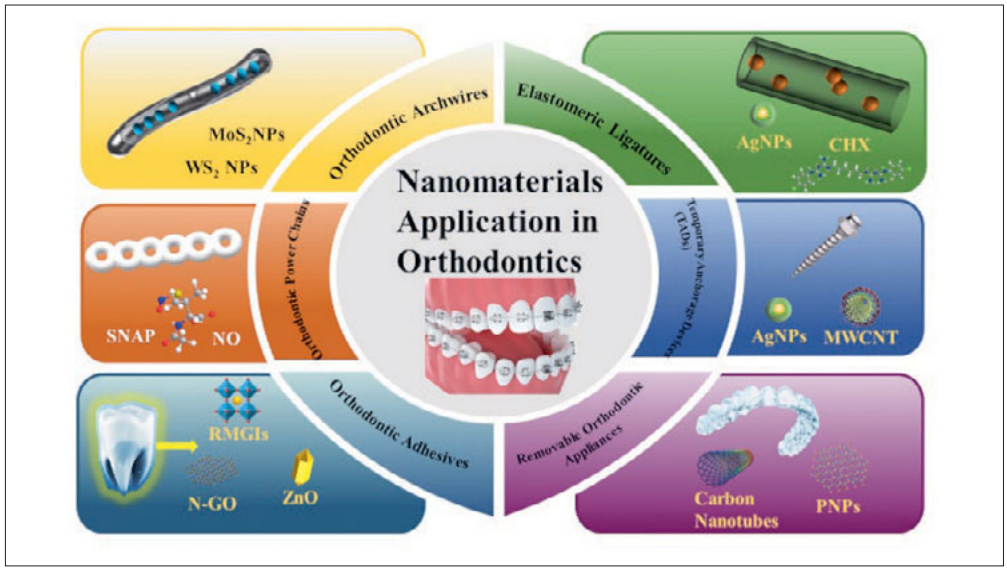

Fig 1 Application of nanomaterials in orthodontics.

to conventional materials allow them to be used in a broad range of clinical dentistry applications, such as periodontics, endodontics, oral and maxillofacial surgery, prosthetics, preventive dentistry and orthodontics[11].

This article focuses on recent innovative applications of nanotechnology in orthodontics, including nanocoating of orthodontic archwires[6,12-16], nanoparticle (NP)-delivering elastomeric ligatures[17-20], property-enhanced power chains via nanoimprinting[8,21], integration of NPs in orthodontic adhesives, and use of shape memory nanocomposite polymers[22-33]. Temporary anchorage devices (TADs) modified with nanotechnology and nanoelectromechanical systems (NEMS) improve orthodontic tooth movement[34-44].

Nanotechnology has optimised the properties of orthodontic materials significantly, reduced treatment duration, increased patient satisfaction and brought numerous benefits to orthodontics; however, further research is necessary to investigate the biocompatibility and cytotoxicity of NPs[45]. There is an urgent need to conduct safety risk assessments and prevent potential side effects.

Application of nano-optimised materials in orthodontics

Generally, nano-optimised materials, as compared to conventional orthodontic materials, exhibit superior properties for clinical use in orthodontics, particularly in terms of antibacterial characteristics and enhanced mechanical properties (Table 1). The predominant applications in orthodontics are outlined in Fig 1.

Nanocoating of orthodontic archwires and brackets

A primary challenge faced in orthodontic biomechanics is reducing friction between the archwire and the bracket. Employing excessive orthodontic force to counteract friction may lead to root resorption and anchorage loss, necessitating the minimisation of friction between the archwire and bracket to expedite tooth movement and shorten the treatment duration[12].

NiTi archwires boast the highest corrosion resistance, shape memory effect and superelasticity; however, prolonged use may result in erosion by active saliva, and the subsequent release of nickel ions could be harmful, as it could cause local swelling, taste disorders, allergies and even the induction of pre-malignant lesions in the oral mucosa structure[13,46].

To mitigate friction between the archwire and bracket and minimise the release of sensitising nickel ions, NP coatings on archwires have emerged as an effective solution. MoS_2 (molybdenum disulphide) and WS_2 (tungsten disulphide) nanomaterials are particularly well-suited for coating applications due to their lubricating properties[6]. They feature a unique layered structure, consisting of a hexagonal crystal (H) of the metal Mo/W intercalated between two layers of anionic sulphur atoms, yielding synthetic 2H-MoS_2 and 2H WS_2, respectively. Van der Waals forces between the synthesised layers are relatively weak, resulting in an unstable structure that readily bends and folds in on itself. As observed in transmission electron microscopy images of these NPs, the structures exhibit concentric layers encircling a hollow core measuring 10 to 100 nm. This distinctive structure renders the WS2/MoS2 nanocoat-

Table 1 Optimised properties of orthodontic materials through nanotechnology support applications in orthodontics.

Applications in orthodontics	Current limiting factors	Optimised properties of orthodontic materials	Materials in nanotechnology	Reference
Orthodontic archwires and brackets	Friction between archwires and brackets; release of potential hazards	Lower friction; limited release of sensitising metal; antibacterial and anti-inflammatory properties	MoS_2 NPs, WS_2 NPs	6
			Clove and cardamom reinforced ZrO_2 NPs	14
			TiO_2 NPs	13, 15
			Graphene sheets embedded carbon (GSEC)	12, 16
			Ag NPs	15
Elastomeric ligatures	Accumulation and retention of bacterial plaque; degradation of mechanical properties caused by bacterial by-products	Long-term antibacterial properties; embedded NPs in the elastomeric matrix	Ag NPs	19, 20
			Chlorhexidine hexametaphosphate (CHX-HMP)	17, 18
Orthodontic power chains	Discolouration; reduction of elasticity	Hydrophobic property; maintained surface and mechanical properties; antimicrobial properties	Anodic aluminium oxide (AAO)	8
			S-Nitroso-N-acetyl penicillamine (SNAP)	21
TADs	Bacterial colonisation around miniscrews; weakened intimacy and stability to the bone caused by autoclaving; excessive bone resorption and inhibiting bone regeneration caused by titanium-type materials; low survival rates and removal torque values (RTVs)	Antibacterial properties; enhanced matrix mineralisation; rapid osseointegration on the bone–implant surface; better biocompatible properties; reduced pressure-holding effect; enhanced early osseointegration; higher bone–implant contact (BIC) ratio	Ag NPs	43, 44
			Multiwalled nanotubes (MWCNTs)	42
			Hydroxyapatite (HA)	41
			HA/collagen nanocomposite (Hap/Col)	40
			Polyetheretherketone-nano HA (PEEK-HA)	39
			TiO2 nanotube	38
			Drug-loaded nanotube arrays	37
			ZnO NPs	36
Orthodontic adhesives	Accumulation of oral biofilm; enamel demineralisation, white spot lesions	Reduced surface roughness; enhanced long-term optical properties; superior mechanical properties; stronger antimicrobial and remineralisation capabilities	Ag NPs	29
			Chitosan NPs (CNPs)	28
			Cinnamon NPs	27
			ZnO NPs	26
			NPs of calcium fluoride (nCaF2) and dimethylamino hexadecyl methacrylate (DMAHDM)	25
			Nano-HA	23, 24
			Nano-graphene oxide (N-GO)	22
Removable orthodontic appliances	Contain bacteria caused by surface porosities; increased risk of candidiasis and stomatitis	Improved mechanical properties and thermal conductivity; antimicrobial properties	Nano-graphene	63
			Carbon nanotubes	64
			Propolis NPs (PNPs)	60
			Gold nanoclusters	65

ing an exceptional solid lubricant, transforming sliding friction into rolling friction while maintaining stable tribological properties under high loads[6]. Previous research has demonstrated that the sliding friction resistance of archwires coated with Ni-P and inorganic fullerene-like WS_2 (IF) NPs was significantly diminished in an oral environment simulated with deionised water[47]. Archwires coated with cobalt and IF-WS_2 NPs, produced via electrochemical codeposition, displayed a 66% reduction in the friction coefficient compared to their uncoated counterparts[6].

In the most recent study, graphene sheets embedded carbon (GSEC) film coating demonstrates a low friction coefficient (0.12) and exceptional wear resistance[12]. As the substrate bias voltage increases from +5 to +50 V, the local microstructures of carbon films fabricated using the electron cyclotron resonance plasma sputtering system transition from amorphous carbon to graphene nanocrystallites. The development of a salivary adsorbed layer and graphene sheet-rich tribofilm on the contact interfaces contributes to the enduring low friction performance of GSEC film-coated archwires (exceeding 30 days)[12].

Given the toxicity of WS_2, alternative NPs, such as carbon nitride (CNx), ZnO and nanoceramics, have been employed. Recent research has revealed that coatings of clove and cardamom-reinforced zirconium oxide NPs exhibit strong antibacterial and anti-inflammatory properties. Notable antimicrobial activity against *Lactobacillus*, *Streptococcus mutans*, *Staphylococcus aureus* and *Candida albicans* has been detected. Furthermore, minimal cytotoxicity suggests improved applicability for orthodontic archwires; however, due to the absence of in vivo testing, additional studies are required to confirm clinical efficacy[14]. Silver- and nitrogen-doped TiO_2 NPs have also demonstrated antimicrobial capabilities when applied to orthodontic archwires[13].

Delivering NPs from elastomeric ligatures

Orthodontic elastomeric ligatures, composed of polyurethane or latex, offer advantages such as ease of application, diverse colour options, reduced patient discomfort and cost-effectiveness[48]; however, they are prone to bacterial plaque accumulation and retention, which can result in enamel demineralisation and white spot lesions. Moreover, bacterial by-products compromise the ligatures' mechanical properties. During orthodontic treatment, elastomeric ligatures firmly secure the archwires within the brackets. These ligatures can function as a carrier scaffold for NP delivery, embedding an elastic matrix with anti-inflammatory/antibiotic drug molecules and anti-cariogenic fluoride[49].

Prior research has demonstrated the synthesis of silver NPs (AgNPs) in situ on orthodontic elastomeric ligatures, providing antibacterial properties against *Streptococcus mutans*, *Staphylococcus aureus*, *Lactobacillus casei* and *Escherichia coli*[20]. NPs enhance the permeability of bacterial cytoplasmic membranes, disrupting the bacterial envelope by continuously releasing silver ions. Additionally, they interact with sulphur and phosphorus within DNA, hindering DNA replication, cell reproduction and protein synthesis[50]. Consequently, silver NPs eliminate microorganisms effectively and reduce the enamel demineralisation rate. Furthermore, elastomeric ligatures incorporating AgNPs can release silver ions for up to 4 months, providing long-lasting antibacterial effects with excellent biocompatibility and no adverse impact on mechanical properties[19].

Recent in vitro studies have demonstrated that chlorhexidine hexametaphosphate (CHX-HMP) enables a sustained release of soluble chlorhexidine (CHX) from orthodontic elastomeric ligatures for over 8 weeks following ethanol pre-treatment, without compromising the mechanical properties of the ligatures[18]. As a cationic compound possessing broad-spectrum antibacterial activity, CHX has emerged as a crucial component in oral preparations, such as mouthrinse and oral gel, as well as an antimicrobial agent. In the form of digluconate salt (CHXdg), CHX is highly soluble in water, thus facilitating its incorporation into water-based local preparations. In contrast, CHX-HMP exhibits lower solubility, which results in sustained release. Although CHX concentrations in the oral environment are insufficient to present a health risk, it is important to consider the potential for drug resistance and adverse reactions when designing NP delivery systems to achieve targeted release[17].

Orthodontic power chains: Nanoimprinting and nitric oxide–releasing technologies

Since the 1960s, power chains have been widely employed in orthodontic treatment. Primarily composed of polyester or polyether polymers derived from rubber, these chains are connected by urethane bonds [-(NH)-(CO)-O-]. In clinical orthodontic practice, they are valued for their flexibility, cost-effectiveness and adjustability; however, their susceptibility to changes in the oral environment (temperature, pH and moisture absorption) can lead to discolouration and diminished elasticity due to water absorption. To address these issues, some researchers have fabricated nanostructures on orthodontic power chains using nanoimprinting with anodic aluminum oxide (AAO) templates as mould inserts. Subsequent surface treatments revealed an increased contact angle of power chains (from 80 to 130 degrees), causing them to transition from hydrophilic to hydrophobic. Surface modification resulted in a 2% decrease in water absorption rate, enhancing surface properties and reducing colour adhesion on the orthodontic power chains[8].

A study conducted by Warden et al[21] explored the potential of a novel nitric oxide (NO)–releasing polymer and nanotechnology. NO is an endogenous gaseous free radical with antimicrobial properties, demonstrated to prevent biofilm formation and effectively inhibit the growth of oral microorganisms such as *Streptococcus mutans* and *Lactobacillus casei*. S-Nitroso-N-acetyl penicillamine (SNAP) serves as a synthetic NO donor that can be successfully integrated into elastomeric power chains. These chains release NO within 3 days, suppressing *Streptococcus mutans* growth for the initial 24 to 48 hours; however, the number of SNAP molecules loaded into the chains and the polymer network interactions within the material may impact the elastic properties of the chains. Future research should aim

to extend NO release duration, minimise cytotoxicity and preserve the mechanical properties of orthodontic power chains[21].

Nanotechnology as applied in TADs

TADs, comprising miniscrews, mini-plates and prosthodontic dental implants, are well-suited for most orthodontic applications due to their ability to withstand forces up to 300 g[51]. Although TADs offer absolute anchorage control with minimal patient compliance and enable the reciprocal force of anterior retraction to be transmitted to the alveolar bone without affecting the posterior teeth[39], their high failure rate (15% to 30%) poses a challenge for clinicians[36,42]. An ideal TAD should possess two key features: reduced insertion torque to minimise bone damage, and improved holding power to prevent premature loss[52]. Factors such as bone integration at the bone–implant interface and bacterial colonisation around the micro-implants may influence the characteristics of TAD and the treatment outcomes achieved. Consequently, minimising inflammation is crucial for the long-term stability and success of implants.

Autoclaving, which employs steam within the range of 121°C to 134°C, has become the most prevalent and reliable method for sterilising instruments and devices used in medical treatment; however, repeated autoclaving can alter the microstructure of TADs, diminishing their integration and stability with the bone. After assessment, AgNPs solution demonstrated antibacterial activity against *Porphyromonas gingivalis* comparable to that of autoclaving, while also preventing damage to the devices caused by autoclaving[43]. By coating micro-implants with an AgNP-coated biopolymer (Ti-BP-AgNP), research has shown that the modified implants exhibited strong antibacterial properties (against *Streptococcus mutans*, *Streptococcus sanguini*, and *Aggregatibacter actinomycetemcomitans*), confirming their potential as excellent implantable biomaterials[44].

One study aimed to evaluate the impact of surface roughness and carboxyl functionalisation of multi-walled carbon nanotubes (MWCNTs) combined with collagen coatings on titanium substrates, as well as their subsequent effects on osteoblast responses. The results indicated that both MWCNT and MWCNT-COOH (which renders the surface hydrophilic and wettable) coatings increased surface roughness and enhanced osteoblast (MC3T3-E1) proliferation and differentiation, and matrix mineralisation in vitro; however, the latter proved more effective. Earlier research corroborated the cytocompatibility of MWCNTs, suggesting that their functionalisation warrants further investigation[42]. Additionally, nanotopography and hydroxyapatite (HA) have been demonstrated to synergistically promote osteoblast adhesion, proliferation, differentiation and osseointegration. Following anodic oxidation (AO) with HA coating, the modified TADs' surfaces exhibited upregulated gene expression of osteogenic and adhesion markers such as osteopontin (OPN), osteocalcin (OCN), vinculin and collagen type 1 (COL)[41].

In conjunction with these previous findings, a recent study reported that the thickness of a bone-like hydroxyapatite/collagen nanocomposite (Hap/Col) coating can be controlled effectively using a modified electrophoretic deposition (EPD) technique incorporating Mg^{2+} ions, resulting in higher adhesive strength[40]. The Hap/Col-coated Ti substrate prepared by EPD shows promising potential for subperiosteal TAD applications, promoting rapid osseointegration at the bone–implant interface[40].

ZnO NPs induce bacterial cell death by deactivating respiratory chain enzymes and increasing reactive oxygen species (ROS) production. A recent in vitro study demonstrated that orthodontic miniscrews (OMSs) coated with ZnO NPs exhibited greater antibacterial activity than those coated with Ag/HA NPs[36]. Furthermore, ZnO NP-coated OMSs displayed lower cytotoxicity and enhanced cytocompatibility compared to Ag/HA NP-coated counterparts, as evidenced by in vitro tests on fibroblasts, osteocytes, osteoblasts, and oral epithelial cells[36]. These findings suggest that ZnO NPs hold promise for minimising inflammation around OMSs[36].

Although titanium-based materials are frequently employed in TADs, they can cause excessive bone resorption and impede bone regeneration. Polyetheretherketone (PEEK) offers high biocompatibility with the human body, preventing allergic reactions to TADs and reducing the pressure-holding effect, and thus serves as a valuable alternative TAD material. Molecular docking investigations revealed that the docking of PEEK and HA exhibits a higher binding affinity for osteogenic markers related to osseointegration, such as insulin-like growth factor-1 (IGF-1) and alkaline phosphatase (ALP), rendering it a more suitable biomaterial for osseointegration than either PEEK or nano-HA alone; however, further in vitro, in vivo and clinical studies are required to examine the biological, chemical and mechanical properties of PEEK and HA combinations extensively[39].

A recent study demonstrated that, under controlled TAD design, loading protocols, and surgical and placement techniques, and accounting for random host factors, the survival rates and removal torque values (RTVs) of nanoporous surfaces (100.0%) surpassed

those of microporous surfaces (83.3%)[38]. TiO_2 nanotubes can be fabricated on OMS surfaces through anodic oxidation in a fluoride-containing electrolyte at low voltage. The nanoporous surface structures enhance early osseointegration by facilitating protein adsorption, osteoblast adhesion and bone tissue healing[38]. This enhancement may be attributed to the greater TiO_2 thickness, which provides improved hydrophilicity, increased surface area, enhanced surface roughness and superior biomechanical stability. To validate the findings of this study, further histological, biological and clinical investigations are necessary[38].

In an in vivo pilot study, nanotube arrays embedded with recombinant human bone morphogenetic protein-2 (rhBMP-2) or ibuprofen were placed on rabbit tibiae[37]. Eight weeks post-implantation, the bone–implant contact (BIC) ratio of ibuprofen-loaded OMSs (71.6%) was significantly higher than that of conventional OMSs (44.3%). The results indicated that TiO_2 OMS surfaces with nanotube arrays can function as drug carriers and that ibuprofen-loaded nanotube arrays can promote osteointegration of OMSs[37]. In addition to drug-loaded nanotube arrays, nanoscale structures for precise and painless drug delivery offer substantial potential in orthodontic applications. In a separate study, microneedle patches were employed to monitor bacterial growth by releasing specific, titrable antimicrobials or NPs that inhibited the proliferation of targeted bacteria[53].

Introduction of nanofillers or NPs to orthodontic adhesives

Bonding is the most prevalent technique for attaching brackets and bonds to teeth surfaces due to its simplicity and aesthetic appeal; however, orthodontic adhesives possess a greater retention capacity for cariogenic streptococci compared to bracket materials. White spot lesions, resulting from the accumulation of oral biofilm around orthodontic brackets and composite materials, are a common issue in orthodontic treatment, complicating oral hygiene maintenance for patients. Consequently, antimicrobial agents, such as fluoride, chlorhexidine and benzalkonium chloride, have been incorporated into orthodontic adhesives[27].

Nanocomposites have gained recognition as adhesives suitable for clinical use. The anticipated properties of nano-adhesives encompass high antimicrobial activity, biocompatibility, elevated translucency, superior polish, wear resistance and the elimination of the need for separate etching[54]. In comparison to traditional orthodontic adhesives, the integration of nanofillers contributes to reduced surface roughness, enhanced long-term optical properties and improved mechanical properties, such as compressive and tensile strength, due to the high surface–volume ratio of nanofillers[10]. A nano-scratch experiment conducted on enamel demonstrated that nanocomposite adhesives and conventional adhesives exhibited similar tribological behaviours (including wear resistance, scratch hardness and friction coefficient). Given its impact on enamel following bracket removal, as well as its mechanical and physical characteristics and bond strength, nanocomposite adhesive emerged as the preferred choice for bonding orthodontic brackets[55].

However, in vitro experiments revealed that the shear bond strength (SBS) decreased following the incorporation of AgNPs (0.3% (w/w)), though it remained acceptable (exceeding the clinically recommended bond strength of 5.9 to 7.8 MPa)[29]. The studies encompassed in this systematic review and meta-analysis confirmed that orthodontic adhesives containing AgNPs exhibited significant antibacterial activity. Nonetheless, due to the lack of standardised protocols in the in vitro models, a degree of heterogeneity was present between the studies. The long-term efficacy of orthodontic adhesives with incorporated AgNPs in a simulated oral environment warrants further investigation[56].

Studies have suggested that varying concentrations of chitosan NPs (CNPs) exhibit different antibacterial effects against the multispecies biofilm of cariogenic bacteria on orthodontic primer in a rat model. Orthodontic primers incorporating 10% CNPs maximally inhibited *S. mutans*, *S. sanguinis* and *L. acidophilus* for up to 7 days. Given the competition between *S. mutans* and *S. sanguinis* in the oral cavity, the presence of the latter can reduce the count of *S. mutans*. Results demonstrated that orthodontic primers containing a 5% concentration of CNPs exerted a non-selective inhibitory effect against *S. mutans* and *S. sanguinis*; however, as the CNP concentration increased from 1% to 10%, the SBS experienced an insignificant decrease[28].

Incorporating a 1.3% mass fraction of zinc oxide NPs or 3% cinnamon NPs into orthodontic adhesives has been demonstrated to enhance their antibacterial properties and hinder biofilm formation without compromising the adhesives' properties or SBS[26,27]. Moreover, this modification reduced the development of caries lesions around brackets during orthodontic treatment. As nano-zinc oxide and nano-chitosan particles have both exhibited antibacterial properties, a novel study sought to combine them in varying proportions to capitalise on their synergistic benefits[57]. In vitro experimental findings revealed that incorporating

1% and 5% zinc oxide and chitosan particles did not adversely affect the SBS of adhesives[57].

Fluoride, a dual-functional anti–caries lesion agent, has been utilised in resin-modified glass ionomer cements, although its concentration of released fluoride is insufficient for long-term suppression of biofilm metabolism. For the first time, 20 wt% NPs of calcium fluoride ($nCaF_2$) and 3 wt% dimethylamino hexadecyl methacrylate (DMAHDM) were integrated into orthodontic cement. The results indicated that the innovative orthodontic cement increased enamel hardness by 56%, decreased lesion depth by 43%, reduced biofilm CFU by 3log and significantly diminished metabolic activity, polysaccharide production and acid production. Assuming no adverse effects on bracket-enamel SBS and biocompatibility, the novel nanostructured orthodontic cement possesses superior antimicrobial and remineralisation capabilities compared to commercial orthodontic cement, potentially decreasing the incidence of enamel demineralisation, white spot lesions and caries lesions during orthodontic treatment[25].

Recent research has focused on the development of novel remineralisation techniques as alternatives to fluoride. Upon using nano-hydroxyapatite on demineralised enamel, researchers observed higher SBS values than in untreated samples, albeit still lower than those in normal enamel[23]. Furthermore, the microhardness values increased to 278.97 VHN following remineralisation treatment, compared to 238.76 VHN for demineralised enamel. Based on these findings, employing biomimetic nano-hydroxyapatite on demineralised enamel surfaces during orthodontic treatment is advisable within certain limitations; however, complete remineralisation treatment should be avoided prior to bonding to prevent a decrease in SBS[23]. Not only does nano-hydroxyapatite remineralise demineralised enamel, but it also enhances the mechanical properties of orthodontic adhesives when combined with a conventional Heliosit adhesive resin at specific concentrations. A conventional Heliosit/Ivoclar Vivadent adhesive containing 2% wt calcium hydroxyapatite NPs demonstrated improvements in both the degree of conversion (DC) and SBS; however, incorporating 4% wt NPs led to a reduction in the DC and a decrease in the SBS of orthodontic adhesives. The primary cause of these reductions was the agglomeration of calcium hydroxyapatite NPs, which interfered with light penetration through the adhesive layer, resulting in a substantial decline in the photopolymerisation process[24].

Apart from the aforementioned nanofillers and their various combinations, numerous novel NPs can enhance the performance of orthodontic adhesives. Nano-graphene oxide (N-GO) is known to exhibit reduced toxicity, an improved surface–volume ratio, superior mechanical properties and cost-effectiveness. A study employed scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and Zeta potential analysis to confirm the successful synthesis of N-GO, and concluded that the incorporation of N-GO is beneficial for orthodontic adhesives[22]. SBS testing and the adhesive remnant index (ARI) were used to assess the physical-mechanical properties of N-GO-incorporated adhesives[22]. The results indicated that adhesives containing 5 wt% N-GO significantly enhanced SBS without negatively impacting the ARI. In the disk agar diffusion test, N-GO addition was found to impart antimicrobial and anti-biofilm properties against *S. mutans* to the adhesives; however, as the proportion of N-GO in the orthodontic adhesives increased, the mean SBS decreased despite a notable inhibition of *S. mutans* growth[22]. Clinical trials should be conducted to verify the anti-caries properties of N-GO-incorporated adhesives[22].

Removable orthodontic appliances with NPs

Removable orthodontic appliances, such as expanders, functional appliances and retainers, are frequently constructed from cold-cure acrylic resins[58]. These resins primarily consist of polymethyl methacrylate (PMMA)[59], a material favoured for its affordability, low weight and aesthetic appeal[60]; however, PMMA removable appliances are prone to surface porosities, which can harbour *Streptococcus spp.*, *Lactobacillus spp.*, *Enterobacteriaceae* and non-streptococci anaerobic bacteria. Patients who wear removable orthodontic devices tend to exhibit elevated levels of *C. albicans* in their saliva, increasing their risk of developing candidiasis and stomatitis. Furthermore, there is a direct correlation between removable orthodontic appliances and an increase in periodontal pathogenic microorganisms[61]. Although mechanical cleaning can help mitigate biofilm formation, it has proven to be largely ineffective in fully eliminating microorganisms, as they can infiltrate PMMA to depths of 1 to 2 mm. The efficacy of antibacterial solutions largely relies on patient compliance, which can be compromised among children, underscoring the need for greater focus on the self-sterilizing properties of PMMA. To imbue acrylic resins with bactericidal activity, NPs such as silver, platinum, titanium dioxide (TiO_2) and zinc oxide (ZnO) have been incorporated into the materials; however, the potential release of metal ions and their impact on the biocompatibility of acrylic appliances, as well as the resistance mechanisms of

microorganisms, limit the long-term use of metal NPs[62].

In recent studies, nano-graphene and carbon nanotubes have been incorporated into polyurethane resins to enhance properties such as mechanical strength and thermal conductivity[63,64]. Given the successful application of propolis in preventing dental caries and gingivitis demonstrated in earlier studies, propolis NPs (PNPs) were introduced into PMMA. The findings indicate that a 1% concentration of PNPs potentially offers the most effective anti-biofilm performance for clinical use, exhibiting antimicrobial properties against *S. mutans*, *S. sanguinis*, *L. acidophilus* and *C. albicans*[60]. Aligners coated with quaternary ammonium (QA)-modified gold nanoclusters (QA-GNCs) can efficiently inhibit the adhesion of cariogenic pathogenic *S. mutans* and the formation of biofilm for at least 3 months[65].

Future applications of nanotechnology in orthodontics

Shape memory polymers

Shape memory polymers (SMPs) are materials with the ability to recall macroscopic or equilibrium shapes. They can be manipulated and set to temporary or dormant shapes under specific temperatures and pressures and can revert to their original shape upon suitable stimulation, such as heat, electric and magnetic fields, water, light or physiological triggers (pH, body temperature, specific ions or enzymes)[33]. Advantages like simple installation, effortless shape adjustment, light weight, comfort and aesthetic appeal render SMPs a promising material for use in orthodontics[32]. Incorporating graphene NPs and carbon nanotubes into SMPs can boost their mechanical properties and thermal conductivity[30,31]. Nanotechnology-modified SMPs can deliver light and continuous force for extended periods, alleviate patient discomfort and maintain an aesthetically pleasing appearance.

Biological nanoelectromechanical systems

A significant issue in orthodontics is the extended treatment duration. Research on animals has demonstrated that electricity can effectively expedite tooth movement, elevate cAMP and cGMP concentrations in osteoblasts and periodontal ligament cells, and hasten the synthesis and secretion associated with bone remodelling. Microfabricated biocatalytic fuel cells can generate electricity in the oral environment to facilitate orthodontic tooth movement. Using glucose as a fuel source and immobilising enzymes on electrode surfaces for electricity production presents challenges, such as limited lifespan and suboptimal power density. A range of nanostructured materials, including mesoporous media, NPs, nanofibres and nanotubes, have been identified as efficient hosts for enzyme immobilisation. Integrating these nanostructured conductive materials can enhance the activity and stability of immobilised enzymes and improve the power density of biofuel cells[34,35].

Orthodontic nanorobots

Nanorobotics is the field of designing and constructing nanorobots, which consist of components at or near the nanometre scale. Nanorobots can expedite tooth movement using NEMs and nano low-intensity pulsed ultrasound devices. Furthermore, integrated nanomechanical sensors enable clinicians to apply force precisely. Nanorobots can be administered through mouthrinse or toothpaste, providing continuous calculus debridement and safe self-inactivation upon ingestion. Additionally, nanosensors have been examined and validated for remote, objective monitoring of wear and adherence to orthodontic removable appliances[66].

Conclusion

In recent years, nanotechnology has impacted the field of stomatology significantly, leading to substantial innovation and benefits. Numerous dental nanomaterials have emerged and demonstrated immense potential in orthodontics, particularly in enhancing the mechanical and antimicrobial properties of orthodontic appliances. The integration of constantly evolving nanotechnology into clinical orthodontic treatments still draws attention. Although nanotechnology undoubtedly offers advantages in making orthodontic treatment more comfortable, rapid, straightforward and aesthetically pleasing, cytotoxicity and biocompatibility must be considered carefully when introducing new nanomaterials. The oral environment is dynamic, and the nanoscale size of NPs could potentially disrupt biomolecules, cells and human organs, or even induce oxidative stress that impairs human cellular mitochondrial function. The potential toxicity of nanomaterials is not yet fully understood; thus, further investigation is necessary to gather information on the long-term in vivo performance and safety of nanomaterials in orthodontics. Besides ensuring human safety, the environmental impact of nanomaterials should also be addressed. Future research should focus on controlling the release of nanomaterials in a targeted and quantitative manner, thereby maximising

their efficacy and safety in the context of nanotechnology applications.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Yi Lin WANG contributed to drafting the manuscript; Dr Zhi Jian LIU contributed to supervision and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Stem Cells from Human Exfoliated Deciduous Teeth Alleviate High-Altitude Cerebral Oedema by Shifting Microglial M1/M2 Polarisation

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Objective: *To explore the high-efficiency and low-risk prevention and treatment strategies for stem cells from human exfoliated deciduous teeth (SHED) for high-altitude cerebral oedema.*
Methods: *A low-pressure and low-oxygen tank mimicking high-altitude conditions was used to establish the high-altitude cerebral oedema animal model. The preventive effects of SHED for cerebral oedema were then evaluated by haematoxylin and eosin (H&E) and histological staining. In vitro, SHED was co-cultured with BV-2 to analyse the effects of SHED by western blot and immunofluorescence staining.*
Results: *SHED can prevent and treat cerebral oedema in a high altitude rat animal model. Mechanistically, SHED treatment can protect brain cells from apoptosis induced by high altitude condition. Moreover, SHED treatment can inhibit M1-type polarisation and promote M2-type polarisation of microglia cells via the suppression of hypoxia inducible factor (HIF)-1α-mediated extracellular signal-regulated kinase (ERK) signalling activated in high altitude condition.*
Conclusion: *SHED treatment can relieve high-altitude cerebral oedema via inhibiting HIF-1α-mediated ERK signalling, which indicates that SHED is a promising alternative strategy to prevent and treat high-altitude cerebral oedema.*
Key words: *cerebral oedema, microglia, polarisation, SHED, stem cells*
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In the case of high altitude, low air pressure, thin air and low oxygen content, a series of physiological and pathological changes occur, known as acute mountain sickness (AMS). Cerebral oedema usually occurs in high-altitude areas above 4,000 metres and is the final stage of acute high-altitude reaction[1]. Within 24 hours, the patient may fall into a coma or die due to a cerebral haemorrhage[2]. The underlying mechanisms that cause high-altitude cerebral oedema remain unclear. Cerebral hypoxia is one of its main initiating factors[3]. This condition can cause brain swelling and thus increase brain size in high-altitude areas; cerebral blood flow also increases, which leads to increased intracranial pressure and more severe swelling of brain tissue[4,5]. Several prevention and treatment options are available for AMS and cerebral oedema, including acclimatisation, slow ascent[6], oxygen uptake, nutritional support and drug therapy[7]. Drugs, such as carbonic anhydrase inhibitors, medroxyprogesterone and glucocorticoids, are used to treat cerebral oedema by improving oxygenation and reducing the inflammatory response[6]; however, carbonic anhydrase inhibitors, medroxyprogesterone and glucocorticoids show variable side effects. As such, it is urgent to explore the high-efficiency and low-risk treatment strategies for cerebral oedema.

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Stem cells from human exfoliated deciduous teeth (SHED) are derived from the pulp of naturally replaced primary teeth; they are readily available, non-invasive and less subject to ethical controversy[8]. SHED have shown profound proliferation and multi-lineage differentiation capacity. They also exhibit therapeutic effects on multiple diseases, such as Alzheimer's disease, ischemic brain injury, Parkinson's disease, osteoarthritis and amyotrophic lateral sclerosis[9-12]. SHED have been found to demonstrate profound effects on neuronal diseases[13-15], which may be due to their embryonic neural crest origin and neurotrophic properties[16]. SHED may be a promising strategy for preventing and treating cerebral oedema that needs to be illustrated.

A variety of immune cells in brain tissue participate in the regulation and repair of brain tissue homeostasis, among which microglia, the macrophages in brain tissue, play an important role[17,18]. Macrophages can be activated by different stimulus factors into two different forms based on polarisation: M1 and M2 types[19]. M1-type macrophages can induce the production of a variety of inflammatory factors, such as inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-18 and CD86, which have proinflammatory effects[20]. M2-type macrophages can exert regulatory or inhibitory effects against proinflammatory and cellular immune responses. The polarisation of macrophages is reversible and plays an important role in disease development, reversal and treatment. Injury-related factors, such as ischemia and hypoxia, can induce M1-type polarisation of microglia and enhance the phagocytic ability of microglia and thus cause endothelial injury[21-23]. Thus, how to regulate the polarisation of macrophages to inhibit inflammation and promote tissue healing has become an important topic in the treatment of diseases[24-27]. Whether SHED treatment can be used to manipulate the polarisation of microglia remains unknown.

In the present study, we found that SHED showed preventive and therapeutic effects on cerebral oedema by controlling the polarisation of microglia, which is mediated by hypoxia-inducible factor (HIF)-1α/extracellular signal-regulated kinase (ERK) signalling.

Materials and methods

The study was approved by the Animal Use and Care Committee of Peking University (LA2020-124). All institutional and national guidelines for the care and use of laboratory animals were followed.

Animal model

Male Sprague-Dawley rats (8 to 10 weeks old) were purchased from Vital River Laboratory Animal Technology (Beijing, China). The rats were randomly assigned to three groups: the control group, normoxia; the high-altitude group, placed in a low-pressure oxygen tank (FENGLEI, Shanghai, China) for 1 week; and the SHED infusion group, with SHED (5×10^6) suspended in 500 µl, then the cells were injected into the tail vein over the course of 1 minute. One week after the stem cell injection, the rats in the SHED infusion and high-altitude groups were placed in the low-pressure oxygen tank for 1 week. The environment mimicking high altitude was set at an altitude of 5,000 metres, a velocity of 10m/s, humidity of 60% to 70%, and a temperature of $25^0C \pm 2^0C$ with a 12-hour light-dark cycle and ad libitum access to feed and water.

Microglia culture

The microglia BV-2 were purchased from Procell Life Science&Technology (Wuhan, China) and cultured in BV-2 Cell Culture Medium (CM-0493, Procell) supplemented with 10% (vol/vol) foetal bovine serum (FBS, Procell), and 1% (vol/vol) penicillin/streptomycin solution (P/S solution, Procell) at 37^0C in a humidified atmosphere of 5% CO_2/95% air.

SHED and microglia co-culture

For the SHED co-culture group, SHED were co-cultured with microglia by transwell assay. In the transwell plates, 2×10^5 BV-2 cells were plated in the lower chamber and 1×10^5 SHED were plated in the upper chamber, and BV2 specific medium was used as the co-culture medium.

Brain water content analysis

Brains were collected after anaesthesia with 1% sodium pentobarbital, and the wet weight was immediately determined using a precision electronic balance. The samples were then dried at 105^0C to a constant weight. The brain water content (BWC) was calculated according to the following formula: BWC(%) = ((wet weight−dry weight)/wet weight) × 100%.

Haematoxylin and eosin staining

The brain samples were fixed in 4% paraformaldehyde for 24 hours at 4^0C, dehydrated through a graded alcohol series and embedded in paraffin. Sections with a thick-

ness of 6 μm were obtained and stained with haematoxylin and eosin (H&E) according to the manufacturer's instructions to observe the morphology of the cortex and medulla. Photomicrographs were captured using a digital camera attached to a light microscope (Olympus, Tokyo, Japan).

Immunofluorescence

The cells were cultured on chamber slides, fixed in 4% paraformaldehyde for 24 hours at 4^0C, then washed three times with phosphate-buffered saline (PBS, pH 7.4), immersed in 5% bovine serum albumin solution for 30 minutes and incubated with primary antibodies diluted in PBS overnight at 4^0C. Next, the sections were washed with PBS and incubated with secondary antibodies for 60 minutes in the dark. The nucleus was stained with DAPI mounting medium and the slides were observed using an upright fluorescence microscope (Olympus).

Deparaffinised sections were treated with 5% bovine serum albumin (BSA) for 30 minutes, then the sections were incubated with BAX (50599-2-lg, Proteintech, Rosemont, IL, USA) and IBA-1 (1022-5, Santa Cruz Biotechnology, CA, USA) primary antibodies overnight at 4°C, respectively. The samples were then incubated with secondary antibodies for 60 minutes in the dark. The nucleus was stained with DAPI mounting medium and the slides were observed using a fluorescence microscope.

Western blot

Proteins were extracted from cells by using RIPA lysis and extraction buffer. Protein concentration was determined using the bicinchoninic acid (BCA) method. 20 μg protein was separated by the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method, then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in tris-buffered saline with 0.1% Tween 20 detergent (TBST) buffer (150 mM NaCl, 50 mM Tris, pH 7.5, with 0.1% Tween 20) containing 5% BSA (v/v) at room temperature for 1 hour. Next, the membranes were incubated with primary antibodies (rabbit polyclonal anti-iNOS, ARG-1, HIF-1α, p-ERK and β-actin; 1:1,000) overnight at 4^0C, then then the membranes were incubated with the HRP-conjugated secondary antibody for 1 hour at room temperature. The protein band was visualised using chemiluminescence with a ChemiDoc MP system (Bio-Rad, Hercules, CA, USA). β-actin was used as a control, and the density of protein bands was semi-quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction

The total RNA of cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The complementary DNA (cDNA) was synthesised using a reverse transcription system (Toyobo, Osaka, Japan) and quantitative polymerase chain reaction (qPCR) was carried out using SYBR Green PCR master mix (Applied Biosystems, Waltham, MA, USA) on an ABI 7900 fast real-time PCR system (Applied Biosystems). The gene expression levels were normalised to the internal controls (β-actin) and the relative expression levels were evaluated using the $2^{-\Delta\Delta CT}$ method. The primers for iNOS, TNF-α, ARG-1 and IL-10 are listed in Table S1 (provided on request).

Immunohistochemistry staining

For immunohistochemistry staining analysis, deparaffinised sections were incubated with 3% H_2O_2 for 15 minutes and then treated with 5% BSA for 10 minutes. Next, the sections were incubated with iNOS (18985-1-AP, Proteintech), ARG-1 (D4E3M, Cell Signaling Technology, Danvers, MA, USA), HIF-1α (20960-1-AP, Proteintech), cleaved CASPASE-3 (9664S, Cell Signaling) and ERK (4695, Cell Signaling) primary antibodies overnight at 4°C, respectively. Then, the samples were incubated with HRP-conjugated secondary antibodies and visualised with DAB substrate kit staining (ZLI-9017, ZSGB-Bio, Beijing, China). The nucleus was stained with hematoxylin and the images were photographed by microscope (Olympus).

Determination of apoptosis

Apoptosis in brain tissues was detected by terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end labelling (TUNEL) staining. Paraffin-embedded sections were deparaffinised and digested with 20 μg/mL proteinase K for 30 minutes at room temperature. After washing with PBS, the slides were incubated with the TUNEL reaction overnight at 4°C and visualised with DAB substrate kit staining. TUNEL+ cells were observed and counted in at least three different fields per section.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, NY, USA). Independent unpaired two-tailed Student *t* tests were used to analyse

the comparisons between two groups. The differences among more than two groups were assesses with a one-way analysis of variance (ANOVA) followed by a Fisher least significant difference post hoc test. The level of statistical significance was set at $P < 0.05$.

Results

SHED infusion alleviated cerebral oedema

To analyse whether SHED infusion can attenuate cerebral oedema, we injected 1×10^6 SHED into rats placed in a low-pressure oxygen tank setting at a 5,000-metre altitude environment to mimic high-altitude conditions (Fig 1a). The BWC of rats in the high-altitude group was significantly higher than that of rats in the control group, and SHED infusion decreased the BWC elevated by hypoxia stimulation (Fig 1b).

We then analysed the rats' blood and the results showed that the red blood cell specific volume (haematocrit, HCT), haemoglobin (HGB) and red blood cell (RBC) count in the high altitude and SHED infusion groups were significantly higher than those in the control group (Figs 1c to 1e). These results indicated that the number and density of erythrocytes in the blood increased under the high-altitude environment, and the oxygen uptake ability enhanced to adapt to it.

Next, the HE staining results showed that karyopyknotic, cell oedema and vacuolar degeneration of brain stromal cells were detected in the high-altitude group. The number of blood vessels in the medulla and the circumference of blood vessels increased in the high-altitude group. After SHED infusion, cell oedema reduced significantly (Fig 1f). These results indicated that SHED infusion can alleviate cerebral oedema at high altitude and reduce BWC.

SHED infusion reduced apoptosis of brain cells

Low oxygen levels may lead to cell apoptosis[28]. TUNEL staining showed that the ratio of TUNEL positive cells increased significantly in the high-altitude group compared with the control group, and decreased in the SHED infusion group (Figs 2a to c). Caspase-3 is one of the key enzymes of apoptosis. Immunohistochemical results of brain tissue paraffin sections showed that the expression of cleaved Caspase-3 increased significantly in the high-altitude group and decreased after SHED infusion (Figs 2a to c). The immunofluorescence co-staining of microglia marker IBA-1 and apoptotic markers BAX in brain tissue sections showed that the positive sites of IBA-1 overlapped with the positive sites of BAX in the high-altitude group, suggesting that apoptosis occurred in microglia cells (Fig 2d). Microglia cells were cultured in a low oxygen incubator to mimic a high-altitude low oxygen environment, and the results showed that the ratio of apoptotic cells increased significantly in the high-altitude low oxygen group. Then, the ratio of cell apoptosis induced by low oxygen increased after SHED co-culturing (Fig 2e). These results indicate that SHED can prevent cell apoptosis caused by high-altitude conditions in vivo and in vitro.

SHED treatment suppressed M1-type polarisation of microglia in an environment mimicking high altitude and low oxygen

Microglia are a kind of macrophage in the nervous system and are involved in the regulation of nervous system homeostasis[17,18]. Regulation of microglial polarisation may be involved in the pathology of and therapy for neurological diseases[24-27]. First, we analysed the expression of iNOS, which is a representative marker of M1-type polarisation. The results showed that the ratio of iNOS positive cells increased significantly in the high-altitude group compared with the control group and decreased significantly in the SHED infusion group (Fig 3a). Next, we cultured microglia in a low-oxygen incubator, which mimicked a high-altitude, low-oxygen environment, and the qPCR results revealed that the mRNA expression levels of M1-type polarisation markers iNOS and TNF-α in the high-altitude group were significantly higher than those in the control group. On the other hand, the expression levels of these genes decreased in the SHED co-culture group (Figs 3b and c). Moreover, the expression of iNOS in the high-altitude group was significantly higher than that in the control group, but the iNOS expression in the SHED co-culture group was lower than that in the high-altitude group, as assessed by Western blot and immunofluorescence staining (Figs 3d and e). These results indicate that hypoxia stimulation can promote the transformation of microglial cells to M1-type, which was attenuated by SHED treatment.

SHED treatment promoted M2-type polarisation of microglia in an environment mimicking high altitude and low oxygen

The proportion of arginase-1 (ARG-1) positive cells increased significantly after SHED infusion compared with the control and high-altitude groups (Fig 4a). We then analysed the effects of SHED on M2-type polarisation in microglia using a co-culture system, and found

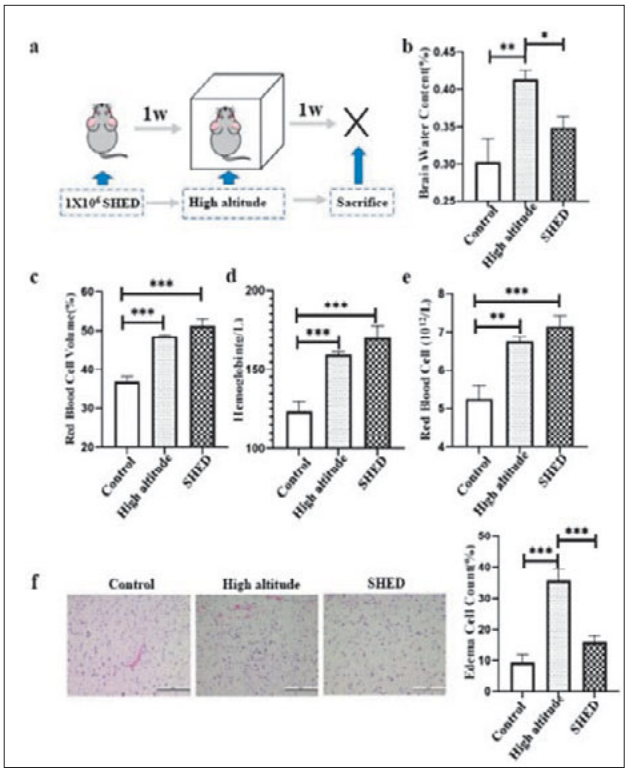

Fig 1 SHED infusion showed therapeutic effects on hypoxic high altitude cerebral oedema in vivo. **(a)** The schema of high-altitude and SHED therapy rat models (n = 5). **(b)** Quantitative analysis of BWC in the control, high-altitude and SHED infusion groups. **(c)** Red blood cell volume in the control, high-altitude and SHED infusion groups. **(d)** Haemoglobin in the control, high-altitude and SHED infusion groups. **(e)** Red blood cells in the control, high-altitude and SHED infusion groups. **(f)** Representative images of HE staining of brain tissue in the control, high-altitude and SHED infusion groups. Scale bars 200 μm. Data are presented as mean ± standard deviation (*P < 0.05, **P < 0.01, ***P < 0.001).

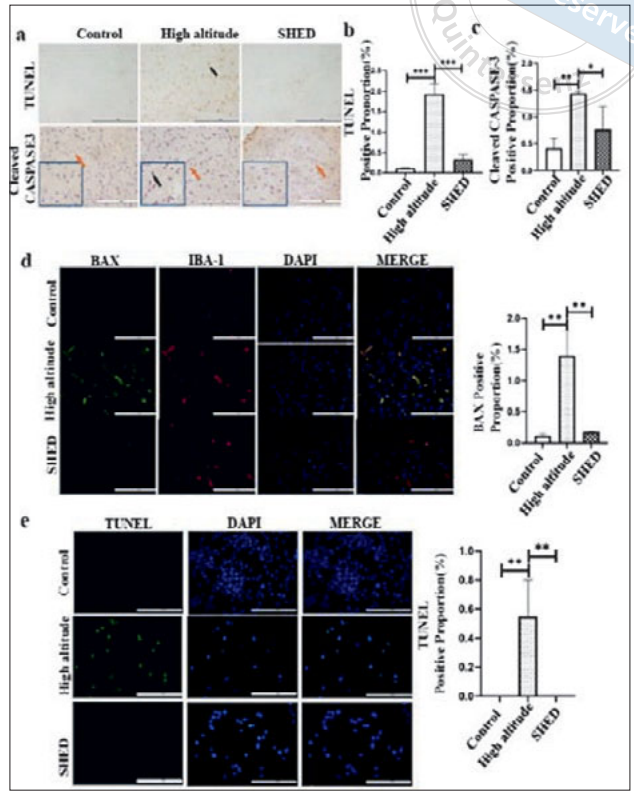

Fig 2 SHED infusion reduced brain cell apoptosis. **(a)** Representative images and quantification of TUNEL (upper panel) and cleaved Caspase-3 (lower panel) staining of brain tissue in the control, high-altitude and SHED infusion groups (scale bars 200 μm). **(b and c)** Quantitative analysis of TUNEL **(b)** and cleaved Caspase-3 **(c)** level in the control, high-altitude and SHED infusion groups. **(d)** Expression of BAX and IBA-1 in the control, high-altitude and SHED infusion groups, analysed by immunofluorescence (scale bars 200 μm). **(e)** Representative images and quantification of TUNEL staining in BV-2 microglia in the control, high-altitude and SHED infusion groups (scale bars 200 μm). Data are presented as mean ± standard deviation (*P < 0.05, **P < 0.01, ***P < 0.001).

that the mRNA expressions of M2-type polarisation markers ARG-1 and IL-10 in the SHED co-culture group were significantly higher than those in the control and high-altitude groups (Figs 4b and c). Moreover, the protein expression of ARG-1 in the SHED co-culture group was significantly higher than that in the control and high-altitude groups, as assessed by Western blot and immunofluorescence staining (Figs 4d and e).

SHED treatment reduced HIF-1α expression in an environment mimicking high altitude and low oxygen

Next, we analysed the expression of HIF-1α in an environment mimicking high altitude and low oxygen and found that the expression of HIF-1α in the high-altitude group increased significantly, attenuated by SHED treatment analysed by qPCR and immunofluorescence staining (Figs 5a to c). YC-1, an inhibitor of HIF-1α protein, was used to treat microglia to verify the role of HIF-1α. The results showed that the elevated gene expressions of M1-type polarisation markers iNOS and TNF-α in the high-altitude group decreased significantly after YC-1 treatment (Fig 5d). Immunofluorescence staining results also revealed that the expression of iNOS, which was elevated in the high-altitude group, was decreased after HIF inhibitor YC-1 treatment (Figs 5e and f). Meanwhile, the expressions of ARG-1 and IL-10 related with M2-type polarisation increased significantly in the YC-1 treatment group compared with the high-altitude group

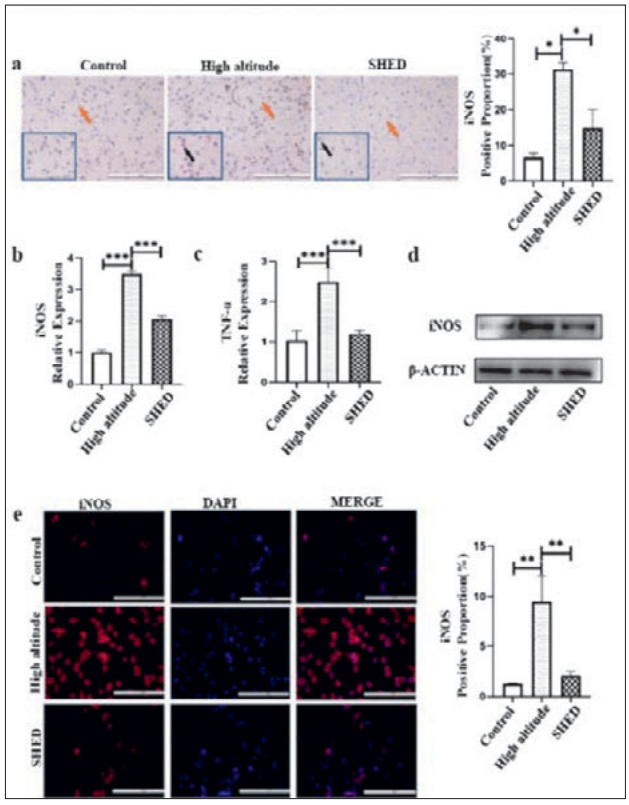

Fig 3 SHED treatment dampened the M1-type polarisation phenotype of BV-2 microglia in an environment mimicking high altitude and low oxygen. **(a)** Expression of iNOS in the control, high-altitude and SHED co-culture groups as assessed by immunohistochemical staining (scale bars 200 μm). **(b and c)** Expression of iNOS and TNF-α in BV-2 microglia in the control, high-altitude and SHED co-culture groups, assessed by qPCR. **(d)** Expression of iNOS in BV-2 microglia in the control, high-altitude and SHED co-culture groups was assessed by Western blot. **(e)** Expression of iNOS in BV-2 microglia in the control, high-altitude and SHED co-culture groups as assessed by immunohistochemical staining (scale bars 200 μm). Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

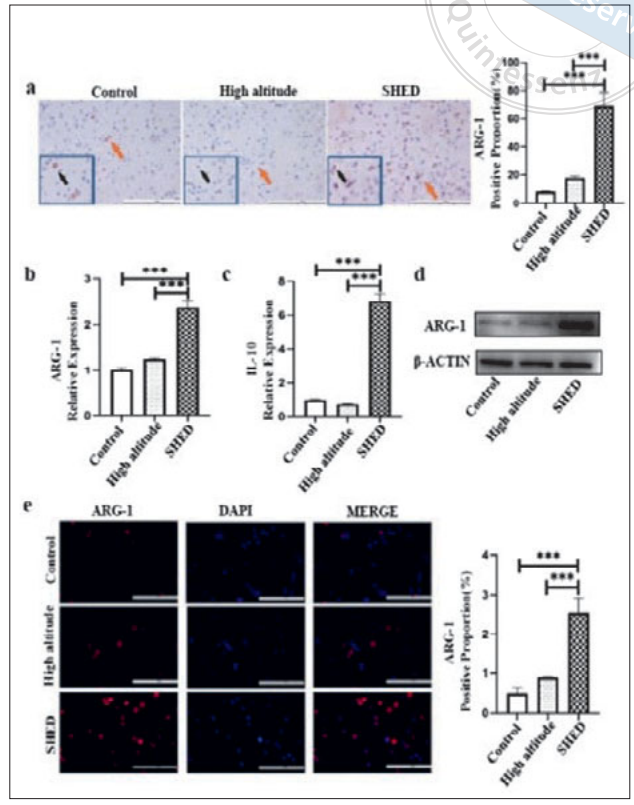

Fig 4 Stem cell therapy promoted the M2-type polarisation phenotype of BV-2 microglia in an environment mimicking high altitude. **(a)** Expression of ARG-1 in the control, high-altitude and SHED co-culture groups, as assessed by immunohistochemical staining (scale bars 200 μm). **(b and c)** Expression of ARG-1 and IL-10 in BV-2 microglia in the control, high-altitude and SHED co-culture groups, analysed by qPCR. **(d)** Expression of ARG-1 in BV-2 microglia in the control, high-altitude and SHED co-culture groups, analysed by Western blot. **(e)** Expression of ARG-1 in BV-2 microglia in the control, high-altitude and SHED co-culture groups, as assessed by immunohistochemical staining (scale bars 200 μm). Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

(Fig 5g), and immunofluorescence staining results showed the expression of ARG-1 in the YC-1 treatment group was significantly higher than that in the high-altitude group (Figs 5h and i). These results indicated that hypoxia induced HIF-1α to regulate microglia polarisation, which can be restored by SHED treatment.

SHED treatment alleviated the activation of ERK signalling pathway in an environment mimicking high altitude and low oxygen

HIF-1α activation interacts with multiple signalling pathways, such as the ERK signalling pathway[29,30]. In this study, the expression of phosphorylated ERK (p-ERK) increased significantly in the high-altitude group but decreased after co-culture with SHED, as assessed by immunofluorescence staining and Western blot (Figs 6a to c). To verify the role of the ERK signalling pathway, we used U0126, an inhibitor of this pathway, to treat microglia in an environment mimicking high altitude and low oxygen, and the results showed the decreased gene expression of M1-type polarisation markers iNOS and TNF-α (Fig 6d). The elevated expression of iNOS in the high-altitude group was inhibited after U0126 treatment, as assessed by immunofluorescence staining (Fig 6e). Meanwhile, the gene expression level of M2-type polarisation markers ARG-1 and IL-10 increased significantly after U0126 treatment (Fig 6f). The expression of ARG-1

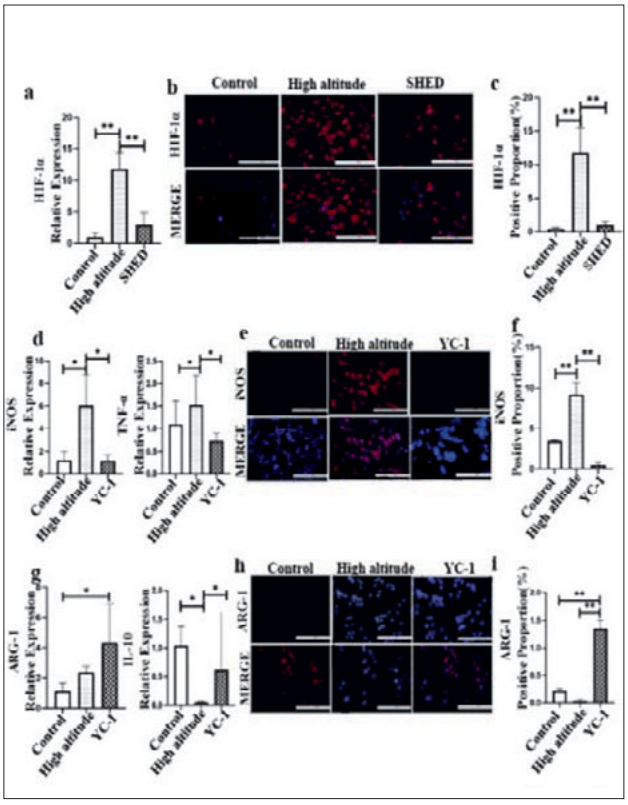

Fig 5 SHED treatment reduced the expression of HIF-1α. **(a)** Expression of HIF-1α in BV-2 microglia in the control, high-altitude and SHED co-culture groups, as assessed by qPCR. **(b and c)** Expression of HIF-1α in BV-2 microglia in the control, high-altitude and SHED co-culture groups, analysed by immunofluorescence staining (scale bars 200 μm). **(d)** Expression of iNOS and TNF-α of BV-2 microglia in the control, high-altitude and YC-1 groups analysed by qPCR. **(e and f)** Expression of iNOS in BV-2 microglia in the control, high-altitude and YC-1 groups (scale bars 200 μm). **(g)** Expression of ARG-1 and IL-10 expression in BV-2 microglia in the control, high-altitude and YC-1 groups, analysed by qPCR. **(h and i)** Expression of ARG-1 in BV-2 microglia in the control, high-altitude and YC-1 groups, as assessed by immunohistochemical staining (scale bars 200 μm). Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

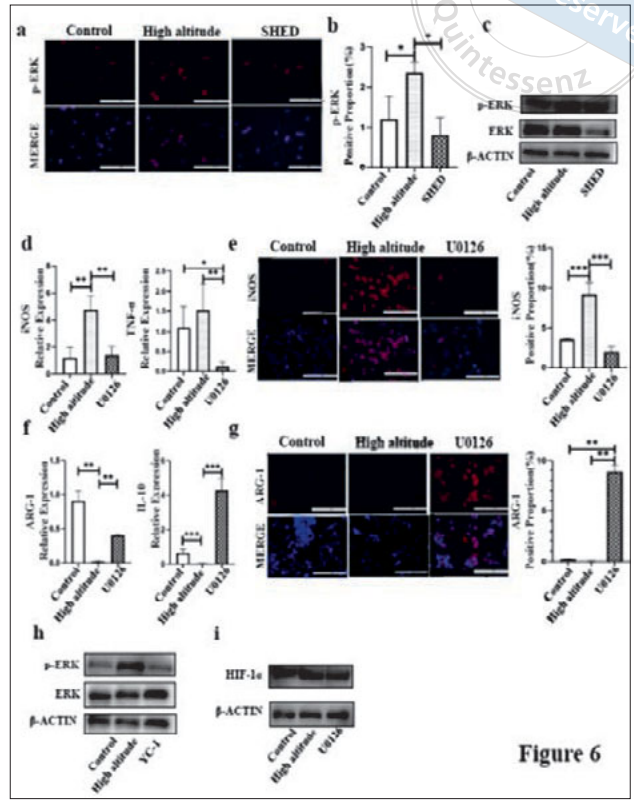

Fig 6 SHED treatment inhibited the ERK signalling pathway activated by an environment mimicking high altitude and low oxygen. **(a and b)** Expression and quantification of p-ERK in BV-2 microglia in the control, high-altitude and SHED co-culture groups, assessed by immunohistochemical staining (scale bars 200 μm). **(c)** Expression of p-ERK in BV-2 microglia in the control, high-altitude and SHED co-culture groups analysed by Western blot. **(d)** Expression of iNOS and TNF-α in BV-2 microglia in the control, high-altitude and U0126 groups analysed by qPCR (U0126 is the inhibitor of the ERK pathway). **(e)** Expression and quantification of iNOS in BV-2 microglia in BV-2 microglia in the control, high-altitude and U0126 groups (scale bars 200 μm). **(f)** Expression of ARG-1 and IL-10 in BV-2 microglia in the control, high-altitude and U0126 groups analysed by qPCR. **(g)** Expression and quantification of ARG-1 in BV-2 microglia in BV-2 microglia in the control, high-altitude and U0126 groups analysed by immunohistochemical staining (scale bars 200 μm). **(h)** Expression of p-ERK and ERK in BV-2 microglia in BV-2 microglia in the control, high-altitude and YC-1 groups analysed by Western blot. **(i)** Expression of HIF-1α in BV-2 microglia in the control, high-altitude and U0126 groups. Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

increased significantly after U0126 treatment compared with that in the high-altitude group (Fig 6g).

Next, we analysed whether the ERK signalling pathway is regulated by HIF-1α. The findings showed that HIF-1α inhibitor YC-1 treatment inhibited the activated ERK signalling under high altitude (Fig 6h), whereas ERK inhibitor U0126 treatment failed to suppress the elevated level of HIF-1α induced by high altitude hypoxia stimulation (Fig 6i). Thus, SHED can attenuate high-altitude cerebral oedema by inhibiting the M1-type polarisation of microglia and promoting M2-type polarisation, which is mediated by the HIF-1α/ERK signalling pathway (Fig 7).

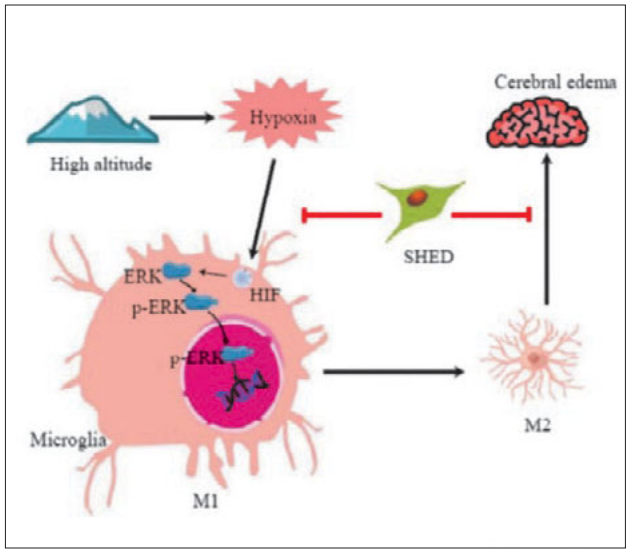

Fig 7 The schema showed that SHED treatment can relieve high-altitude cerebral oedema by inhibiting HIF-1α mediated ERK signalling activation in a hypoxic environment mimicking high altitude.

Discussion

The human body may undergo a series of physiological and pathological changes during sudden exposure to a high-altitude environment due to the thin air and low oxygen content[1,2]. The main symptoms of acute altitude sickness are headache, nausea and vomiting. If the acute altitude sickness is not treated in time, it may develop and lead to cerebral oedema or death, which is the terminal stage of acute altitude sickness. The aetiology of and treatment strategy for cerebral oedema remain to be studied. Mesenchymal stem cells have shown profound therapeutic effects on neuronal injury, such as rescuing injured neurons exposed to oxygen-glucose deprivation by inhibiting the inflammatory cytokine TNF-α[31]. In this study, we established a high-altitude cerebral oedema model to mimic a high-altitude, low-oxygen environment. The results showed that the BWC, brain oedema and cell apoptosis of brain tissues increased significantly under high altitude conditions, whereas SHED infusion can relieve cerebral oedema and reduce the proportion of apoptosis and oedema of brain cells to effectively reduce the occurrence of cell oedema. To the best of the present authors' knowledge, this study is the first report about the prevention and therapeutic effects of SHED on high-altitude cerebral oedema, suggesting that SHED is a promising strategy for preventing and treating cerebral oedema.

Mesenchymal stem cells can induce the polarisation of macrophages to a M2-type phenotype[32] and play a therapeutic role in the cardiovascular, autoimmune and central nervous systems and in the event of inflammatory disease[33,34]. Microglia, one of the main types of cells that regulate the inflammatory response after brain injury, play an important role in high-altitude sickness[35]. Microglia may differentiate into two forms: the pro-inflammatory M1 type and anti-inflammatory M2 type. Under hypoxic and ischemic conditions, microglia are prone to polarise into the M1 type, which releases pro-inflammatory cytokines, leading to secondary brain injury[36]. In the case of ischemia and hypoxia, M2-type polarisation of microglia can activate peroxisome proliferator–activated receptor gamma and promote microglia to release anti-inflammatory cytokines, which alleviates brain injury[37,38]. Regulation of microglial polarisation from M1 to M2 type may be the key to treating ischemic encephalopathy[39]. SHED have shown a profound capacity for proliferation and multi-lineage differentiation. Studies have found that SHED can affect the local microenvironment of bone regeneration, secrete anti-inflammatory factors and promote angiogenesis through the paracrine effect[40]. It has been reported that SHED and their conditioned media can be effective against neurodegeneration through multiple mechanisms, including cell replacement, paracrine effects, angiogenesis, synaptogenesis, immunomodulation and apoptosis inhibition[41]; however, the specific mechanism of SHED remains to be studied. Thus, we speculated that SHED could regulate the inflammatory environment of brain cells and relieve the symptoms of brain cell oedema. In our study, when microglia co-cultured with SHED, the mRNA and protein levels of M1-type polarisation marker molecules decreased compared with groups not co-cultured with SHED in the high-altitude conditions, whereas the levels of M2-type polarisation marker molecules increased significantly. Therefore, after hypoxia stimulation, SHED can inhibit M1-type polarisation of microglia and promote M2-type polarisation, which alleviates the occurrence of cerebral oedema.

Hypoxic conditions can activate the ERK signalling pathway and lead to a series of diseases[42]. The regulation of the ERK signalling pathway can play an important role in hypoxic disease treatment[43]. In our study, the expression of p-ERK significantly increased under hypoxia in vitro and decreased after co-culturing with SHED. The in vivo experiments indicated that the expression level of ERK increased significantly under hypoxia stimulus. These results suggest that the ERK signalling pathway regulates microglial polarisation to control high-attitude cerebral oedema. Whether other pathways participate in this process needs to be investigated further.

HIF-1α is an oxygen-sensitive transcriptional activator. Under hypoxia, HIF-1α induces regulation of angiogenesis, glucose metabolism, cell proliferation and apoptosis. Regulation of HIF-1α has become a therapeutic target for numerous diseases. HIF-1α knockdown reduces ischemic injury, which suggests that HIF-1 may contribute to tissue damage during cerebral ischemia[44]. In this study, the expression of HIF-1α increased significantly in the high-altitude group and decreased after co-culturing with SHED. Moreover, we observed that ERK signalling activation was regulated by HIF-1α. The M1-type polarisation induced by hypoxia stimulus was inhibited after ERK or HIF inhibitor treatment. These results indicate that ERK and HIF signalling may be potential targets for cerebral oedema treatment, but further investigations are still needed.

Conclusion

In this study, SHED displayed profound preventive and therapeutic effects on high-altitude cerebral oedema through regulating M1/M2 polarisation of microglia, which was controlled by HIF-1α/ERK signalling. It provides new evidence for the prevention and treatment of high-altitude cerebral oedema in the future.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Yi Ming WANG performed the experiments and drafted the manuscript; Drs Yi Kun ZHOU, Chun Shan HAN, Liu Jing CHEN and Zi Meng ZHUANG offered experimental advice; Drs Rui Li YANG and Wei Ran LI designed the study. All the authors approved the submission.

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Saliva Levels of Adrenergic Receptors in Relation to Psychological Factors in Patients with Oral Lichen Planus

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Objective: *To evaluate the saliva level of α1 and β1 adrenergic receptors (ARs) in oral lichen planus (OLP) patients.*
Methods: *This case-control study included unstimulated saliva samples from 33 OLP patients (14 erosive, 19 non-erosive) and 33 healthy controls. All participants were evaluated on psychological conditions via the Depression, Anxiety and Stress Scale – 21 items (DASS 21). The saliva levels of α1 and β1 ARs was measured by enzyme-linked immunosorbent assay (ELISA). Data were analysed with a t test using SPSS 25 (IBM, Armonk, NY, USA).*
Results: *The saliva levels of α1 and β1 ARs of OLP patients (both erosive and non-erosive forms) were significantly higher than in healthy controls. Stress levels in patients with both forms of OLP were significantly higher than in the healthy group. There was a positive correlation between salivary α1 and β1 ARs and stress, and this positive correlation was also seen for saliva β1 ARs between anxiety or depression. The saliva level of α1 ARs was inversely correlated with unstimulated salivary flow rates ($r = -0.246$; $P = 0.046$).*
Conclusion: *This study indicated that OLP patients with both erosive and non-erosive forms have higher psychological stress and saliva levels of α1 and β1 ARs than healthy controls; however, the role of α1 and β1 ARs as salivary markers with regard to the development, severity of symptoms and outcome of OLP needs further investigation.*
Key words: *adrenergic receptors, oral lichen planus, saliva, stress*
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One of the most common, immune-mediated inflammatory diseases in the oral mucosa is oral lichen planus (OLP), which occurs as the result of autoreactivity of cytotoxic T lymphocytes attacking the basal keratinocytes[1]. Middle-aged women are the most commonly affected population, and the skin, vagina, oesophagus and larynx can also be affected[2].

The aetiology of OLP is not yet fully known; however, psychological disorders such as depression, anxiety and stress contribute towards its development and can exacerbate the lesions[3]. Previous studies established that OLP patients have higher levels of depression, anxiety and stress than the general population[4]. So far, researchers have used a variety of tools to investigate the relationship between psychological factors and lichen planus, such as standard questionnaires[5], examining the polymorphism of genes involved in the stress pathways[6], and measuring the level of biological markers such as cortisol, alpha-amylase and oxidative stress in various mediums[7].

Evaluation of psychological factors in lichen planus patients through self-reporting is unreliable, because patients can easily hide emotional changes or refuse to see a psychologist or psychiatrist for diagnosis or treatment due to embarrassment or side effects of medication[4]. Thus, the use of objective indicators with quan-

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titative measurement capability should be considered.

Activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis and their specific receptors following stress is one of the proposed hypotheses in OLP[8,9]. α and β adrenergic receptors (ARs) are from G protein-coupled receptors. The catecholamines that are released in response to stress bind to these receptors. In some autoimmune diseases, the quantitative and qualitative changes of ARs on the surface of different cells have been reported, although no such reports are available on OLP patients[10]. Also, α1 and β1 ARs may be involved in associated symptoms, such as xerostomia[3]. The association between OLP and hyposalivation and/or xerostomia has been documented[11,12].

In addition, OLP has been introduced as a potentially malignant disorder, but the exact underlying mechanism initiating malignant transformation in OLP is not clear[13,14]. Activation of the sympathoadrenal system by stress can have a role in cancer initiation, progression and metastases mainly via ARs, although this relationship is highly speculative[15].

Considering the possible role of psychological stress, the sympathetic nervous system and ARs in the aetiopathogenesis of immune-mediated inflammatory diseases such as OLP and its related symptoms, this study aimed to evaluate the level of α1 and β1 ARs in the saliva of OLP patients.

Materials and methods

This case-control study was approved by the Ethics Committee of the School of Dentistry at Tehran University of Medical Sciences, Tehran, Iran (TUMS.DENTISTRY.REC.1396.4182). Thirty-three patients with definite clinical and histopathological diagnoses of OLP were selected among those referred to the Oral and Maxillofacial Medicine Department of School of Dentistry of TUMS from 5 April 2016 to 20 December 2017. A total of 33 age- and sex-matched healthy persons (Table 1) who did not have any clinical signs/symptoms of gingival inflammation or other type of oral lesion and were willing to participate in this study comprised the control group. All participants signed written informed consent forms before participating, and all research was conducted in accordance with the Declaration of Helsinki. Of all the OLP patients, 12 had erosive-atrophic, 18 had reticular, 2 had bullous and 1 had papular OLP. The authors evaluated AR levels in three groups: control, erosive OLP (erosive-atrophic and bullous, n =14), and non-erosive OLP (reticular and popular, n = 19). The inclusion criteria were definite clinical diagnosis of OLP based on the presence of bilateral reticular lesions in the oral mucosa, and fulfilment of the modified World Health Organisation histopathological criteria for OLP (well-defined band-like zones of inflammatory infiltration limited to the superficial part of the connective tissue, including mainly mature lymphocytes and vacuolar degeneration of the basal layer of the epithelium). The exclusion criteria were history of systemic or local treatments in the past 3 months, smokers, patients with systemic diseases such as cardiovascular diseases, hypertension, renal or hepatic diseases, diabetes mellitus, malignancies or neuromuscular disorders, with a history of medication intake, particularly medication affecting the ARs in the past 3 months, and pregnant or nursing women. In addition, all participants were evaluated for psychological conditions via the Depression, Anxiety and Stress Scale – 21 Items (DASS 21)[16]. Several previous studies had evaluated the psychological profile of OLP patients using DASS-2117-19, and this questionnaire has also been used in several previous studies to assess the psychological effects of drugs with an affinity to the ARs[20]. The scale can quantify the severity of depression, anxiety and stress. The validity and reliability of this questionnaire in the Persian language were examined by Samani and Jokar[21] in 2010.

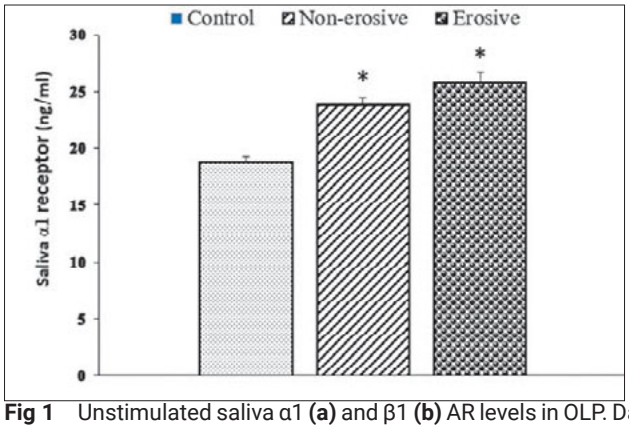
Each of the DASS subscales consists of seven questions, and the final score is obtained through the sum of the scores for the related questions. Each question is scored from 0 (“does not apply to me at all”) to 3 (“perfectly true for me”).

Saliva sample collection

Unstimulated saliva samples were collected from all participants from 9 a.m. to 12 p.m. They were instructed to refrain from eating for 2 hours before saliva sampling, then sit on a chair and spit into a plastic vial as often as possible for 5 minutes. The salivary flow rate was calculated by dividing the saliva volume (in millilitres) by time (in minutes). Hyposalivation was diagnosed when the unstimulated salivary flow rate was less than 0.1 ml/min[22].

Evaluation of the severity of OLP lesions and disease activity

To evaluate the severity of oral lichen planus lesions (severity score), the Thongprasom scoring system, as referenced in Thongprasom et al[23], was used. In this system, 0 indicates the absence of lesions, 1 denotes mild white lesions without erythematous areas, 2 indicates white striae with atrophic lesions smaller than 1 cm, 3



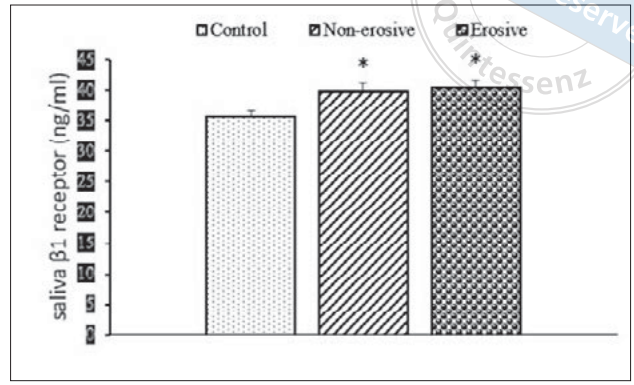


Fig 1 Unstimulated saliva α1 **(a)** and β1 **(b)** AR levels in OLP. Data are expressed as mean ± standard error of the mean and analysed using a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test. *$P < 0.05$ compared with healthy controls and non-erosive OLP, respectively.

corresponds to white striae with atrophic lesions larger than 1 cm, 4 denotes lesions with ulcerated areas less than 1 cm and 5 indicates lesions with ulcerated areas above 1 cm. The pain intensity (pain score) of OLP patients was also measured using a visual analogue scale (VAS), from 0 to 10.

Laboratory procedures

After centrifuging the samples for 10 minutes at 2000 rpm, the purified saliva (supernatant) was poured into 1-ml microtubes, kept at −20°C (as frozen) and sent to a laboratory to measure the level of α1 and β1 ARs within 24 hours. The measurements were made using the enzyme-linked immunosorbent assay (ELISA) kit for β1 and α1 ARs respectively (USCN Life Science, Wuhan, China) according to the manufacturer's protocol to determine the exact level of ARs in each individual.

Statistical analysis

The collected data were analysed using SPSS version 25.0 (SPSS, IBM, Armonk, NY, USA). A Shapiro-Wilk test was used to check normality and the level of significance was $P > 0.05$, thus they had normal distribution. The results were presented as mean ± standard error or median ± interquartile range and analysed using a *t* test, chi-square test and one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls as post hoc test, Kruskal-Wallis test, and Spearman and Pearson correlation coefficient. The level of significance was $P < 0.05$.

Results

There was no significant difference in age between the OLP (46.2 ± 2.2 years) and control (45.5 ± 2.2 years) groups ($P = 0.808$).

The level of α1 and β1 ARs in unstimulated saliva were significantly higher in OLP patients compared with the healthy controls ($P < 0.05$), but there were no significant differences in saliva levels of α1 and β1 ARs between erosive and non-erosive forms of OLP (Fig 1). Statistical analysis of the results showed that unstimulated salivary flow rates were significantly lower in OLP patients with the erosive form than in healthy controls ($P = 0.005$); however, there was no significant difference between patients with the non-erosive form of OLP and healthy controls (Fig 1). The stress score was significantly higher in both forms of OLP than in the control group, and was also higher in patients with the erosive form than those with the non-erosive form (Table 2). There was no significant difference in anxiety and depression between the three groups (Table 2).

The level of α1 and β1 ARs in saliva had no significant association with sex ($P > 0.05$). The level of α1 and β1 ARs in saliva had no significant correlation with age either ($P > 0.05$).

Saliva concentrations of β1 ARs were significantly correlated with pain, severity, stress, anxiety and depression. The saliva level of α1 ARs was significantly correlated with stress, pain and severity, but not with anxiety and depression (Table 3).

The unstimulated salivary flow rate was inversely correlated with the saliva level of α1 ARs ($r = −0.246$; $P = 0.046$) but not with the level of β1 ARs ($r = −0.099$; $P = 0.433$)

Table 1 Age and sex of participants in the two study groups

Variable	Control	OLP
N	33	33
Age (y)	45.5 ± 2.2	46.2 ± 2.2
Sex (M/F)	12/21	12/21

F, female; M, male.

Table 2 Comparison of variables between the OLP and control groups.

Variable	Control (n = 33)	Non-erosive OLP (n = 19)	Erosive OLP (n = 14)	F	*P* value
Unstimulated flow rate (ml/min)	0.43 ± 0.03	0.33 ± 0.04	0.28 ± 0.02*	4.566	0.014[a]
Anxiety (score)	4 ± 9	8 ± 5	9 ± 12	NA	0.137[b]
Depression (score)	4 ± 8	8 ± 14	7 ± 10	NA	0.474[b]
Stress (score)	0 ± 1	1 ± 3*	2 ± 1*#	NA	0.000[b]

Data are expressed as **(a)** mean ± standard error of the mean or **(b)** median ± interquartile range and analysed using a **(a)** one-way ANOVA followed by a Student-Newman-Keuls post hoc test or **(b)** a Kruskal-Wallis test. * and # indicate $P < 0.05$ compared with the control and non-erosive OLP groups, respectively. The level of significance was $P < 0.05$. NA, not applicable.

Table 3 Correlation between stress, anxiety and depression with saliva levels of α1 and β1 ARs. Data were analysed using a Spearman correlation test.

Variable	Stress	Anxiety	Depression	Severity score	Pain score
Unstimulated saliva α1 AR (ng/ml)	r = 0.418; *P* = 0.001*	r = 0.188; *P* = 0.131	r = 0.058; *P* = 0.645	r = 0.706; *P* = 0.000*	r = 0.445; *P* = 0.000*
Unstimulated saliva β1 AR (ng/ml)	r = 0.526; *P* = 0.001*	r = 0.355; *P* = 0.003*	r = 0.464; *P* = 0.001*	r = 0.404; *P* = 0.001*	r = 0.395; *P* = 0.001*

Discussion

The present findings revealed the obviously higher saliva levels of α1 and β1 ARs in OLP patients compared with healthy controls. The main sources of the whole saliva content are from saliva secreted from the salivary glands, exfoliated oral mucosa cells and substances that enter the saliva from the bloodstream. The molecules that are expressed in different parts of salivary glands, either parenchyma or ducts, can be secreted in saliva[24]. Thus, the increased saliva levels of ARs in OLP patients can be caused by overexpression of ARs in the salivary gland cells and upregulation of ARs in the peripheral blood cells or tissue of OLP lesions. Determining the exact source of the AR in saliva requires further research across multiple mediums simultaneously.

Saliva as a diagnostic medium is superior to other mediums in several ways. Saliva sampling is non-invasive, safe, simple and inexpensive. It can also be repeated without causing discomfort to the patient[14]. Whole saliva is an accessible, valuable biofluid that contains components derived from various sources such as serum and mucosal surfaces[25].

OLP, as an immune-mediated inflammatory disease, has a complex, multifactorial pathogenesis. Physiological and psychological stressors are the major environmental aetiological factor involved in autoimmune diseases[26]. The present findings confirmed the results of previous studies regarding the substantial relationship between psychogenic stress and OLP[4]. We also demonstrated that unstimulated saliva levels of α1 and β1 ARs have a positive correlation with stress, whereas anxiety and depression had a positive correlation with β1 ARs only. The sympathoadrenal axes are the two main hormonal pathways that are activated alone or together in response to stress[27].

OLP is most common in middle-aged women. Most OLP patients have experienced several stressful events in their lifetime and have an elevated hypothalamic-pituitary-adrenal (HPA) response and cortisol levels similar to those under chronic stress[9,28,29], which can cause an imbalance in the autonomic nervous system (ANS)[30]. The sympathetic nervous system (SNS) is a part of the ANS in which there is a strong correlation between the ANS and the immune system. The binding of epinephrine to ARs alters the function of immune

cells and the secretion of inflammatory mediators such as TNF-α, IL-6 and IL-10. Several pieces of evidence confirm the role of these cytokines in the aetiopathogenesis of immune-mediated inflammatory diseases, including OLP[31]. Hence, these inflammatory factors can be considered as an intermediate link between the sympathetic nervous system and the development of autoimmune diseases[32].

Given these preconditions, in the present study, we showed the relation between the essential components of the SNS (ARs) and OLP.

A three-way association between psychological factors, the ANS and the immune system has been demonstrated in the development and severity of a variety of autoimmune diseases such as rheumatoid arthritis[32,33].

Psychosomatic disorder is a specific term to define clinical symptoms in a single organ system that are due to emotional factors, usually through autonomic nervous system innervation. In 2001, Bailoor and Nagesh[34] classified OLP as an oral psychosomatic disorder. Stress leads to the release of noradrenaline and adrenaline. This causes the activation of the SNS, both centrally and peripherally[35]. Several studies have demonstrated overactivity of SNS and higher levels of noradrenaline and adrenaline in OLP patients[36]. Activation of the sympathetic α/β-AR, mainly in innate immune cells, can result in either reduced or increased inflammatory cytokine production depending on timing and local noradrenaline concentration and influence the development and severity of inflammation[22]. Chronic stress can cause immune activation and suppresses immune-protective parameters that increase the risk of inflammatory/autoimmune disease such as OLP[37].

Along with this phenomenon, we also reported a significant relationship between the level of stress and the severity of lichen planus lesions. The higher incidence of major depression, anxiety and mood disorders by upregulating inflammatory cytokines such as IL-17 can be effective in the development and severity of inflammatory diseases[38]. The moderating role of immunosuppressive drugs and parasympathetic stimulation on psychological symptoms and autoimmunity confirms this[39]. In the present study, the positive correlation between stress, anxiety and depression with saliva levels of α1 and β1 ARs supports the above. Like in other immune-mediated inflammatory/autoimmune diseases, such as systemic sclerosis, an overactive SNS is associated with decreased parasympathetic system function[40]. Previous studies have shown downregulation of muscarinic receptors in OLP patients[41]. Overexpression of ARs may reflect increased SNS activity in OLP patients. Upregulating the activity of the SNS by increasing the production of salivary mucin via β1 ARs and proteins via ARs α1 and β1 and vasoconstriction can play a role in reducing the salivary flow rate in OLP patients[42]. The results of the present study also showed a negative correlation between salivary flow rate and AR levels.

In addition, changes in the levels of ARs can be effective in malignant transformation in OLP. Once the catecholamine neurotransmitter binds to an adrenoreceptor, it causes the initiation of signalling pathways that cause uncontrolled cell proliferation, invasion, migration and metastasis that play the main role in carcinogenesis[43]. Several studies have reported overexpression of α1 and β1 ARs in oral squamous cell carcinoma tissue compared with normal mucosa[44,45].

The results of the present study present an opportunity for better understanding of the interaction between the ANS, inflammation and the onset and severity of autoimmune diseases. Disruption of this three-way cycle through a variety of physiological and psychological therapies such as medications, stress reduction protocols and lifestyle changes can be considered in the treatment of immune-mediated inflammatory diseases, including OLP. Further studies with special attention paid to histological analysis are needed to confirm the overexpression of α1 and β1 ARs in OLP.

Conclusion

This study indicated that OLP patients with both erosive and non-erosive forms have higher psychological stress and saliva levels of α1 and β1 ARs than healthy controls; however, the role of α1 and β1 ARs as a saliva marker in the development, severity of symptoms and outcome of OLP needs further investigation.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Narges GHOLIZADEH and Nafiseh SHEYKHBAHAEI designed the study, collected and analysed the data and drafted the manuscript; Drs Arvin REZAYI and Iraj MIRZAII-DIZGAH collected and analysed the data. All authors reviewed and approved the manuscript.

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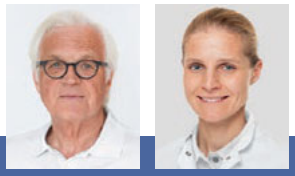

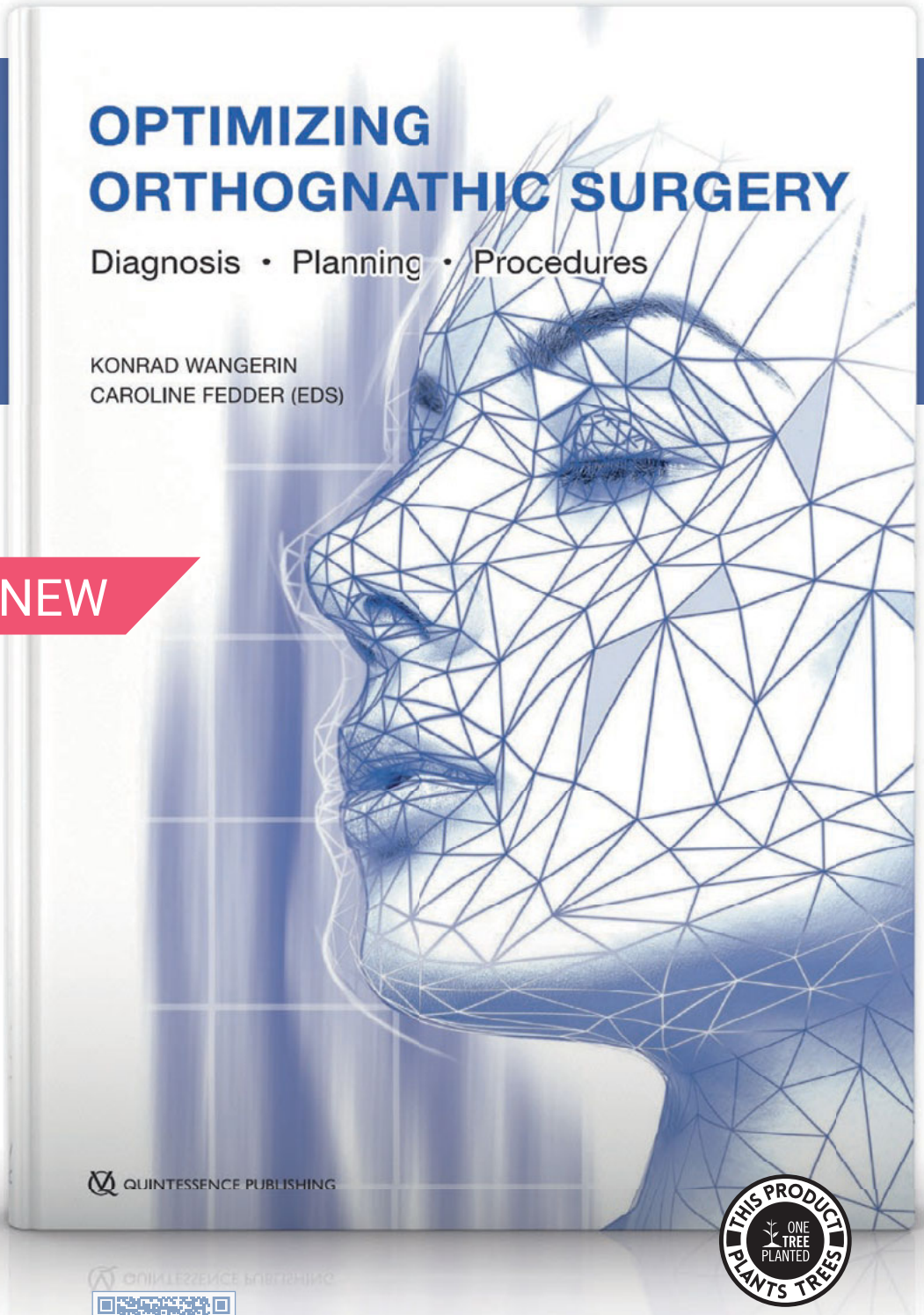

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Oral Health Status of Disabled Children and Adolescents in China

Lu GAO[1], Xue Nan LIU[2]

Objective: *To investigate the dental caries status and periodontal status of disabled children and adolescents aged 0 to 18 years in China and provide suggestions for future policies.*
Methods: *The cross-sectional survey included disabled children and adolescents aged 0 to 18 years in welfare institutions across 10 provinces in eastern, central and western regions in China, between November 2013 and May 2015. Oral health examination included dental caries status and periodontal status. The mean score for decayed, missing and filled teeth (dmft/DMFT) due to caries in primary/permanent dentition, gingival bleeding rate and calculus rate were recorded and statistically evaluated. Data analysis was performed using SPSS 24.0 software (IBM, Armonk, NY, USA), and the level of statistical significance was $P < 0.05$.*
Results: *The mean dmft/DMFT values of for groups aged 0 to 5, 6 to 12 and 13 to 18 years were 1.48 ± 3.00, 2.19 ± 2.94 and 1.78 ± 2.93, respectively. The mean scores for the groups aged 0 to 5 ($P < 0.05$) and 13 to 18 years ($P < 0.01$) showed a significant difference among different disability types. A significant difference between sexes was found only in the group aged 0 to 5 years ($P < 0.05$). Gingival bleeding rates were 13.70% for the group aged 0 to 5 years, 24.81% for the group aged 6 to 12 years and 42.06% for the group aged 13 to 18 years, and calculus rates were 5.48%, 22.41% and 47.62% for the three age groups, respectively. The gingival bleeding rate for all three age groups showed a significant difference between different disability types ($P < 0.01$), whereas calculus rates a showed significant difference only in the groups aged 6 to 12 and 13 to 18 years ($P < 0.01$).*
Conclusion: *The prevalence of dental caries, gingival bleeding and calculus in disabled children and adolescents in China is high. Disability type was strongly associated with oral health status. Specially designed oral health education and training are necessary for clinical professionals and caregivers.*
Key words: *calculus, China, dental caries, disabled children and adolescents, gingival bleeding*
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Oral health plays an important role in function and aesthetics, as well as growth and development for children and adolescents. According to the Second National Sampling Survey of China on Disabled Population conducted in 2006, there were 2.46 million disabled children and adolescents aged 6 to 14 years, accounting for 2.96% of the total disabled population[1]. In general, children with disabilities suffer from worse oral conditions and higher incidence of untreated caries, gingivitis and periodontal disease than those of the non-disabled population[2-6].

Dental caries is the most common oral disease among children and adolescents in the world, affecting eating function and patterns, development of permanent dentition and general health in the long term[7].

Table 1 Demographic characteristics of disabled Chinese children and adolescents aged 0 to 18 years.

Variable		n	%
Sex (n = 1355)	Male	828	61.1
	Female	527	38.9
Age, y (n = 1379)	0–5	157	11.4
	6–12	589	42.7
	13–18	633	45.9
Disability type (n = 1306)	Visual	243	17.6
	Hearing	316	22.9
	Verbal	124	9.0
	Physical	94	6.8
	Intellectual	513	37.2
	Mental	16	1.2

Periodontal disease is also one of the most prevalent oral health diseases worldwide, affecting not only the oral cavity but also systematic chronic diseases like infective endocarditis and diabetes mellitus[8].

China is the largest developing country in the world and has made great efforts regarding social and economic growth and development in the past decade, yet the oral health status of disabled children and adolescents has still lacked multiregional data up to now. The aim of this study was to investigate the dental caries and periodontal health status of disabled children and adolescents in China aged 0 to 18 years in order to provide suggestions for future policies and actions to better benefit this population.

Materials and methods

Ethical approval (approval no. PKUSSIRB-20130069) was obtained from the Institutional Review Board of Peking University Hospital of Stomatology. Written informed consent was obtained from the children's guardians before the survey.

Study design

Disabled children and adolescents aged from 0 to 18 years were recruited as participants in this study. The sample size was estimated based on calculation formulae. The prevalence of dental caries was estimated as 50% based on the reference[9]. The 95% confidence interval (CI) was set at 10% with two sides. To account for the stratification factor and an anticipated response rate of 90%, the minimum sample size was 954. A convenience sampling method was adopted for this survey. All the participants who met the criteria for disability were selected from 25 special education schools or welfare institutions in 10 provinces/autonomous regions, namely Guangdong, Guangxi, Heilongjiang, Liaoning, Xinjang, Gansu, Shanxi, Anhui, Fujian and Guizhou.

Clinical examination

The oral examination included dental caries experience and periodontal health status. Dental caries experience was measured using the decayed, missing and filled teeth (dmft/DMFT) index according to World Health Organisation (WHO) criteria[10]. For periodontal status, gingival bleeding and calculus status were recorded. An artificial light source, planar mouth mirror and Community Periodontal Index probe were used for oral examination. The disability type was also recorded according to the criteria in the Second National Sampling Survey of China on Disabled Population.

Before the study, all examiners were trained on the inspection standard. After training, a consistency test with the reference examiner and the examiner's own standard was performed. One or two examiners selected from each province attended the training programme and then conducted all the clinical examinations in their own province. Only examiners with a consistency test result higher than 0.8 could take part in further examinations. The survey was conducted from November 2013 to May 2015.

Statistical analysis

Data analysis was performed using SPSS 24.0 software (IBM, Armonk, NY, USA). A descriptive analysis of each factor involved was performed for demographic characteristics, dt/DT, mt/MT, ft/FT, dmft/DMFT, caries prevalence, gingival bleeding rate and calculus rate. Furthermore, the mean value of dmft/DMFT was compared among all categories of involved factors using non-parameter tests (Mann-Whitney test for variables

Table 2 Caries prevalence and dt, mt, ft, dmft [should these be written in capitals?] for children aged 0 to 5 years.

Variable	dt		mt		ft		dmft	Caries prevalence (%)
	Mean ± SD	Ratio (%)	Mean ± SD	Ratio (%)	Mean ± SD	Ratio (%)	Mean ± SD	
Total	1.48 ± 3.00	100.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	1.48 ± 3.00	30.60
Male	1.65 ± 3.05	100.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	1.65 ± 3.05	34.00
Female	1.20 ± 2.93	100.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	1.20 ± 2.93	25.00
Visual	0.90 ± 1.45	100.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	0.90 ± 1.45*	30.00
Verbal and hearing	1.33 ± 2.72	100.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	1.33 ± 2.72	30.40
Physical	5.90 ± 5.72	100.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	5.90 ± 5.72	60.00
Intellectual and mental	0.75 ± 1.94	100.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	0.75 ± 1.94	25.00

*Difference among groups: $P < 0.05$.

Table 3 Caries prevalence and dt + DT, mt + MT, ft + FT, dmft + DMFT of children aged 6 to 12 years.

Variable	dt + DT		mt + MT		ft + FT		dmft + DMFT	Caries prevalence (%)
	Mean ± SD	Ratio (%)	Mean ± SD	Ratio (%)	Mean ± SD	Ratio (%)	Mean ± SD	
Total	2.12 ± 2.88	96.80	0.05 ± 0.41	2.28	0.02 ± 0.23	0.91	2.19 ± 2.94	56.54
Male	2.23 ± 2.96	97.38	0.04 ± 0.28	1.75	0.02 ± 0.19	0.87	2.29 ± 3.00	57.34
Female	1.95 ± 2.78	94.66	0.08 ± 0.56	3.89	0.03 ± 0.29	1.46	2.06 ± 2.88	54.95
Visual	2.04 ± 2.35	98.08	0.03 ± 0.21	1.44	0.01 ± 0.09	0.48	2.08 ± 2.36	63.48
Verbal and hearing	2.40 ± 3.44	98.77	0.01 ± 0.16	0.41	0.01 ± 0.12	0.41	2.43 ± 3.46	57.14
Physical	2.63 ± 3.09	97.41	0.07 ± 0.44	2.59	0.00 ± 0.00	0.00	2.70 ± 3.27	63.04
Intellectual and mental	2.15 ± 2.84	93.89	0.09 ± 0.59	3.93	0.05 ± 0.35	2.18	2.29 ± 2.95	57.33

Table 4 Caries prevalence and DT, MT, FT and DMFT of adolescents aged 13 to 18 years.

Variable	DT		MT		FT		DMFT	Caries prevalence (%)
	Mean ± SD	Ratio (%)	Mean ± SD	Ratio (%)	Mean ± SD	Ratio (%)	Mean ± SD	
Total	1.54 ± 2.61	86.52	0.14 ± 0.64	7.87	0.10 ± 0.48	5.62	1.78 ± 2.93	50.90
Male	1.38 ± 2.27	90.12	0.10 ± 0.59	6.41	0.05 ± 0.27	3.47	1.74 ± 2.57*	48.30
Female	1.83 ± 3.09	82.99	0.21 ± 0.71	9.43	0.17 ± 0.69	7.58	2.63 ± 3.94	55.50
Visual	1.90 ± 2.42	90.63	0.05 ± 0.32	2.23	0.15 ± 0.61	7.14	2.09 ± 2.80**	65.40
Verbal and hearing	0.88 ± 1.52	79.25	0.20 ± 1.08	17.92	0.03 ± 0.23	2.83	1.10 ± 1.89	38.50
Physical	1.72 ± 2.17	91.67	0.16 ± 0.45	8.33	0.00 ± 0.00	0.00	1.37 ± 3.06	65.60
Intellectual and mental	1.20 ± 2.45	84.62	0.06 ± 0.55	8.92	0.03 ± 0.23	6.46	1.88 ± 2.46	56.50

*Difference among groups: $P < 0.05$; **Difference among groups: $P < 0.01$.

with two categories and Kruskal-Wallis test for these factors with three or more categories). The gingival bleeding rate and calculus rate were compared using a chi-square test.

Results

A total of 1379 disabled children and adolescents (mean age 11.48 ± 4.27 years) underwent oral health examination. The demographic information for participants enrolled in the study is shown in Table 1.

Tables 2 to 4 show the prevalence of dental caries, mean dmft/DMFT values and the subgroup (dt/DT, mt/MT and ft/FT) values in the groups aged 0 to 5, 6 to 12 and 13 to 18 years under different stratification of sex and disability type. The results found that 30.60% of disabled children aged 0 to 5 years, 56.54% of children aged 6 to 12 years and 50.90% of adolescents aged 13 to 18 years experienced dental caries. The mean DMFT values in the groups aged 0 to 5, 6 to 12 and 13 to 18 years were 1.48 ± 3.00, 2.19 ± 2.94 and 1.78 ± 2.93, respectively.

A significant difference between sexes was only found in the group aged 0 to 5 years ($P < 0.05$). Both the mean dmft value for the group aged 0 to 5 years ($P < 0.05$) and that for the group aged 13 to 18 years ($P < 0.01$) showed a significant difference among different disability types.

The periodontal status of the disabled children and adolescents included in this study is shown in Figs 1 to 4. As per the results, 13.70% of the group aged 0 to 5 years, 24.81% of that aged 6 to 12 years and 42.06% of

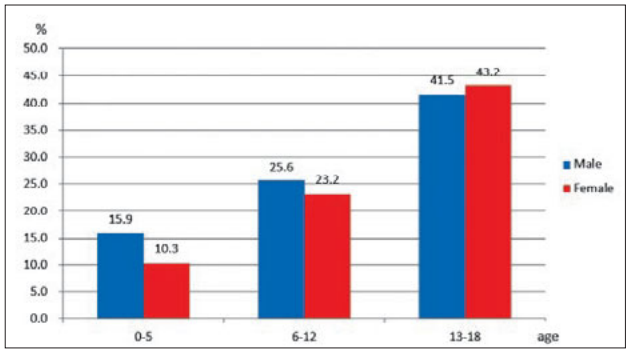

Fig 1 Gingival bleeding rate for children and adolescents aged 0 to 5, 6 to 12 and 13 to 18 years by sex.

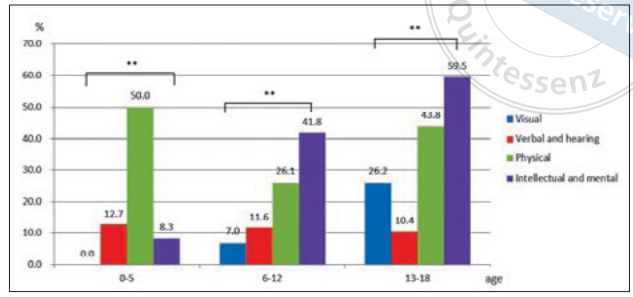

Fig 2 Gingival bleeding rate for children and adolescents aged 0 to 5, 6 to 12 and 13 to 18 years by disability type. **Difference among groups: $P < 0.01$.

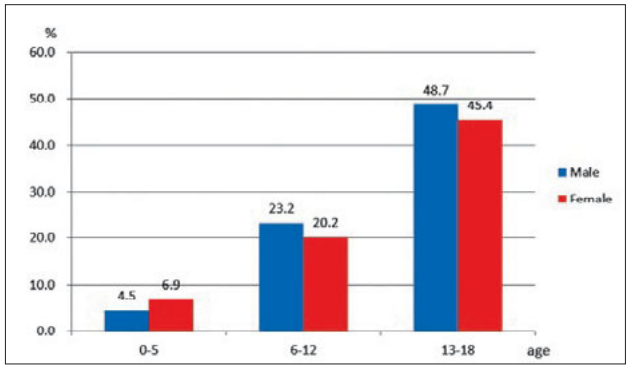

Fig 3 Dental calculus rate for children and adolescents aged 0 to 5, 6 to 12 and 13 to 18 years by sex.

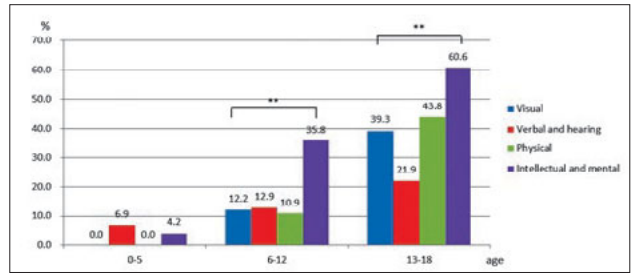

Fig 4 Dental calculus rate for children and adolescents aged 0 to 5, 6 to 12 and 13 to 18 years by disability type. **Difference among groups: $P < 0.01$.

that aged 13 to 18 years suffered from gingival bleeding. The gingival bleeding rate increased with age. Participants who were physically disabled reported a worse gingival bleeding rate than those with other disability types in the group aged 0 to 5 years, as well as the intellectually and mentally disabled group in the children and adolescents aged 6 to 12 and 13 to 18 years ($P < 0.01$).

The calculus rates for the three age groups were 5.48%, 22.41% and 47.62%, respectively, also showing an increasing trend with age. Of the participants aged 6 to 12 and 13 to 18 years, the intellectually and mentally disabled group reported a worse calculus rate than other disability types ($P < 0.01$).

Discussion

According to an estimate from the WHO[11], the proportion of the population living with disabilities or special needs in developing countries amounts to 12%, whereas the same indicator in developed countries is approximately 10%. This is the first study to conduct a large-scale oral health survey covering different types of disabled children and adolescents from eastern, central and western regions of China. The study used data sourced from special education schools or welfare institutions in 10 provinces. Descriptive and univariate analyses of the oral health condition were used to provide a necessary basis for the health authorities to improve the dental health of disabled children and adolescents in China.

The prevalence of caries in disabled children and adolescents (6 to 12 years 56.54%, 13 to 18 years 50.90%) was higher than that in children without disabilities (12 years 38.5%, 15 years 44.4%)[12]. This result was similar to the findings of previous studies[2,4,11,13] and may be related to their disabilities relating to cognition, memory, communication and manual dexterity, as well as a lack of sufficient oral health promotion and treatment resources. Diet is likely the main reason, since sweets are given by parents to children with disabilities mostly as a reward for behaviour control. Moreover, the proportion of dt/DT for both primary and permanent teeth (0 to 5 years 100.00%, 6 to 12 years 96.80%, 13 to 18 years 86.52%) remained at a higher level than those without

disabilities (3 to 5 years 96.6%, 12 years 83.4%, 15 years 81.3%)[12,14] indicating that children with disabilities have insufficient treatment for caries.

Disabled children and adolescents in all age groups suffered from periodontal problems and their periodontal health became worse as they got older.

The significant difference in caries status recorded in the present study between male and female patients aligns with that found in national surveys[12]. A possible explanation for the higher level of dental caries in female patients is the increased frequency of snacking.

The findings indicated that disability type was strongly associated with not only the dmft of primary teeth and DMFT of permanent teeth, but also the prevalence of gingival bleeding and calculus. Children with intellectual, mental, visual and physical disabilities have poorer oral health status than those with verbal and hearing disabilities. There are many possible explanations for this. It may because it is more difficult for individuals with intellectual, mental and visual disabilities to access social and dental care resources, meaning they are at a disadvantage when it comes to understanding the importance of oral hygiene. Such barriers make it more difficult for intellectually and mentally disabled people to comprehend complex tasks. Regarding physically disabled people, handling instruments, like toothbrushes or floss, is more difficult than it is for others.

Disabled individuals are at a high risk of health problems, especially young children and adolescents who largely rely on family and social support[15]. The oral health status of children with special needs is initially similar to that of normal children, but their diet, eating patterns, medication needs, physical limitations and lack of oral cleaning ability, as well as the attitude of their caregivers, contribute to a subsequent decline in their oral health status[16]. The reduced access to oral health care for disabled children and adolescents results from financial, social and physical barriers, as well as their inability to cooperate during treatment or routine oral health care[4,7,11,13,17].

To promote the oral health of children and adolescents with disabilities, a targeted approach is necessary. Educational activities are the most common method and should be adapted to individuals' needs based on their type of disability. Hartwig et al[18] carried out a quasi-experimental study of the effects of oral health educational intervention for children and adolescents with neurological and psychomotor disabilities in southern Brazil. The clinical examination showed a significant decrease in dental debris and gingival indices compared with baseline after supervised tooth brushing and educational activities[18]. Alwadi et al[19] described different inclusive methods to allow children to share their opinions on oral health in the study, mainly through children's knowledge and their oral health practices and experiences of visiting dental clinics, as well as the physical barriers they experienced and their positive and negative feelings about oral health. Their findings highlight that it is possible to include disabled children in the oral health process is possible, and this will help to transform services and reduce such children's oral health inequalities[19]. Sardana et al[16] delivered specially designed oral health education and performed two different motivational techniques using tactile or auditory sensations. A significant improvement in mean plaque and gingival scores was noted at the 6-month evaluation, indicating that tactile and auditory measures were effective in educating and motivating visually impaired children regarding daily oral hygiene maintenance[16].

Clinical professionals play an important role in treatment success and sustainable monitoring of oral health status for disabled children and adolescents. To ensure the dental workforce is able to respond to the needs of those with disabilities, education is essential[20]. Education in tooth brushing and oral health knowledge for disabled children and their caregivers, regular oral examination and fluoride treatment have been carried out, but relevant training for clinical professionals in special skills required to deal with children and adolescents with special needs is still insufficient[17].Wilson et al[20] carried out a survey and discussion on final-year dental students' insight into disability-related issues. The results highlighted the importance of tailoring education for medical students and young clinical professionals to increase their capability and confidence in treating and helping disabled children and adolescents more effectively[20]. The ability to provide systematic and targeted oral health services for disabled children and adolescents is in urgent need of strengthening. Performing dental treatment under general anaesthesia can improve the oral quality of life in children with disabilities and their families significantly. Notable progress has been made in China regarding general anaesthesia techniques[21].

Caregivers play an essential role in the daily care and education of disabled children and adolescents[22]. It is thus extremely important to alert caregivers about the importance of prophylactic and therapeutic measures of oral health[3,13]. Abullais et al[13] carried out a questionnaire-based study to evaluate caregivers' knowledge of, attitude towards and practices employed in oral healthcare at centres for the intellectually disabled in southern Saudi Arabia. They found that the care-

givers' knowledge and attitude needed improvement and that they should be advised to participate in training programmes on the importance of preventive oral health services and dental treatments[13]. Zeng et al[3] used a questionnaire to analyse caregivers' oral health knowledge and attitudes in Shanghai city and found their knowledge to be low; thus, tailored educational programmes should be carried out for caregivers to strength their oral health education, especially in suburban areas.

The present study has some limitations. First, limited by the characteristic of the disabled children and adolescents, a randomised sampling method was unable to be used for the study design. Second, only univariate analysis was applied, which could not avoid mutual effects between different variables. Third, the social and economic status, oral health habits and other information relating to the participants requires further analysis. Nevertheless, the results reflect the oral health status of and associated factors for disabled children and adolescents to some extent.

Conclusion

The prevalence of dental caries of disabled children and adolescents in China is high and was higher than the mean level in the fourth national survey. All age groups of disabled children and adolescents suffered from periodontal problems and their periodontal health became worse as they got older. Disability type was strongly associated with dental caries, gingival bleeding and calculus, all of which may be related to a lack of sufficient oral health promotion and treatment resources. Specially designed oral health education and training for clinical professionals and caregivers are important and indeed necessary to improve the oral health status of disabled children and adolescents.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Lu Gao participated in the data analysis and drafted the manuscript; Dr Xue Nan LIU designed the study, collected the data and revised the manuscript. Both authors read and approved the final manuscript for submission.

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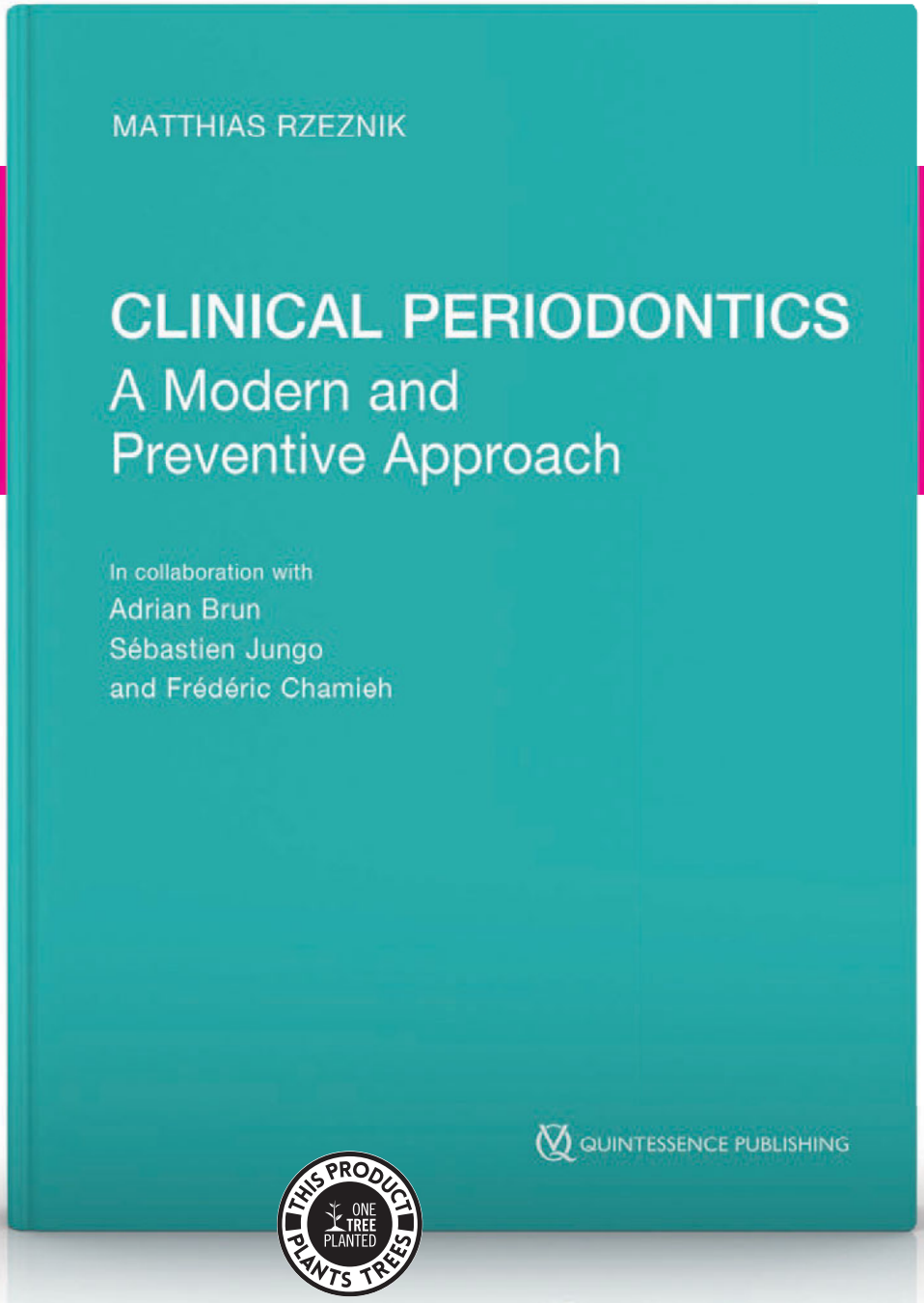

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Effect of Dentine Conditioning with Silver Diamine Fluoride on Wettability of Root Canal Sealers

Surmayee SINGH[1], Rajat KUNDRA[1], Sajan Daniel GEORGE[2], Swithin HANOSH[2], Prasanna NEELAKANTAN[3], Manuel S THOMAS[1]

Objective: *To test the null hypothesis that dentine treatment with silver diamine fluoride (SDF), a potent antimicrobial agent, following use of proteolytic and chelating agents does not influence the wettability of an epoxy resin (AH Plus, Dentsply Sirona, Charlotte, NC, USA) and a tricalcium silicate sealer (BioRoot RCS, Septodont, Saint-Maur-des-Fossés, France).*

Methods: *Seventy-two intraradicular dentine specimens were divided into six groups based on the final irrigation solutions used: 2.5% sodium hypochlorite (NaOCl) and 17% ethylenediaminetetraacetic acid (EDTA) (NaOCl-EDTA) (group 1); NaOCl-EDTA-NaOCl (group 2); NaOCl-EDTA followed by 3.8% SDF, NaOCl-EDTA-SDF (group 3); NaOCl-EDTA-NaOCl-SDF (group 4); SDF (group 5) and saline (group 6). After irrigation, the specimens were divided into subgroups according to the sealer used, AH Plus or BioRoot RCS. Contact angles were measured using a contact angle analyser. The data were analysed using an independent t test, one-way analysis of variance (ANOVA) and Tamhane T2 post hoc test, with the level of significance set at $P < 0.05$.*

Results: *In the epoxy resin sealer group, dentine surfaces treated with only SDF showed the lowest contact angle. This was significantly less than the groups in which NaOCl was used as the final irrigant ($P < 0.05$). In the tricalcium silicate–based sealer group, the groups treated with SDF showed significantly greater contact angles when compared to the control group ($P < 0.05$).*

Conclusion: *It was concluded that SDF conditioning of dentine favours the wettability of epoxy resin sealer but is detrimental to the wettability of tricalcium silicate sealer.*

Key words: *bioceramic sealer, contact angle, epoxy resin sealer, ethylenediaminetetraacetic acid, silver diamine fluoride, sodium hypochlorite*

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Root canal disinfection aims to eliminate debris, microorganisms and toxins from the root canal system to facilitate healing of the periradicular tissues. This is primarily achieved using various root canal irrigation solutions. The currently recommended irrigation regimen involves a sequence of proteolytic irrigants such as sodium hypochlorite (NaOCl) and demineralising/chelating agents such as ethylenediaminetetraacetic acid (EDTA). To further improve the antimicrobial effect of this sequence, some studies recommend final rinsing with sodium hypochlorite or antiseptics such as chlorhexidine[1,2]. Despite this, it has been shown that biofilms can recover on irrigated dentine[3,4]. Thus, practitioners are constantly searching for alternative and supplementary irrigants to be used after NaOCl-EDTA.

Silver diamine fluoride (SDF), a potent antimicrobial agent, is a Food and Drug Administration (FDA)-approved treatment for preventing dental caries. The original formulation, i.e., 38% w/v Ag $(NH_3)F_2$, consists of 24.4% to 28.8% (w/v) silver, 5.0% to 5.9% fluoride and around 8% ammonia[5]. SDF has been shown to eradicate biofilm, prevent collagenolytic activity in dentine and

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restore dentine microhardness[5,6]. Its antimicrobial action is attributed to silver and fluoride ions which can interfere with microbial cell division, cellular respiration, metabolism, biofilm formation and adhesion[7]; however, this formulation causes blackish discolouration of the teeth. As such, a 1:10 diluted version of SDF has been proposed as a root canal disinfectant[8]. The diluted SDF solution has demonstrated good penetrability[8], acceptable biocompatibility[9], bactericidal action[7,8,10] and fungicidal action[11] as well as substantivity[12]. Thus, 3.8% SDF can be considered the last solution to be used within the root canal to maximise canal disinfection.

On the other hand, irrigating sequences alter the compositional and surface characteristics of dentine, which influence the interactions between it and root canal sealers[13]. Specifically, it has been shown that irrigants influence the wettability and adhesion of root canal sealers[14,15]. The relative surface free energy, also known as the wetting ability of a material on the dentine surface, influences the material's spreadability, adaptation and adhesion to dentine[14,16]. Surface free energy is measured and expressed in terms of the contact angle between a liquid and a solid surface. The contact angle and surface free energy are inversely related. Therefore, if the value of the contact angle is low, the surface free energy (wettability) will be greater[17]. Close adaptation of the root canal filling to the dentine is considered to provide a durable seal that can entomb bacteria and prevent microbial recolonisation[18]. Although the prospect of using SDF as a root canal disinfectant appears promising, little is known about its impact on the wettability of root canal sealers. Thus, the present study aimed to assess the wettability of an epoxy resin and tricalcium silicate sealer on dentine surfaces treated with diluted SDF following the use of a proteolytic and chelating agent. The null hypothesis tested was that dentine treatment with 3.8% SDF following the use of a proteolytic and chelating agent does not influence the wettability of an epoxy resin and a tricalcium silicate sealer.

Materials and methods

Study design and approval

The in vitro study was initiated after obtaining ethical clearance from the Institutional Ethics Committee (reference no. 21030).

Sample size calculation

In reference to the article by Gandhi et al[15], and with 5% alpha error, 80% power of the study and a clinically significant difference of fifteen units, the required sample in each group for this study was determined to be six using PASS 11.0.7 (NCSS, LCC, Kaysville, UT, USA).

Tooth selection and preparation

Prior to the experiment, 36 premolars with single root canals extracted for orthodontic treatment were cleaned and stored in 0.2% sodium azide (Sigma Aldrich, St Louis, MO, USA) at 4^{0}C until use. The teeth included had a mature apex and no cracks or apical resorption. Teeth with root canal filling and calcified canals were excluded from the study.

Specimen preparation

A low-speed diamond disc (Diamond Disk DA0001 4A, Toboom, Shanghai, China) under water coolant was used to decoronate the teeth at the cementoenamel junction and for apical third resection. The radicular sections were then split longitudinally in a buccolingual direction into 72 halves, then a 600-grit silicon carbide paper under distilled water was used to polish these sections for 15 seconds to produce a uniform smooth surface for analysis and standardise the thickness of the smear layer. The dimension of the dentine disc was maintained at around 5 × 5 × 2 mm (width × length × thickness).

Experimental groups

The dentine discs (n = 72) were randomly divided into six groups and the dentine specimens were immersed in the irrigating solution corresponding to 2 ml per sample as described below. In groups 3, 4 and 5, the dentine specimens were saturated with 3.8% SDF and spread on the surface using a microtip brush (Denmax International, Bengaluru, India). The details of the irrigants and sealer used in the study are shown in Table 1. The groups were as follows:

- Group 1: 2.5% NaOCl for 1 minute followed by 17% EDTA for 1 minute (NaOCl-EDTA);
- Group 2: 2.5% NaOCl for 1 minute, followed by 17% EDTA for 1 minute, then a final flush of 2.5% NaOCl for 1 minute (NaOCl-EDTA-NaOCl);
- Group 3: 2.5% NaOCl for 1 minute, followed by 17% EDTA for 1 minute, then application of 3.8% SDF for 3 minutes (NaOCl-EDTA-SDF);
- Group 4: 2.5% NaOCl for 1 minutes, followed by 17%

Table 1 Details of the materials used in the present study.

Material	Abbreviation	Manufacturer	Composition and Usage
Sodium hypochlorite	NaOCl	Vishal Dentocare Pvt, Ahmedabad, India (lot no. VM-122)	The samples were immersed in 2.5% NaOCl for the specified time
Ethylenediaminetetra-acetic acid	EDTA	B.N Laboratories, Mangalore, India (batch no. 23)	The samples were immersed in 17% EDTA for the specified time
Silver diamine fluoride	SDF	Saforide RC, Bee Brand Medico Dental, Osaka, Japan) (manufacturing no. 912 GA)	3.8% SDF [$Ag(NH_3)F_2$] was applied on the samples using a microtip brush for the specified time
Epoxy resin–based sealer	ER	AH Plus (Dentsply, Sirona, Charlotte, NC, USA) (lot no. 2204000468)	Paste A (epoxide paste): diepoxide, calcium tungstate, zirconium oxide, silica, iron oxide pigment; Paste B (amine paste): Dibenzydiamine, aminoadamante, trycyclodecane-diamine, calcium tungstate, zirconium oxide, silica, silicone oil. 1:1 ratio of both pastes was mixed on a paper pad to a homogenous consistency
Tricalcium silicate sealer	C3S	BioRoot RCS (Septodont, Saint Maur-des-Fosses, France) (lot no. B28766)	Powder: tricalcium silicate, zirconium oxide and povidone; liquid: aqueous solution of calcium chloride and polycarboxylate. One scoop of powder is mixed with 5 drops of liquid to form a smooth paste.

EDTA for 1 minute, 2.5% NaOCl for 1 minute, and then a final application of 3.8% SDF for 3 minutes (NaOCl-EDTA-NaOCl-SDF);
- Group 5: 3.8% SDF application for 3 minutes (SDF);
- Group 6: Saline application for 3 minutes.

The treatment of the dentine discs was performed by a single operator. Following irrigation, the dentine discs were submerged in deionised water for around 2 minutes to remove the test solutions. The specimens were then dried with absorbent paper to remove any excess water without drying the specimen. Following this, the twelve specimens in each group were randomly divided into two subgroups with six samples each, based on the sealer tested. Both epoxy resin (ER) sealer (AH Plus, Dentsply Sirona, Charlotte, NC, USA) and tricalcium silicate sealer (C3S) sealer (BioRoot RCS, Septodont, Saint-Maur-des-Fossés, France) were manipulated according to the manufacturer's instructions (Table 1).

Contact angle measurement

The specimens were stabilised on a glass slab using cyanoacrylate glue (Feviquik, Pidilite, India). Following this, 0.2 ml AH Plus and BioRoot RCS sealer was placed over the tooth specimen. A calibrated micropipette (Eppendorf, Hamburg, Germany) was used to control the volume of sealer. The image of the droplets was captured using contact angle analyser (Holmarc contact angle meter, Opto-Mechatronics, Kochi, India), then the static contact angle made by the sealers on the intraradicular dentine after 1 minute was evaluated using Contact Angle Meter software version 5.0.0.0 (Holmarc Opto-Mechatronics, Kochi, India.)[15]. All the measurements in duplicate were taken at a room temperature of approximately 22^0C.

Statistical analysis

The contact angle values obtained were tabulated and subjected to statistical testing using statistical software (SPSS version 20.0.0, IBM, Armonk, NY USA). The normality of the data was tested using a Shapiro-Wilk test. Since the values were normally distributed, an independent *t* test was used to compare the contact angle of two root canal sealers specific to each irrigation strategy. A one-way analysis of variance (ANOVA) and a Tamhane T2 post hoc test were used to determine the level of significance for each root canal sealer in relation to various irrigation protocols evaluated. The level of statistical significance was set at $P < 0.05$.

Results

ER sealer

Treatment with SDF alone (group 5) resulted in the lowest contact angle for AH Plus. This was significantly less than dentine treated with NaOCl-EDTA-NaOCl (group 2) ($P = 0.03$), or the former followed by SDF (group 4) ($P = 0.008$) (Table 2 and Fig 1). Group 3 (NaOCl-EDTA-SDF) demonstrated a significantly lower contact angle than group 2 ($P = 0.000$) and group 4 ($P = 0.000$). Groups in which NaOCl was used after EDTA (groups 2 and 4)

Table 2 Wetting angle of ER sealer and tricalcium silicate sealer on intraradicular dentine after treating the dentine surface with various final irrigation regimen.

Group	Contact angle, ^{0} (mean ± standard deviation)	
	ER sealer	C3S sealer
Group 1: NaOCl-EDTA	36.8 ± 4.8^{AB}	40.1 ± 4.3^{a}
Group 2: NaOCl-EDTA-NaOCl	36.6 ± 1.9^{A}	57.7 ± 8.0^{ab}
Group 3: NaOCl-EDTA-SDF	27.5 ± 1.6^{B}	53.7 ± 4.8^{b}
Group 4: NaOCl-EDTA-NaOCl-SDF	38.9 ± 1.8^{A}	47.3 ± 3.6^{ab}
Group 5: SDF	26.9 ± 3.6^{B}	58.9 ± 5.6^{b}
Group 6: Saline	35.5 ± 4.5^{A}	47.5 ± 2.9^{ab}

A,BWithin the column means the mean value of the contact angle of ER sealer without a common superscript indicated a significant difference ($P < 0.05$) between the groups.

a,bWithin the column means the mean value of the contact angle of C3S sealer without a common superscript indicated a significant difference ($P < 0.05$) between the groups.

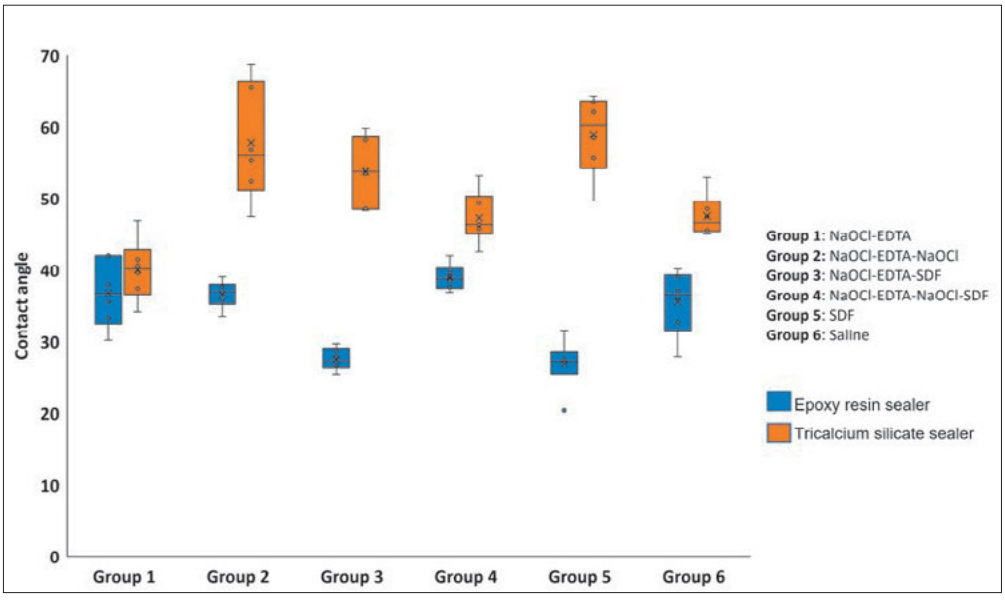

Fig 1 Contact angle measurement of root canal sealer tested following various irrigation protocols.

showed poor wetting characteristics in the ER sealer group.

C3S sealer

Dentine treated with NaOCl-EDTA (group 1) showed the smallest contact angle of all the groups. It was significantly lower than group 3 (NaOCl-EDTA-SDF) ($P = 0.027$) and group 5 (3.8% SDF only) ($P = 0.006$) (Fig 1 and Table 2). The application of SDF resulted in an increase in the contact angle of BioRoot RCS sealer.

ER vs C3S sealer

The contact angles made by AH Plus sealer were smaller than those made by BioRoot RCS sealer in all groups. The difference was significant in all groups except group 1.

Discussion

This study assessed the effect of SDF on the wettability of an ER and C3S sealer and found that SDF treatment of dentine improved the wettability of the former but was detrimental to that of the latter; thus, the null hypothesis was rejected. In the ER sealer group, dentine treated with NaOCl after the traditional irrigation approach using NaOCl and EDTA (groups 2 and 4) showed the least wettability regardless of final use of SDF. It is now well known that AH Plus, the ER sealer used in this study, chemically bonds to the amide groups in dentinal collagen. It has been demonstrated that NaOCl-treated dentine exhibits a reduction in the intensity of collagen amide peaks and an increase in the apatite/collagen ratio, confirming degradation of the collagen matrix[19]. Ramirez-Bommer et al[20] showed that

the presence of mineralised matrix offers some protection against this degradation. They found that NaOCl-EDTA-NaOCl increased collagen depletion on dentine surfaces approximately fourfold compared with NaOCl alone[20]. Thus, the excessive degradation of collagen by NaOCl after the removal of the protective hydroxyapatite by EDTA could be the reason for the poor spreading of the ER sealer.

Interestingly, in groups where SDF was applied to dentine samples not treated with NaOCl as the final irrigant, wetting of the ER sealer was significantly greater. SDF is known to interact with hydroxyapatite, resulting in the formation of silver phosphate and calcium fluoride[21]. Additionally, silver ions can be reduced by proteins (collagen), resulting in the metallic silver–collagen complex[22]. Therefore, it could be speculated that the silver–collagen complex stabilises the dentine collagen and this could have encouraged better interaction with the AH Plus sealer.

On the other hand, tricalcium silicate–based sealers have been proposed to bond to the dentine surface through a process called biomineralisation, whereby nucleation of carbonated apatite occurs at the sealer–dentine interface[23]. The reaction between SDF and hydroxyapatite that leads to the formation of silver phosphate and calcium fluoride precipitate[21] may render the calcium and phosphate ions unavailable for the biomineralisation process. This may have resulted in the poor spreading and adaptation of the tricalcium silicate sealer after SDF application (groups 3 and 5).

The wettability of the tricalcium silicate sealer was significantly enhanced by using the commonly recommended final irrigation protocol for removing the smear layer, NaOCl followed by EDTA (group 1). Removal of the smear layer has been shown to improve the micromechanical interactions of the calcium silicate–based sealer with dentinal tubules and thus provided better sealer–dentine interaction in the short term[24]; however, final EDTA rinsing is detrimental to the adhesion and biomineralisation of tricalcium silicate–based materials[25,26] and this may impact the sealer–dentine interface in the long term. Similar to the ER sealer group, the contact angle of the tricalcium silicate sealer on the dentine surface with NaOCl rinsing (group 2) was relatively higher, though not statistically significant. The reason for this needs to be investigated further.

Regardless of the final irrigation protocol used, BioRoot RCS sealer showed poor wettability compared to AH Plus sealer. This could be attributed to the higher surface tension, intermolecular attraction and increased viscosity of the tricalcium silicate sealer. Although these results are in accordance with others[15,27,28], they are contrary to the findings of Ha et al[14]. The bioceramic sealers used in the study by Ha et al14 were all supplied in paste form rather than BioRoot RCS, which is available in powder and liquid form for controlled hydration. This, as well as the difference in methodology, could have contributed to the contrasting results. In the present study, the wettability of the root canal sealer was measured by the contact angle made by the sealer on a flat radicular dentine surface, whereas Ha et al[14] evaluated the wetting properties of the root canal sealers by testing the materials as solids and measuring the contact angles of two probe liquids on their surfaces.

Contrary to the study by de Assis et al[29], the control group (group 6), i.e., the group where the smear layer was left intact, showed no significant influence on the wettability of both sealers assessed. This could be attributed to variation in the surface roughness of the dentine disc prepared and the thickness of the smear layer produced, which in turn can influence the surface free energy of the dentine surface.

A limitation of this study was that mechanical instrumentation of the root canals was not performed; rather, the surface was polished with fine sandpaper discs to achieve standardised surfaces between the experimental groups. Additionally, the dentine specimens were dipped in irrigating solutions, unlike in a clinical situation where only one surface is exposed to the irrigants. As a result, outcomes may have been overestimated. Nonetheless, this study provides valuable evidence on the effect of SDF on the wettability of sealers. Additionally, studies on the chemical interaction of SDF with bioactive materials such as tricalcium silicates is an important area for future research.

Conclusion

The results of the present study demonstrated that the wettability of ER-based and tricalcium silicate sealers was influenced by the final irrigation protocol used. SDF was shown to improve the wettability of AH Plus sealer, whereas it had a detrimental effect on the wettability of BioRoot RCS. Additionally, both sealers tested in this study exhibited poor wetting characteristics when NaOCl was used as the final irrigant.

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Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Surmayee SINGH, Sajan Daniel GEORGE, Prasanna NEELAKANTAN and Manuel S THOMAS contributed to the study design, manuscript draft and revision; Drs Rajat KUNDRA and Swithin HANOSH contributed to the experimental work, data collection and manuscript revision.

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