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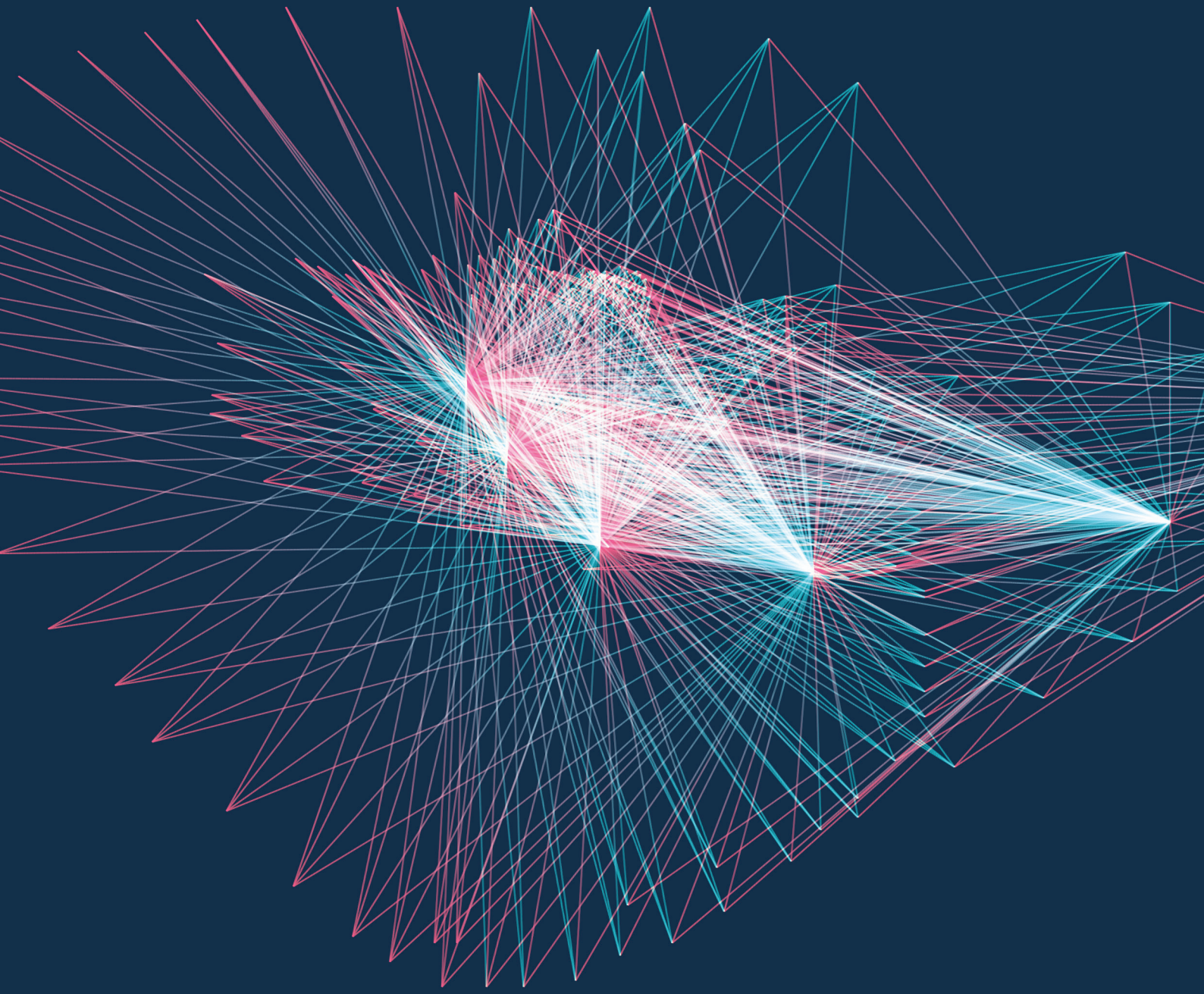

Chiang Mai University's Faculty of Dentistry publishes academic research articles in the newly titled - **Oral Sciences Reports,** which was previously known as *Chiang Mai Dental Journal (CMDJ)*. The journal was originally established for the purposes of publishing academic research articles by the Faculty of Dentistry at Chiang Mai University in 1977. In the current report, editors and experts in their respective fields review articles received from authors prior to being published to ensure that the content of all articles is up-to-date, universal, logical, and in accordace with academic principles so the reader can apply knowledge and cite works in the development of dentistry for the purposes of advancing future research while being beneficial to patients and society.

At present, Oral Sciences Reports openly receives all submissions through an online journal review process system. The new online system also allows reviewers and researchers an ability to read 3 issues each year.

Aim and Scope of the journal

To compile research and content that is up to date and usable to all branches of dentistry and related fields. The articles in Oral Sciences Reports are fundamental research work, including original articles, review articles, case reports/ series, short communications, and letters to the editor.

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Accepted articles will be fairly reviewed by the editors and experts with full transparency through the following process.

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2. The articles will be considered and reviewed through a non-bias process by concealing the names of authors and related persons in the considered documents while also concealing the names of the experts and reviewers who review the articles (double-blind review).
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4. Authors of articles are responsible to review and verify the accuracy of the text, images, tables in the articles before publication.
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Editorial Board

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Oral Sciences Reports invites the following submissions:

1.	Original Articles	Original contributions of research reports or unpublished recent academic research to the development and applications in dentistry and related fields. The original article must not exceed 4000 words in length and must contain no more than 10 figures and tables in total.
2.	Review Articles	Comprehensive reviews of special areas of focus in dentistry and related fields. Articles that contain important collected data from numerous books or journals and from the writer's experience. Information should be described, reviewed, compared, and analyzed. The review article must not exceed 4000 words in length and must contain no more than 10 figures and tables in total.
3.	Systematic Reviews	Clearly formulated reviews that uses systematic and reproducible methods to identify, select and critically appraise all relevant research, and to collect and analyze data from the studies that are included in the review.
4.	Case Reports/Series	Original findings that highlight novel technical and/or clinical aspects in dentistry and related fields which include clinical symptoms, diagnosis, patient care, treatment, follow-up, and evaluation. The report must not exceed 2500 words in length and must contain no more than 5 figures.
5.	Letters to the Editor	Commentaries on published papers in the journal and other relevant matters that must not exceed 1000 words in length
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7. Supplementary data (if applicable)

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3. Permission must be obtained for use of copyrighted material from other sources (including the internet).
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- EU Clinical Trials Register
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The clinical trial registration number and name of the trial register should be included in Materials and Methods of the manuscript. For epidemiological observational trials, authors of epidemiological human observations studies are required to review and submit a 'strengthening the reporting of observational studies in Epidemiology' (STROBE) checklist and statement. Compliance with this must be detailed in Materials and Methods.

D. Systematic Review

The abstract and main body of the systematic review should be reported using the PRISMA for Abstract and PRISMA guidelines respectively. Authors submitting a systematic review should register the protocol in one of the readily-accessible sources/databases at the time of project inception and not retrospectively (e.g. PROSPERO database, OSF registries). The protocol registration number, name of the database or journal reference should be provided at the submission stage in Materials and Methods. A PRISMA checklist and flow diagram (as a Figure) should also be included in the submission material.

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All texts in the submitted manuscript are required to be inclusive language throughout that acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (for instance by using 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess'). Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, religion, culture, or any other characteristic.

A. Title page

The title page will remain separate from the manuscript throughout the peer review process and will not be sent to the reviewers. It should include these following details:

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B. Abstract

Abstract must not exceed 250 words with concise and informative explanations about the article. Authors must prepare an abstract separately from the main manuscript using Microsoft Word processing software (.doc or .docx). Please avoid references and uncommon abbreviations, but if essential, abbreviations must be defined at their first mention in the abstract itself. Abstract structure of the original articles must consist of ‘Objectives, Methods, Results, and Conclusions’.

Abstract of other types of submitted articles should be summarized in one paragraph. Up to five keywords relevant to the articles must be provided and arranged in alphabetical order.

C. Manuscript

Oral Sciences Reports adheres to a double-blinded review. The main body of the paper (including the references, figures, tables and any acknowledgements) must not include any identifying information, such as the authors' names. The layout of the manuscript must be as simple as possible with double-spaced, single column format with Sans Serif font and uploaded as an editable Microsoft Word processing file (.doc or .docx). Complex codes or hyphenate options must be avoided, but the emphatic options such as bold face, italics, subscripts, and superscripts, etc. are encouraged.

1. Original article

• *Introduction* should include literature reviews of previous studies, research questions, and the rationale for conducting the study. The Introduction should not be too long and should be easy to read and understand while avoiding a detailed literature survey or a summary of the results.

• *Methods* should provide sufficient details in a logical sequence to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized and indicated by a reference. If quoting directly from a previously published method, use quotation marks and cite the source. Any modifications to existing methods should also be described.

• *Results* should show the data gained from the study's design in text, tables and/or illustrations, as appropriate, and be clear and concise.

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• *Conclusions* refers to a summary of the study or research results.

• *Acknowledgments:* Please specify contributors to the article other than the authors accredited. Please also include specifications of the source of funding for the study.

Formatting of funding source:

This work was supported by the 1st organization name [grant numbers xxxx]; the 2nd organization name [grant number yyyy]; and the 3rd organization name [grant number zzzz].

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant or funding from funding agencies in the public, commercial, or not-for-profit sectors.

• *References* should be confined to documents relating to the author's article or study. The number should not exceed 80, placed in order and using numbers which are superscripted and put in parentheses, starting with number 1 in the article and in reference document's name. (see 'References' section for more information regarding reference formatting)

2. Review articles should be divided into Introduction, Review and Conclusions. The Introduction section should be focused to place the subject matter in context and to justify the need for the review. The Review section should be divided into logical sub-sections in order to improve readability and enhance understanding. Search strategies must be described, and the use of state-of-the-art evidence-based systematic approaches is expected. The use of tabulated and illustrative material is encouraged. The Conclusion section should reach clear conclusions and/or recommendations on the basis of the evidence presented.

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• Results should present in structured fashion (e.g. results of the search process, characteristics of the included studies, results of primary meta-analysis, additional analysis, publication bias, quality of evidence).

• Discussion should summarize the results, highlighting completeness and applicability of evidence, quality of evidence, agreements and disagreements with other studies or reviews, strength and limitations, implications for practice and research.

• Conclusion(s) should reach clear conclusions and/or recommendations on the basis of the evidence presented.

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There are some necessary considerations which should be comprehended and consistent throughout the article:

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2. Mathematical expressions: the numbers identifying mathematical expressions should be placed in parentheses after the equation, flush to the right margin; when referring to equations within text, use the following style: Eq. (5), Eqs. (3-10), [see Eq. (4)], etc.

3. Nomenclature: abbreviations and acronyms should be spelled out the first time they are used in the manuscript or spelled out in tables and figures (if necessary). Units of measure and time require no explanation. Dental nomenclature in the manuscript should be complete words, such as maxillary right central incisor. Numbering of teeth from pictures or tables should follow the FDI two-digit system.

4. Units: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Figures should be prepared and submitted separately from the main manuscript. Color artworks are encouraged at no additional charge. Regardless of the application used other than Microsoft Office, when the electronic artwork is finalized, please ‘save as’ or ‘export’ or convert the images to **EPS, TIFF, or JPEG format with the minimum resolution of 300 dpi.** Keep the artwork in uniform lettering, sizing, and similar fonts. Please do not submit graphics that are too low in resolution or disproportionately large for the content. Authors must submit each illustration as a separate file.

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Citation in text

Any citations in the text should be placed in order and using numbers which are superscripted and put in parentheses. Please ensure that all citations are also present in the reference list consecutively in accordance with their appearance in the text.

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All references should be brought together at the end of the paper consecutively in accordance with their appearance in the text and should be in the Vancouver reference format. Please follow these examples of correct reference format below:

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1.1. One to six authors

Author(s) – Family name and initials. Title of article. Abbreviated journal title. Publication year;volume (issue):pages.

Example:

Parvez GM. Pharmacological activities of mango (Mangifera Indica): A review. J Pharmacognosy Phytother. 2016;5(3): 1-7.

Or

Choi YS, Cho IH. An effect of immediate dentin sealing on the shear bond strength of resin cement to porcelain restoration. J Adv Prosthodont. 2010;2(2):39-45.

Or

Firmino RT, Ferreira FM, Martins CC, Granville-Garcia AF, Fraiz FC, Paiva SM. Is parental oral health literacy a predictor of children's oral health outcomes? Systematic review of the literature. Int J Paediatr Dent. 2018;28(5):459-71.

1.2. More than six authors

Author(s) – Family name and initials of the first six authors, et al. Title of article. Abbreviated journal title. Publication year;volume(issue):pages.

Example:

Vera J, Siqueira Jr JF, Ricucci D, Loghin S, Fernández N, Flores B, et al. One-versus two-visit endodontic treatment of teeth with apical periodontitis: a histobacteriologic study. J Endod. 2012;38(8):1040-52.

1.3. Article in press

Authors separated by commas – Family name and initials. Title of article. Abbreviated journal title in italics. Forthcoming - year of expected publication.

Example:

Cho HJ, Shin MS, Song Y, Park SK, Park SM, Kim HD. Severe periodontal disease increases acute myocardial infarction and stroke: a 10-year retrospective follow-up study. J Dent Res. Forthcoming 2021.

2. *Books*

2.1. Book with author (s)

Author(s) – Family name and initials (no more than 2 initials with no spaces between initials)– Multiple authors separated by a comma. After the 6th author add - "et al". Title of book. Edition of book if later than 1st ed. Place of publication: Publisher name; Year of publication.

Example:

Sherwood IA. Essentials of operative dentistry. Suffolk: Boydell & Brewer Ltd; 2010.

Or

Abrahams PH, Boon JM, Spratt JD. McMinn's clinical atlas of human anatomy. 6th edition. Amsterdam: Elsevier Health Sciences; 2008.

2.2. Book with no author

Title of book. Edition of book if later than 1st ed. Place of publication: Publisher name; Year of publication.

Note: Do not use anonymous. Please begin a reference with the title of the book if there is no person or organization identified as the author and no editors or translators are given.

Example:

A guide for women with early breast cancer. Sydney: National Breast Cancer; 2003.

2.3. Chapter in a book

Author(s) of chapter - Family name and initials, Title of chapter. In: Editor(s) of book - Family name and initials, editors. Title of book. edition (if not first). Place of publication: Publisher name; Year of publication. p. [page numbers of chapter].

Example:

Rowlands TE, Haine LS. Acute limb ischaemia. In: Donnelly R, London NJM, editors. ABC of arterial and venous disease. 2nd ed. West Sussex: Blackwell Publishing; 2009. p. 123-140.

3. Thesis/dissertation

3.1. Thesis in print

Author - family name followed by initials. Thesis title [type of thesis]. Place of publication: Publisher; Year.

Example:

Kay JG. Intracellular cytokine trafficking and phagocytosis in macrophages [dissertation]. St Lucia, Qld: University of Queensland; 2007.

3.2. Thesis retrieved from full text database or internet

Author - family named followed by initials. Thesis title [type of thesis/dissertation on the Internet]. Place of publication: Publisher; Year [cited date – year month day]. Available from: URL

Example:

Pahl KM. Preventing anxiety and promoting social and emotional strength in early childhood: an investigation of risk factors [dissertation on the Internet]. St Lucia, Qld: University of Queensland; 2009 [cited 2017 Nov 22]. Available from: https://espace.library.uq.edu.au/view/UQ:178027

4. Webpage

4.1. Webpage with author

Author/organization's name. Title of the page [Internet]. Place of publication: Publisher's name; Publication date or year [updated date - year month day; cited date - year month day]. Available from: URL

Example:

American Dental Association. COVID-19 and Oral Health Conditions [Internet]. Chicago: American Dental Association; 2021 Feb 12 [updated 2021 Feb 12; cited 2021 Jun 24]. Available from: https://www.ada.org/en/press-room/news-releases/2021-archives/february/covid-19-and-oral-health-conditions

4.2. Webpage with no authors

Title [Internet]. Place of publication (if available): Publisher's name (if available); Publication date or year [updated date (if available); cited date]. Available from: URL

Example:

Dentistry and ADHD [Internet]. 2019 Jan 15 [updated 2019 Jan 15; cited 2020 Apr 8]. Available from: https://snoozedentistry.net/blog/dentistry-and-adhd/

4.3. Image on a webpage

Author/organization. Title [image on the Internet]. Place of publication: Publisher's name; Publication date or year [updated date; cited date]. Available from: URL

Note: If the image does not have a title - give the image a meaningful title in square brackets.

Example:

Poticny DJ. An Implant-Supported Denture Offers a Number of Advantages [image on the Internet]. Texas: Office of Dan Poticny; 2018 Nov 21 [updated 2018 Nov 21; cited 2019 Aug 30]. Available from: https://www.dfwsmiledoc.com/blog/post/an-implant-supported-denture-offers-a-number-of-advantages.html

5. Government publications/reports

5.1. Reports and other government publications

Author(s). Title of report. Place of publication: Publisher; Date of publication – year month (if applicable). Total number of pages (if applicable eg. 24 p.) Report No.: (if applicable)

Example:

Australian Institute of Health and Welfare. Oral health and dental care in Australia: key facts and figures trends 2014. Canberra: AIWH; 2014.

5.2. Government reports available online

Author(s). Title of report. Report No.: (if applicable). [Internet]. Place of publication: Publisher or Institution; Publication date or year [updated date - year month day; cited date - year month day]. Available from: URL

Example:

World Health Organization. WHO mortality database [Internet]. Geneva: World Health Organization; 2019 Dec 31 [updated 2019 Dec 31; cited 2021 Mar 29]. Available from: https://www.who.int/data/mortality/country-profile

6. Tables/Figures/Appendices

Follow the format of book, journal or website in which you found the table/figure/appendix followed by: table/figure/image/appendix number of original source, Title of table/figure/appendix from original source; p. Page number of table/figure/appendix from original source.

Note: each reference to a different table/figure within the same document requires a separate entry in the Reference list. Please provide permission documents from the original sources.

Example:

Smith J, Lipsitch M, Almond JW. Vaccine production, distribution, access, and uptake. Lancet 2011;378(9789):428-438. Table 1, Examples of vaccine classes and associated industrial challenges; p. 429.

7. Journal abbreviation source

Journal names should be abbreviated according to the Web of Science - Journal Title Abbreviations.

Peer-review Process

Oral Sciences Reports follows a double anonymized review process. Each manuscript will be assigned to at least three expertises for consideration. The identities of both reviewers and authors are concealed from each other throughout the review to limit reviewer bias. To facilitate this, please ensure that the manuscript keeps anonymity before submission such as affiliation, author's gender, country or city of origin, academic status, or previous publication history. Our peer review process is confidential and identities of reviewers are not released. Letters and technical comments are sent to the authors of the manuscript on which they comment for response or refutation, but otherwise are treated in the same way as other contributions with respect to confidentiality.

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Microplastics in Dentistry: A Review of Health and Environmental Risks

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Abstract

Objectives: This narrative review investigated the sources, mechanisms of release, and health implications of microplastics within the dental profession.

Methods: A systematic literature search on Databases PubMed, Scopus, Web of Science and Google Scholar found eight key studies that examined various combinations of terms, including "microplastics" and "nanoplastics" related to "dental materials" and "oral healthcare," as well as their impact on the environment impact accordingly to inclusion/exclusion criteria. Detection techniques such as scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and gas chromatography-mass spectrometry (GC-MS) were commonly applied to characterize the morphology and composition of microplastics.

Results: The main findings indicate that microplastics originate from different sources, including mechanical wear, chemical degradation, and thermal stress, with occupational exposure posing risks to dental professionals, primarily through inhalation of airborne particles. Systemic exposure, resulting from ingestion or mucosal absorption of microplastics, has been linked to immune suppression, oxidative stress, and systemic toxicity. Environmentally, microplastics from dental sources infiltrate wastewater and contribute to aquatic pollution.

Conclusions: This review underscores the importance of adopting sustainable practices, including the use of biodegradable materials and enhanced waste management. Future research should focus on longitudinal studies, bioremediation approaches, and the development of alternative, eco-friendly dental materials.

Keywords: biodegradable materials, dentistry, environmental pollution, health risks, microplastics, occupational exposure, oral healthcare products, oxidative stress, resin-based composites, sustainable practices, wastewater contamination

Introduction

Microplastics (MPs), defined as plastic particles measuring less than 5 mm, have become a major environmental pollutant. The presence of plastics in ecosystems is now recognized as a significant concern, mainly due to the extensive use of plastic items and inadequate waste management strategies.[1] MPs can be categorized into two types: primary MPs, which are produced specifically for industrial or consumer applications (e.g., microbeads in cosmetics and toothpaste), and secondary MPs, which result from the degradation of larger plastic products through environmental weathering processes, including ultraviolet radiation, wind, and wave action. MPs accumulate worldwide, particularly in marine ecosystems, as shown by the Great Pacific Garbage Patch.[2,3]

MPs have been detected in various habitats, ranging from coastal areas to isolated Arctic regions. They have invaded terrestrial ecosystems and been incorporated into soils through agricultural practices, including the use of sewage sludge as fertilizers. Global plastic manufacturing exceeds 460 million metric tonnes yearly, with MPs constituting a substantial portion of this total due to their durability against environmental degradation.[4]

MPs pose significant ecosystem risks, primarily threatening biodiversity and affecting aquatic organisms. Marine organisms often mistake these particles for food, leading to ingestion, bioaccumulation, and trophic transfer through food webs. Moreover, plastics serve as vectors for hazardous chemical pollutants, including persistent organic pollutants (POPs) and heavy metals. These pollutants bind to plastic surfaces and are transferred into living organisms, exacerbating toxicity risks. These interactions disrupt the reproductive and physiological processes of aquatic biota, ultimately leading to ecological consequences.[5]

MPs have garnered considerable attention in public health due to the increasing evidence of their ubiquity in air, water, food, and even human tissues.[6] Human exposure to MPs occurs through multiple routes, including oral ingestion of contaminated water and food, inhalation of airborne plastic fragments, and dermal absorption. Studies have reported MPs in drinking water supplies, seafood, and table salt, emphasizing that humans inadvertently ingest significant MPs annually.[7]

Once ingested, MPs can translocate from the gastrointestinal tract into the circulatory system and potentially accumulate in various organs, including the liver, kidneys, and lungs. Several mechanisms have been proposed for their toxicity, including physical damage (e.g., tissue inflammation, disruption of intestinal permeability), oxidative stress, genotoxicity, and immune responses. Furthermore, microplastics are often associated with plasticizers, flame retardants, and bisphenol A (BPA)—chemicals known for their potential to disrupt the endocrine system and their possible carcinogenicity.

The chronic implications of microplastic exposure remain uncertain, although long-term exposure has been associated with conditions such as respiratory dysfunction, metabolic disorders, and cancer. Investigations into MPs in placental tissues and human breast milk have raised concerns about their effects on fetal development and infant health. Therefore, their role as environmental pollutants of concern necessitates stringent regulatory and mitigation strategies. While chronic toxicity of MPs has received significant attention, emerging evidence also suggests the potential for acute effects. Experimental studies have demonstrated that high concentrations of certain polymer leachates, such as polycaprolactone (PCL), can induce immediate cytotoxic responses in both *in vitro* models and aquatic organisms, indicating that acute exposure may also present clinical and environmental risks.[8,9]

The use of polymer-based biomaterials in dentistry has inadvertently contributed to microplastic contamination in clinical environments and the oral cavity. Dental materials commonly implicated include resin-based composites (RBCs), orthodontic adhesives, dental prosthetics, and oral healthcare products like toothpaste and dental floss. MPs in dentistry can originate through mechanical wear, chemical degradation, or fragmentation of polymers under thermal and pH stress.[8-11]

Orthodontic adhesives, for example, degrade over time due to cyclic mechanical forces during mastication. These forces release microplastic particles into saliva, which can be swallowed or aerosolized during dental procedures. Moreover, polishing and finishing procedures performed on resin composites disintegrate MPs, which are washed away via dental unit waterlines, contributing to wastewater pollution.[12]

The ingestion of MPs from dental sources poses potential risks to the oral cavity, including inflammation

of the gingival tissues, increased risk of periodontitis, and oxidative stress-induced damage to the oral mucosa. MPs can act as carriers for bacteria, heavy metals, and other environmental toxins, potentially exacerbating chronic periodontitis and endodontic infections. Inhalation risks are particularly concerning for dental professionals exposed to airborne MPs during routine procedures, especially in poorly ventilated environments.[13-15] Dental clinics have been shown to contain higher levels of suspended plastic particles compared to other indoor environments.[16-19]

Nanoplastics (NPs) was included to capture studies that discuss particulate matter smaller than one μm, which may be relevant given the potential for fragmentation of dental polymers NPs, defined as plastic particles smaller than 1 μm, represent a critical extension of microplastic research due to their greater mobility, higher surface-area-to-volume ratio, and enhanced potential for tissue penetration. Unlike MPs, which are typically retained in the gastrointestinal tract or excreted, MPs have demonstrated the capacity to cross cellular membranes, accumulate in secondary organs, and interact with biological systems at the molecular level.[20,21]

Given these challenges, this narrative review aims to comprehensively evaluate the role of MPs in dentistry, their mechanisms of release, associated health risks, and possible mitigation strategies. By analyzing current literature, this review aims to raise awareness among clinicians and policymakers regarding the multifaceted impacts of dental MPs, including their clinical release mechanisms, occupational and systemic health risks, and environmental consequences, while proposing sustainable mitigation strategies.

Materials and Methods

Eligibility criteria

Studies were included if they met the following criteria: (1) addressed the presence, release mechanisms, or health/environmental impacts of MPs in dentistry and oral healthcare; (2) focused on microplastics from dental materials or oral care products such as composites, adhesives, toothbrushes, and toothpaste; and (3) were peer-reviewed articles published in English. Studies from related sectors, such as general microplastic pollution or marine environmental contamination, were included only if they had relevant parallels to dentistry. Exclusion criteria included studies lacking direct relevance to MPs in the dental context, as well as non-peer-reviewed reports, editorials, and conference abstracts. Studies were grouped based on their primary focus: microplastic sources, health risks, and environmental impacts.

Information sources

The search for eligible studies was conducted using major academic databases, including PubMed, Scopus, Web of Science, and Google Scholar. Additionally, reference lists from selected articles were manually searched for relevant citations. No date restrictions were applied. The final database search was completed on 31 January 2025. The search incorporated both standardized Medical Subject Headings (MeSH) and general keywords. In dentistry, the progressive breakdown of polymer-based biomaterials may release both MPs and NPs, necessitating a comprehensive approach to evaluating their risks. However, the biological effects and environmental behavior of NPs differ markedly from MPs, and this distinction is maintained in the interpretation of results.

Search strategy

The search strategy employed Boolean operators and keywords related to MPs and dentistry. The search terms included combinations of the following: "microplastics," "nanoplastics," "dental materials," "resin-based composites," "oral healthcare," "orthodontic adhesives," "wastewater contamination," and "occupational exposure." Filters were applied to exclude non-peer-reviewed publications, and only articles in English were considered. Specific search examples include: (microplastics OR nanoplastics) AND ("dental materials" OR "oral healthcare" OR "resin-based composites"). Search strategies were iteratively refined based on initial screening outcomes.

Selection process

The studies were selected following a systematic screening process. The author reviewed the titles and abstracts of the retrieved articles to determine their relevance. Then, full-text articles were evaluated based on the inclusion criteria. No automation tools were used during the screening process. Two authors independently screened the articles and performed data extraction using a standardized form. Discrepancies were resolved through consensus. No third-party adjudicator was required.

Data collection process

The author extracted data using a standardized data

collection form. For each included study, information was extracted on study objectives, materials examined, investigative techniques, key findings, and associated health and environmental outcomes. This information was cross-checked, and discrepancies were resolved through consensus. No direct contact with study investigators was necessary, as all data were publicly available.

Data items

Primary outcomes: Data on the sources, release mechanisms, and health and environmental risks of MPs in the dental profession were sought. Findings related to microplastic ingestion, inhalation, cytotoxicity, and ecological pathways were prioritized for further examination.

Secondary variables: Study characteristics, such as the type of dental materials evaluated (e.g., resin composites, adhesives), microplastic detection methods (e.g., Fourier Transform Infrared Spectroscopy, Scanning Electron Microscopy), and exposure pathways (e.g., occupational, patient-based), were collected. Funding sources and potential conflicts of interest were noted where available to provide context for the study findings.

Synthesis methods

A narrative synthesis was employed to summarize and integrate the findings across the included studies. Key themes were identified by grouping studies into categories based on their focus, including sources of MPs, health effects, and environmental impact. Data were tabulated where applicable to highlight consistent or divergent findings. Due to the heterogeneity of study designs and outcomes, no meta-analysis or quantitative synthesis was performed. Figures and tables were used to present the significant findings visually.

Certainty assessment

As this is a narrative review, no formal assessment of certainty (e.g., GRADE) was performed. However, the strength of the evidence was evaluated qualitatively, considering the consistency of findings across studies, the robustness of the methods used, and their relevance to dental practice.

Results

Overview of literature search and selected studies

A thorough literature search was conducted across major scientific databases to identify relevant studies on microplastics in dentistry, oral healthcare, and the associated health risks. Out of numerous initial results, eight studies were deemed appropriate due to their specific focus on dental biomaterials, microplastic release mechanisms, health impacts, and mitigation measures. Our initial research obtained 10 results; only eight were selected and included in this review. The rationale for excluding two initially identified studies is explained under 'Limitations of Existing Research. The following subsections detail the primary findings of each selected study, and Tables 1 and 2 provide comprehensive summaries.

Table 1: Summary of main outcomes in studies investigating microplastic contamination in dentistry.

Study	Main Outcomes
Saha *et al.*[20]	Immune suppression, tissue fibrosis, systemic toxicity, microplastic release mechanisms through wear, pH, and thermal fluctuations.
Akhtar *et al.*[21]	Increased inhalation risk for dental professionals, higher microplastic pollution in teaching clinics, polyethylene terephthalate and polyethylene dominance.
James *et al.*[22]	Acute toxicity from polycaprolactone microplastic leachates, oxidative stress in mammalian cells, nuclear receptor activation, oligomers identified as key toxic agents.
Divakar *et al.*[23]	Wastewater contamination from orthodontic adhesives, microplastic forms identified as fibers and pellets through mechanical degradation.
Protyusha *et al.*[24]	Significant contribution of toothbrushes and toothpaste to microplastic release, polyethylene and polyamide polymers, exposure quantified through daily and annual estimates.
Odintsov *et al.*[25]	Partial biodegradation of polytetrafluoroethylene and acrylic fluorinated copolymers in marine organisms, persistence and bioaccumulation concerns.
Wang *et al.*[26]	Development of biodegradable alternatives, reduction in microplastic release through plant-based material use.
Mulligan *et al.*[27]	Persistent surface changes in resin-based composite microplastics, monomer elution, long-term environmental persistence in wastewater.

Table 2: Summary of main results.

Study	Materials Studied	Release Mechanisms	Polymers Identified	Health/Environmental Risks
Saha *et al.*[22]	Toothpaste, orthodontic implants	Friction, pH/thermal fluctuations	Polyethylene, thermoplastic polyurethane	Immune suppression, tissue fibrosis, systemic toxicity
Akhtar *et al.*[23]	Clinical and teaching dental units	Aerosolized particles during use	polyethylene terephthalate, polyethylene	Inhalation risks, higher exposure among female workers
James *et al.*[24]	Polycaprolactone, thermoplastic polyurethane plastics	Leaching of oligomers, degradation	polycaprolactone, thermoplastic polyurethane	Acute toxicity, oxidative stress, receptor activation
Divakar *et al.*[25]	Orthodontic adhesives	Mechanical degradation	Polyamide, ester	Environmental pollution via wastewater
Protyusha *et al.*[26]	Toothbrushes, toothpaste, floss	Mechanical abrasion during use	Polyethylene, polyamide, polyethylene terephthalate	High annual microplastic exposure, polymer risk levels
Odintsov *et al.*[27]	polytetrafluoroethylene and acrylic copolymers	Biodegradation in marine organisms	polytetrafluoroethylene, fluorinated copolymers	Potential bioremediation paths, persistence concerns
Wang *et al.*[28]	Biodegradable plant-based materials	Microplastic degradation mechanism	Biodegradable fibers	Sustainable alternatives to plastic dental products
Mulligan *et al.*[29]	Resin-based composites	Grinding, clinical wear	Monomers, methacrylates	Environmental leaching, persistent surface alterations

Detailed results from selected studies

Saha *et al.*,[22] explored the impact of MPs generated by oral care products and dental materials, including toothpaste and orthodontic appliances, on human health. They highlighted mechanical friction, pH variations, and thermal changes as primary factors contributing to the generation of MPs in the oral environment. Released particles were shown to induce immune suppression, tissue fibrosis, and systemic toxicities. The authors emphasized the need for advanced analytical tools to detect MPs more accurately and sustainable strategies to mitigate their environmental release.

Akhtar *et al.*,[23] investigated MPs' abundance and morphological characteristics in dental healthcare units, distinguishing between teaching hospitals and private dental clinics. Their study revealed higher levels of microplastic pollution in teaching environments, with polyethylene terephthalate (PET) being the most common polymer. The average annual inhalation rate of MPs was found to be higher among female dental professionals. This study also calculated the polymer hazard index (PHI), revealing varying risk levels associated with different types of MPs.

James *et al.*,[24] focused on PCL and thermoplastic polyurethane (TPU) plastics, commonly used in dental modeling and prosthetics. Their experiments demonstrated that aqueous leachates from PCL exhibited acute toxicity in zebrafish models and activated nuclear receptors in mammalian cells responsible for oxidative stress. The study suggested that oligomeric compounds and NPs released from PCL were accountable for the observed toxicity, emphasizing the need to reconsider assumptions regarding the inertness of these materials.

Divakar *et al.*,[25] investigated the generation of MPs from orthodontic adhesives using scanning electron microscopy and Fourier-transform infrared spectroscopy (FTIR). The predominant microplastic types identified included fibers, fragments, and pellets, with polymeric signatures such as polyamides and esters. The study acknowledged that although the quantity of microplastic release was minimal compared to other industries, its environmental impact should not be overlooked, especially in clinical wastewater.

Protyusha *et al.*,[26] conducted a comprehensive risk assessment of MPs found in commercially available oral healthcare products, such as toothbrushes, toothpaste, mouthwash, and dental floss. Their results showed that toothbrushes were the most significant contributors to

microplastic pollution, primarily polyethylene and polyamide particles. The risk of microplastic exposure was quantified through daily and annual estimates, with toothbrushes contributing approximately 48,910 particles per individual annually.

Odintsov *et al.,*[27] investigated the biodegradation of polytetrafluoroethylene (PTFE) and acrylic fluorinated copolymers, commonly used in dental applications, within the digestive tracts of marine gastropods. The study demonstrated that specific biological processes facilitate the partial degradation of these plastics, suggesting possible avenues for bioremediation efforts to reduce microplastic waste in aquatic ecosystems.

Wang *et al.,*[28] explored biodegradable alternatives to plastic-based dental materials by developing a biodegradable respirator made of plant fibers and recycled dental floss silk. The study demonstrated that incorporating microplastic degradation mechanisms within the design of new materials could reduce environmental impact.

Mulligan *et al.,*[29] focused on RBCs, highlighting the significant elution of monomers and other microparticulate waste from these materials during clinical procedures. Surface area analyses and FTIR confirmed the persistence of these particles over time, with the potential leaching of harmful compounds into surrounding environments.

Summary of findings

Table 2 summarizes the key characteristics, microplastic sources, and associated risks reported in each included study.

Investigative techniques for microplastic detection

The selected studies' detection and characterization of MPs relied on advanced microscopy, spectroscopy, and chemical analysis techniques. Scanning electron microscopy (SEM) was one of the most commonly used techniques to provide high-resolution images and evaluate the MPs' surface morphology and particle size, as seen in studies like Divakar *et al.,*[25] Mulligan *et al.,*[29] FTIR was frequently employed to identify the detected MPs' chemical composition and polymer types. FTIR works by detecting the vibrational signatures of molecular bonds, allowing precise polymer identification even in complex environments.

In studies where airborne or environmental MPs were of concern, techniques such as attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) were utilized to analyze microplastic particles directly on the sampling surface without extensive preparation. Protyusha *et al.,*[26] employed this method to assess oral healthcare products, while Akhtar *et al.,*[23] used it to characterize particles in dental clinic environments. Gas chromatography coupled with mass spectrometry (GC-MS) and high-resolution two-dimensional gas chromatography (GC×GC-HRT) were used by James *et al.*[24] to identify polymer fragments, additives, and oligomers within aqueous leachates of PCL plastics.

Additionally, Mulligan *et al.,*[29] conducted potentiometric titration techniques and zeta potential analyses to assess the surface charge properties of RBC microparticles. This contributed to understanding their environmental behavior and interaction with the surrounding ecosystems. Combining these techniques provided robust data on particle morphology, size distribution, polymeric content, and the potential biological and ecological impacts. It is essential to note that several studies[24-26] have detected nanoscale polymer fragments, underscoring the need for detection techniques with high resolution, such as GC-MS and zeta potential analysis. These findings emphasize the co-occurrence of micro- and nanoplastics in dental environments. Odintsov *et al.,*[27] employed digestive tract analysis of marine gastropods combined with fluorescence microscopy to evaluate the degradation capacity of polymer particles, focusing on surface erosion and fragmentation patterns. Wang *et al.,*[28] employed scanning electron microscopy and tensile strength testing to evaluate the structure and biodegradability of plant-fiber-based respirator components made from recycled dental floss.

Sources of dental microplastics

MPs originate from commonly used dental materials, including resin-based composites, orthodontic adhesives, dental prostheses, and oral care products. Mechanical processes such as polishing and mastication contribute to fragmentation. Divakar *et al.,*[25] and Protyusha *et al.,*[26] identified polyethylene and polyamide particles as frequent contaminants derived from toothbrushes, toothpaste, and adhesives.

Mechanisms of microplastic release

Microplastic release within dental settings is facilitated by a range of physicochemical and mechanical processes that affect polymer stability. Mechanical abrasion during finishing, polishing, mastication, and ultrasonic

scaling procedures can induce the fragmentation of polymer-based materials, such as resin composites and orthodontic adhesives. Divakar *et al.,*[25] demonstrated that repetitive mechanical forces applied to adhesives produce fibers and pellets, which may be dispersed via aerosols or effluents.

Thermal stress is another prominent factor contributing to polymer degradation. Temperature fluctuations during restorative procedures, particularly when using curing lamps and rotary instruments, promote the breakdown of polymer chains. Saha *et al.,*[30] reported that thermal changes and intraoral pH variations weaken polymer integrity, thereby accelerating microplastic generation. These phenomena are further exacerbated in materials with low cross-linking densities or inadequate polymerization. Chemical degradation, particularly hydrolysis and oxidative stress, also contributes to the formation of MPs. The oral environment, characterized by enzymatic activity, fluctuating pH levels, and constant exposure to saliva, creates favorable conditions for the gradual degradation of polymeric dental materials. Studies such as those by Mulligan *et al.,*[29] have observed that composite materials may elute monomers and microdebris into saliva and rinse water, indicating a progressive degradation pathway that contributes to both patient exposure and environmental contamination.

Discussion

This section critically synthesizes the results, contextualizing the findings within current knowledge and discussing their clinical implications. Redundancies with the results section have been minimized to offer a more evaluative discussion.

Microplastic contamination in dentistry

Microplastic contamination in dentistry reflects a microcosm of the broader global plastic pollution crisis. Several studies, including those analyzed in this review, demonstrate how standard dental procedures and products, ranging from orthodontic adhesives to resin-based composites, contribute to the release of MPs. The fragmentation of plastics during clinical operations, combined with aerosolization and mechanical degradation, highlights dentistry as a significant but overlooked contributor to indoor and environmental plastic pollution.[20]

Studies like those conducted by Protyusha *et al.,*[26] and Saha *et al.,*[22] reveal the multifactorial mechanisms of microplastic release, including frictional forces, thermal stress, and pH fluctuations. These mechanisms illustrate how polymers commonly used in dental materials undergo chemical breakdown, shedding micro- and nanoplastics intraorally and into the surrounding environment. The potential for these particles to be inhaled or ingested during dental treatments raises questions about occupational exposure risks, especially for dental professionals who spend prolonged periods in clinical settings, as demonstrated by Akhtar *et al.*[23]

While dentistry may not match the microplastic output of larger sectors, such as textiles or fisheries, as indicated by Divakar *et al.,*[25] the clinical and localized nature of dental pollution magnifies its impact on specific populations, particularly patients and practitioners. This finding is significant because it highlights the potential for cumulative exposure and underscores the urgent need to explore mitigative measures in this field. The evidence suggests that the health effects of MPs extend beyond localized oral tissues, necessitating a broader interdisciplinary understanding of their biological behavior.

Health implications of dental microplastic exposure

This review highlights one key concern: the health impact of oral and systemic microplastic exposure resulting from dental procedures. Saha *et al.,*[22] reported adverse effects from MPs originating in dental materials, including systemic toxicity and immune suppression. The authors documented immune suppression, tissue fibrosis, and systemic toxicity as critical outcomes of microplastic exposure.

These health risks primarily arise from two factors: the physical properties of MPs (size, shape, and surface area) and their chemical composition, including associated contaminants such as monomers, stabilizers, and plasticizers. Studies like Mulligan *et al.,*[29] and James *et al.,*[24] further reinforce the role of additives and leachates from dental materials as primary drivers of cytotoxicity and oxidative stress—the study by James *et al.,*[24] for example, demonstrated how oligomeric compounds leached from PCL plastics triggered nuclear receptor activation, which is closely linked to inflammatory and metabolic disturbances.

Furthermore, the oral cavity serves as a direct interface for the systemic absorption of MPs. The combination of mechanical friction, salivary flow, and mucosal

permeability creates an environment conducive to the translocation of MPs into deeper tissues. Recent studies have identified MPs in human feces, placental tissues, and even breast milk, suggesting that exposure from multiple routes, including oral pathways, may contribute to cumulative systemic effects[30,31] (Figure 1). While most toxicity data pertain to MPs, NPs have shown unique biological effects, including mitochondrial damage, oxidative DNA damage, and endocrine disruption at lower concentrations.[20,30] Their small size facilitates cellular uptake and translocation to organs such as the brain and placenta, making their impact more insidious and potentially more severe.

Occupational risks for dental professionals

The occupational exposure of dental professionals is a significant yet underexplored issue, as indicated by Akhtar *et al.,*[23] who found a higher prevalence of inhaled MPs among dental workers, particularly in teaching hospitals, compared to private clinics. The increased exposure risk in teaching settings may be attributed to frequent training procedures, higher patient turnover rates, and the extended use of polymer-based materials in restorative and orthodontic treatments.

This observation has important implications for female dental professionals, who, as reported, exhibit slightly higher inhalation rates of airborne MPs due to occupational factors and possibly hormonal differences affecting pulmonary clearance mechanisms. Long-term exposure can lead to respiratory complications, airway inflammation, and systemic absorption of inhaled particles. Thus, there is an urgent need for improved ventilation systems and non-plastic-based protective barriers to mitigate these risks[31] (Figure 1).

Environmental and ecosystem impacts of dental microplastics

MPs generated within dental clinics are not confined to the clinical environment; wastewater discharge systems act as vectors for their release into larger ecosystems. Studies by Divakar *et al.,*[25] and Protyusha *et al.,*[26] have demonstrated how MPs from dental adhesives, toothbrushes, and resin-based composites enter wastewater treatment plants and sub-sequently infiltrate aquatic ecosystems.

Once introduced into the environment, MPs can persist for extended periods, affecting marine and terrestrial organisms. The biodegradation of fluorinated copolymers and PTFE, as examined by Odintsov *et al.,*[27] demonstrated partial degradation in the digestive tract of marine gastropods. Although this finding suggests potential for bioremediation, it also highlights the persistent nature of these materials and their likelihood of bioaccumulation.

Moreover, the ability of microplastics to adsorb environmental toxins, such as POPs and heavy metals, exacerbates their ecological impact. Contaminated MPs can transfer harmful substances through the food chain, posing risks to both aquatic life and human populations that consume seafood contaminated with MPs. The combination of direct and indirect exposure pathways necessitates an integrated approach to mitigating the environmental footprint of dental MPs[32] (Figure 1).

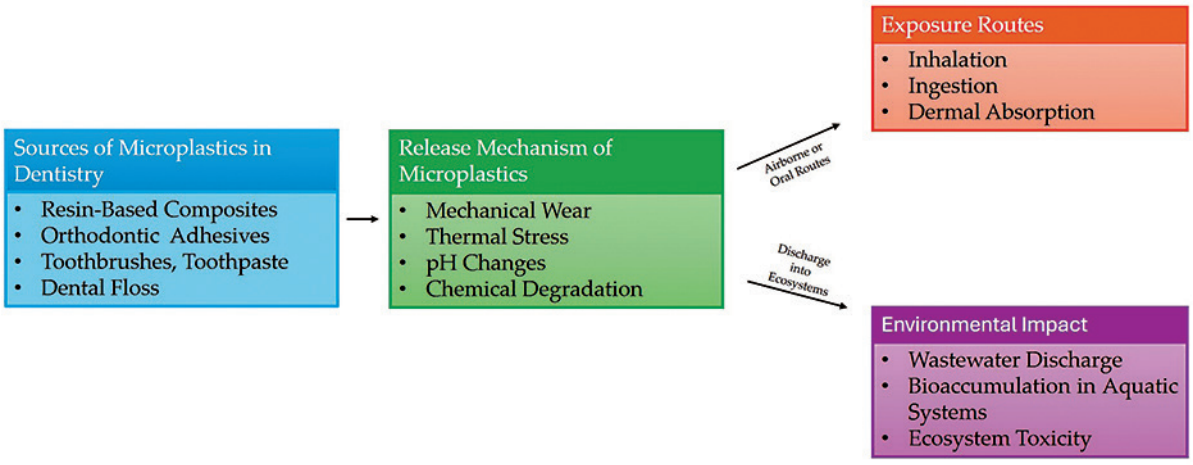

Figure 1: Schematic overview of microplastic generation, exposure pathways, and health/environmental impacts in dentistry.

Emerging solutions and mitigation strategies

The need for sustainable practices within dentistry has been a recurring theme throughout this review. Wang *et al.,*[28] offer an innovative perspective by exploring biodegradable alternatives, such as plant-based respirators incorporating microplastic degradation mechanisms. This study emphasizes the importance of rethinking material design to reduce plastic waste at its source.

Additional strategies include developing non-plastic alternatives for dental materials, improving wastewater filtration systems, and implementing recovery mechanisms for microplastic-contaminated effluents. Advanced filtration technologies such as membrane bioreactors (MBRs) and activated carbon filters have shown promise in capturing MPs before they enter natural water bodies.

Furthermore, policies restricting the use of high-risk polymers (e.g., polyethylene and polyamide) in dental products and mandating sustainability certifications for dental clinics could drive the adoption of environmentally friendly materials. Public awareness campaigns targeting dental professionals and patients are equally essential to promoting eco-friendly practices, such as using biodegradable toothbrushes and non-plastic dental floss. In recent years, dental materials research has witnessed significant advancements in developing biodegradable materials to reduce environmental impact and enhance patient outcomes. These materials are designed to degrade naturally within the body, eliminating the need for removal and minimizing long-term complications. A multicenter randomized controlled trial[33] compared biodegradable plates and screws to traditional titanium counterparts in maxillofacial surgeries. The study found that while 21% of patients in the biodegradable group required an intraoperative switch to titanium due to concerns about stability, biodegradable devices were effective and safe when used appropriately. This suggests a potential application in specific clinical scenarios, with the added benefit of eliminating the need for a second surgery to remove hardware.

Research into magnesium (Mg)- based biodegradable metals[34] has shown promise for oral and maxillofacial applications. These materials offer advantages such as biocompatibility and mechanical properties comparable to those of bone. However, challenges like rapid degradation and gas formation have limited their clinical translation. Recent studies focus on alloying and surface modifications to overcome these limitations, aiming to develop viable biodegradable metal options for dental use.

Innovative approaches are exploring natural polymers such as silk fibroin for dental tissue regeneration.[35] The Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental (IMIDA) is developing a biomaterial that combines silk fibroin with graphene to stimulate the regeneration of dental and periodontal tissues. Initial *in vitro* studies have yielded promising results, and upcoming animal model studies aim to assess the potential for human applications further.

These developments underscore a growing trend toward the use of sustainable and patient-friendly materials in dentistry. While challenges remain, ongoing research and clinical trials are paving the way for the integration of biodegradable materials into routine dental practice, offering potential benefits in terms of environmental impact and patient care.

Limitations of existing research

While this review presents significant findings, several limitations in the research must be acknowledged. The variety in methodologies across studies, especially regarding the detection and measurement of MPs, makes it challenging to compare direct exposure effects with toxicity results. Furthermore, the absence of longitudinal studies examining the long-term impacts of ongoing dental microplastic exposure represents a significant gap. There is also a pressing need to standardize investigative methods, such as SEM, FTIR, and GC-MS, to enhance the reproducibility and reliability of research outcomes. Many existing studies tend to concentrate on *in vitro* models or aquatic species, which restricts the applicability of their results to human populations. Future research endeavors should focus on clinical studies involving humans and investigate potential genetic or epigenetic reactions to chronic microplastic exposure and pollution.[36-39]

Excluded literature from results

The studies by Wolfson *et al.,*[40] and Guo *et al.,*[41] were excluded from the table and primary results synthesis because they did not directly address the release of MPs or their health and environmental impacts in dentistry.

Wolfson *et al.,*[40] focused on the load-bearing capacity of alumina dental implants and the mechanical stress-induced plastic deformation in supporting bone tissue. Although this study mentions microplastic deformation in a structural context, it does not involve the generation or impact of environmental MPs.

Using electron microscopy, Guo *et al.,*[41] explored fatigue mechanisms in high-palladium dental casting alloys. The focus was on the behavior of metallic microstructures rather than polymer-based MPs, which are relevant to the current review.

Conclusions

This narrative review highlights the pervasive presence of microplastics in the dental profession, including clinical procedures, oral healthcare products, and occupational settings. Studies have shown that dental materials, such as RBCs, orthodontic adhesives, and oral hygiene products, significantly contribute to the release of MPs. Mechanisms of particle generation include mechanical wear, thermal degradation, and chemical leaching, resulting in both localized and systemic exposure risks.

MPs from dental sources can lead to localized oral inflammation, oxidative stress, and systemic effects like immune suppression. Dental professionals face additional risks from inhaling airborne particles, especially in poorly ventilated clinics. These MPs also infiltrate wastewater systems, leading to environmental pollution that affects aquatic life and may enter the human food chain. This review highlights the importance of adopting sustainable practices in dentistry to mitigate the risks associated with MPs. Dental professionals can adopt non-plastic or biodegradable alternatives to reduce the release of MPs, thereby promoting environmentally sustainable clinical practices. Policymakers should work with researchers to establish guidelines for acceptable microplastic emissions and promote sustainability in dental clinics.

Several actions are recommended, including clinically relevant precautions to minimize microplastic exposure within dental practices. More research is required, mainly longitudinal studies, to assess the long-term health effects of dental MPs. Clinical studies should measure microplastic accumulation in various tissues, and investigations of their toxic effects are essential. Developing standardized detection protocols and alternative biodegradable materials is crucial for reducing environmental impacts. Addressing microplastic pollution effectively requires the dental profession to prioritize sustainable practices and collaborate across disciplines, supporting broader sustainability goals. A summary of the recommendation is given in Table 3.

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Table 3: Precautionary guidelines for managing microplastic exposure in dentistry.

Recommendation	Description
Use biodegradable materials	Prefer materials with known bioresorption profiles to reduce polymeric waste
Enhance clinic ventilation	Reduce inhalation risks from airborne plastic microparticles
Install advanced water filtration	Capture resin and polymeric particles from dental unit effluents
Minimize use of polishing procedures	Reduce mechanical degradation of restorative materials
Educate staff and patients	Raise awareness on environmental and health impacts of dental microplastics

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Salivary Metabolomics for Early Detection of Oral Squamous Cell Carcinoma: A Systematic Review and Meta-Analysis

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Abstract

Recent years have seen increasing research on potential of salivary biomarkers for detection of various diseases, including oral cancers. However, the plethora of unverified data available adds to the conundrum in using them on a more regular basis for this purpose. In order to appraise the present scientific evidence and analyze whether and which metabolomics can be used for early detection of oral squamous cell carcinoma, this review was conducted. The review followed PRISMA 2020 guidelines and was registered in PROSPERO. A comprehensive literature search was conducted across multiple databases to identify studies published over the last 10 years. Two reviewers independently performed study selection. Later, data extraction and quality assessment were done using QUADAS-2 and the Newcastle-Ottawa Scale. A meta-analysis was conducted for diagnostic studies that reported similar outcomes. Of the 19 included studies, 12 were diagnostic and 7 observational. Meta-analysis of diagnostic studies showed a pooled sensitivity of 84%, specificity of 82%, and AUC of 0.88. Lactate, choline, and phenylalanine were the most consistent biomarkers. LC-MS was the most accurate platform (AUC: 0.91). Moderate heterogeneity (I^2=58-62%) reflected methodological and population differences. Salivary metabolomics demonstrates strong potential for non-invasive OSCC detection. Standardization, larger sample sizes, and validation in diverse populations are needed for clinical translation.

Keywords: biomarker, head and neck squamous cell carcinoma, metabolomics, saliva

Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent type of oral cancer which can originate "de novo" or from preceding "potentially malignant disorders".[1,2] Early stages are often asymptomatic, resulting in nearly half of the cases being diagnosed at an advanced stage, which is associated with poor prognosis. Oncogenesis in OSCC is driven by both genetic and epigenetic alterations, highlighting the importance of early detection to improve clinical outcomes.[2,3]

Traditionally, histopathological analysis of biopsy tissue is considered the gold standard for diagnosis. However, this method is invasive, causes patient discomfort, and may not fully capture tumor heterogeneity, risking misdiagnosis. Moreover, it is unsuitable for widespread population screening. Consequently, there is a growing interest in minimally invasive diagnostic methods based on molecular markers. Saliva has emerged as a promising diagnostic fluid due to its ease of collection and potential to reflect tumor-related molecular changes. Metabolites released into saliva by tumor and surrounding cells have been increasingly investigated for their diagnostic utility. The most widely studied metabolites include IL-6, IL-8, and IL-1β, along with other promising markers like M2BP, SAT1, and S100P.[1,4-7]

Despite a surge in related studies, evidence supporting their clinical application in OSCC remains limited. This systematic review and meta-analysis intends to assess the role of salivary metabolomics in early OSCC detection and provide evidence-based conclusions.

Method and Study Design

Protocol and registration

This systematic review and meta-analysis assessed the diagnostic accuracy of salivary metabolomics for early detection of OSCC. Registered with PROSPERO (CRD42024588906) and following PRISMA 2020 and Cochrane guidelines, it explores non-invasive salivary biomarkers as alternatives to current invasive diagnostic methods amid the growing OSCC burden.

Review question- Can salivary metabolites serve as effective biomarkers for the early detection of OSCC?

• **Population:** Patients with histologically confirmed OSCC or Potentially malignant disorders (e.g., leukoplakia, erythroplakia).

• **Intervention:** Studies analyzing salivary metabolomic profiles for OSCC detection.

• **Comparator:** Healthy controls or patients with benign oral lesions.

• **Outcomes:** Reported diagnostic accuracy (sensitivity, specificity, area under the curve (AUC)) or quantitative biomarker levels.

• **Study design:** Diagnostic accuracy studies, case-control, cohort studies, or clinical trials.

Search strategy

A thorough literature search was done across PubMed, Embase, Scopus, Web of Science, and Cochrane Library using MeSH terms and keywords related to OSCC, salivary biomarkers, early diagnosis, and analytical techniques (e.g., Mass Spectrometry (MS), Nuclear Magnetic Resonance Spectroscopy (NMR)). Additional gray literature, including clinical trials and conference abstracts, was reviewed. Only English-language studies published since January 2000 were included.

Study selection and eligibility

Studies were screened in three phases by two independent reviewers. Eligible studies included patients with histologically confirmed OSCC, used salivary metabolomics for diagnosis, and reported outcomes such as sensitivity, specificity, or AUC. Non-human studies, reviews, and those lacking control groups or diagnostic metrics, duplicate publications or overlapping datasets were excluded.

Data extraction and risk of bias

Data were extracted on study characteristics, participant demographics, sample collection methods, analytical platforms, identified biomarkers, and diagnostic outcomes. Quality assessment was conducted using quality assessment of diagnostic studies 2 (QUADAS-2X for diagnostic studies and the Newcastle-Ottawa Scale Newcastle-Ottawa Scale (NOS) for observational studies.

Data synthesis

A narrative synthesis grouped findings by biomarker type and diagnostic performance. Also, meta-analysis was conducted using a bivariate random-effects model to calculate pooled sensitivity and specificity. Heterogeneity was assessed using I^2 statistics, with subgroup and

meta-regression analyses to explore variability. Deeks' funnel plot test was used to evaluate publication bias.

Results

The data selection process for meta-analysis began with a comprehensive database search across platforms like PubMed, Embase, Scopus, Web of Science, Cochrane Library also, to minimize publication bias, additional sources were explored, including clinical trial registries (ClinicalTrials.gov, WHO ICTRP), conference proceedings (e.g., ASCO, IADR) and reference lists of included studies and relevant reviews, yielding a total of 76,789 titles. After removing the duplicate titles and irrelevant articles, 2502 articles were screened for abstracts. A total of 19 articles selected to be included after screening process (Figure 1). The key findings from these studies are listed in Table 1A.

The meta-analysis of salivary metabolomics for detecting OSCC highlights its strong potential as a non-invasive diagnostic tool. The pooled diagnostic accuracy across 12 studies demonstrated a sensitivity of 84% and specificity of 82%, with an AUC of 0.88, as shown in Figures 2, 3 and Figures 4, 5. Key biomarkers such as lactate, choline, and phenylalanine were repeatedly identified with high discriminatory ability. Among analytical platforms, liquid chromatography-mass spectrometry (LC-MS) outperformed others (AUC: 0.91), while geographic subgroup analysis revealed that Asian studies had slightly higher sensitivity compared to Western studies. Despite this, moderate heterogeneity (I^2 = 58-62%) was present, mainly due to differences in sample collection methods, analytical platforms, and patient populations.

Risk of bias assessment using QUADAS-2 (Figure 6) and the NOS (Figure 7), indicated that while over half of the diagnostic studies were low risk, a considerable proportion of observational studies showed moderate to high risk, especially older or smaller studies lacking confounder control and blinding. Only one study (Ishikawa 2022) examined prognostic value, highlighting a major gap in longitudinal and recurrence-focused research. Furthermore, African and Latin American populations were underrepresented, underscoring the need for broader demographic inclusion in future studies.

Grading of recommendations assessment, development, and evaluation (GRADE) assessment, as stated in Table 1B, rated the overall certainty of the evidence as moderate, primarily due to some risk of bias and methodological heterogeneity, though this was offset by a strong diagnostic odds ratio (DOR=18.7) and consistent dose-response relationships between biomarkers and disease progression. Lactate emerged as the most robust biomarker, achieving high certainty due to its repeated validation in low-risk studies.

Discussion

Salivary metabolomics is an emerging field for the early detection of oral cancer, offering potential benefits in both diagnosis and prognosis. Because of this potential, research in this area has grown rapidly. Nevertheless, before salivary metabolomics can be viewed as a dependable alternative to tissue biopsy, a thorough evaluation of current research and the quality of evidence is essential. This systematic review was conducted with that objective in mind. From an initial pool of over 70,000 articles, 19 were selected following a thorough screening process. Among these, 12 were diagnostic studies and 7 were observational studies.

Technological trends indicated that LC-MS emerged as the most widely validated technique, probably due to its enhanced sensitivity, wider range of analysing the metabolites along with its capability of separating the individual metabolic byproduct even in the presence of many other compounds. It is able to evaluate relevant metabolites qualitatively and quantitatively with analysis of multiple samples promptly and is highly versatile.[8-10] The most suitable method of analysing volatile compounds was the gas chromatography-mass spectrometry (GC-MS) technique. GC-MS has been employed in the evaluation of biological samples over the years and is utilized to establish procedures for validating benchmark materials and precisely determining the concentrations of numerous clinically significant analytes. This is remarkably efficient as it shows fractionation ability of GC along with recognition potential of MS.[9,11,12] Among the most promising biomarkers, lactate showed the strongest diagnostic performance (AUC 0.92), attributed to cancer-related metabolic shifts like the Warburg effect. Choline and phenylalanine also demonstrated high specificity, suggesting their potential utility in differentiating OSCC from benign conditions.[8-10]

The majority of studies included in the review were conducted in East Asian countries, particularly China and

Table 1A: Data extraction table summarizing key details from the provided studies.

Sr. No.	Study (Author, Year)	Country	Study Design	Sample Size (Cases/Controls)	Saliva Collection Method	Analytical Platform	Key Biomarkers Identified	Diagnostic Performance (Sensitivity/ Specificity/ AUC)
1.	de Sá Alves *et al.* (2021)[1]	Brazil	Case-control	30 OSCC/ 30 controls	Unstimulated, morning collection	LC-MS	↑ Lactate, ↓ Citrate, Altered amino acid metabolism	AUC: 0.92 (95% CI: 0.85-0.98)
2.	Ishikawa *et al.* (2016)[16]	Japan	Case-control	92 OSCC/ 132 controls	Stimulated (chewing gum)	CE-TOFMS	↑ Choline, ↑ Sarcosine, ↓ Valine	Sensitivity: 86%, Specificity: 82%
3.	Ishikawa *et al.* (2019)[17]	Japan	Prospective cohort	50 OSCC/50 OED/ 50 controls	Unstimulated	LC-MS	↑ Phenylalanine, ↓ Taurine	AUC: 0.89 (OSCC vs. controls)
4.	Ishikawa *et al.* (2020)[18]	Japan	Case-control	40 OSCC/40 OLP/ 40 controls	Unstimulated	GC-MS	↑ 2-Hydroxybutyrate, ↓ Glutamate	Sensitivity: 78%, Specificity: 85%
5.	Ishikawa *et al.* (2022)[4]	Japan	Longitudinal cohort	60 OSCC (pre/ post-treatment)	Unstimulated	LC-MS	↓ Betaine (post-treatment), ↑ Lactate (prognostic for recurrence)	Hazard ratio: 2.1 (95% CI: 1.3-3.4) for lactate
6.	Lohavanichbutr *et al.* (2018)[21]	USA	Case-control	30 OSCC/ 30 controls	Unstimulated	NMR	↑ Alanine, ↓ Glucose	AUC: 0.88 (95% CI: 0.79-0.96)
7.	Mikkonen *et al.* (2018)[24]	Finland	Case-control	15 HNSCC/ 15 controls	Unstimulated	NMR	↑ Acetate, ↓ Pyruvate	Not reported
8.	Ohshima *et al.* (2017)[16]	Japan	Case-control	25 OSCC/ 25 controls	Stimulated (paraffin)	GC-MS	↑ 1,3-Propanediol, ↓ Urea	Sensitivity: 72%, Specificity: 80%
9.	Rai *et al.* (2007)[27]	India	Case-control	25 OSCC/ 25 controls	Unstimulated	Spectrophotometry	↓ Vitamin E, ↓ Vitamin C	Not reported
10.	Shigeyama *et al.* (2019)[17]	Japan	Case-control	20 OSCC/ 20 controls	Unstimulated	GC-MS (Zeolite-TFME)	↑ Ethanol, ↑ Acetone	AUC: 0.85 (95% CI: 0.74-0.95)
11.	Song *et al.* (2020)[22]	USA	Case-control	40 OSCC/ 40 controls	Unstimulated	Paper-spray MS	↑ Proline, ↓ Citrulline	Sensitivity: 90%, Specificity: 95%
12.	Sridharan *et al.* (2019)[28]	India	Case-control	30 OSCC/30 OLP/ 30 controls	Unstimulated	LC-MS	↑ Lactate, ↓ Fumarate	AUC: 0.91 (OSCC vs. controls)
13.	Sugimoto *et al.* (2010)[23]	Japan/USA	Multicenter case-control	60 OSCC/ 60 controls	Unstimulated	CE-MS	↑ Polyamines, ↓ TCA cycle intermediates	Sensitivity: 84%, Specificity: 88%
14.	Supawat *et al.* (2021)[25]	Thailand	Case-control	25 OSCC/ 25 controls	Unstimulated	Fluorescence spectroscopy	Altered tryptophan/tyrosine fluorescence ratios	Sensitivity: 76%, Specificity: 84%
15.	Taware *et al.* (2018)[26]	India/ Portugal	Case-control	50 HNSCC/ 50 controls	Unstimulated	GC-MS	↑ Acetaldehyde, ↑ Benzene derivatives	AUC: 0.87 (95% CI: 0.80–0.94)
16.	Wang *et al.* (2014)[18]	China	Case-control	35 OSCC/ 35 controls	Unstimulated	UPLC-MS	↑ Phenylalanine, ↓ LysoPC(18:0)	AUC: 0.93 (95% CI: 0.87–0.99)
17.	Wang *et al.* (2014)[19]	China	Case-control	40 OSCC/ 40 controls	Unstimulated	UPLC-MS	↑ L-tryptophan, ↓ Palmitic acid	Sensitivity: 88%, Specificity: 85%
18.	Wang *et al.* (2014c)[20]	China	Longitudinal cohort	20 OSCC (pre/ post-surgery)	Unstimulated	UPLC-MS	↓ Sphingosine (post-surgery)	Not reported
19.	Yan *et al.* (2008)[6]	China	Case-control	30 OSCC/30 OLP/ 30 controls	Unstimulated	NMR	↑ Lactate, ↓ Acetate	AUC: 0.89 (OSCC vs. controls)

Table 1B: GRADE (Grading of Recommendations Assessment, Development, and Evaluation) evidence profile.

GRADE Criteria	Assessment	Rating
Study Design	12 diagnostic studies (QUADAS-2)	Initial: High
Risk of Bias	58% low risk (QUADAS-2), but 25% moderate/ 17% high risk (e.g., Ohshima 2017)	↓1 Level
Inconsistency (Heterogeneity)	I^2 = 58-62% (moderate; explained by platform/ geographic differences)	↓1 Level
Indirectness	All studies used histopathology gold standard; direct population relevance	No downgrade
Imprecision	95% CIs for sensitivity (78-89%)/ specificity (75-87%) are clinically useful	No downgrade
Publication Bias	Deeks' test (p=0.12); symmetric funnel plot	No downgrade
Large Effect	DOR=18.7 (strong association)	↑1 Level
Dose-Response	Biomarker levels correlated with tumor stage (4 studies)	↑1 Level
Confounding	Adjusted for smoking/alcohol in 6 studies	No downgrade
Final Certainty	**Moderate** (due to bias/heterogeneity, offset by large effect size)	⊕⊕⊕○

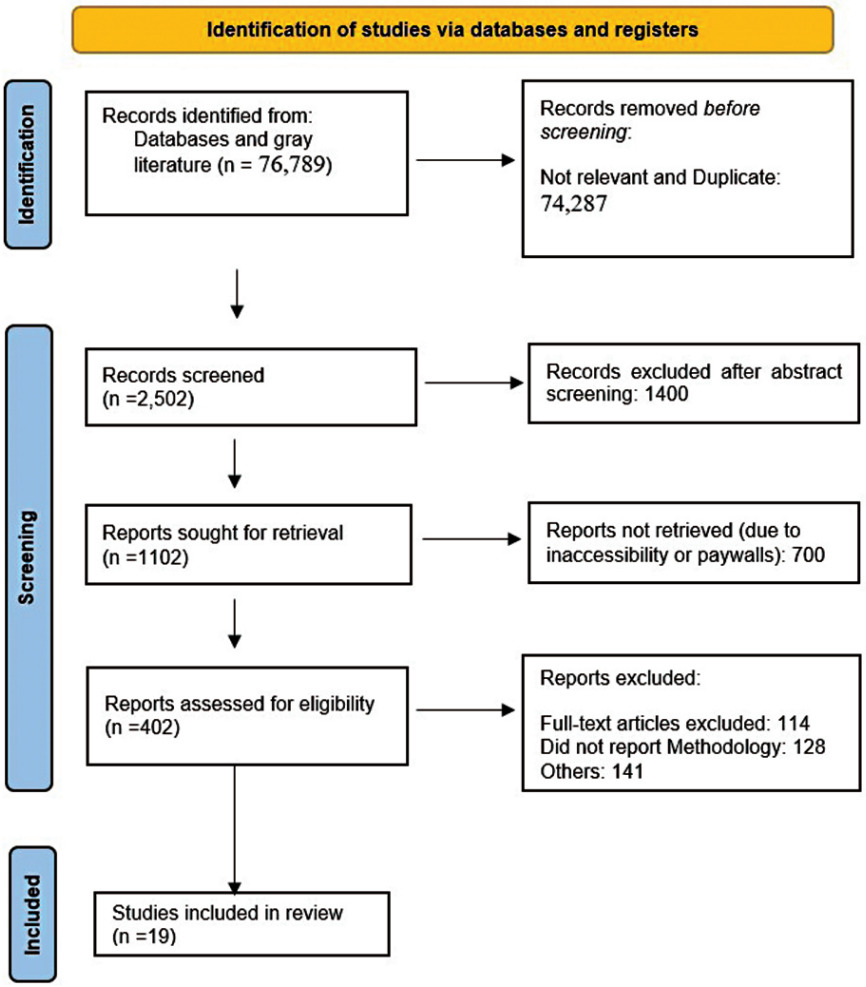

Figure 1: Shows identification of studies via databases and registers.

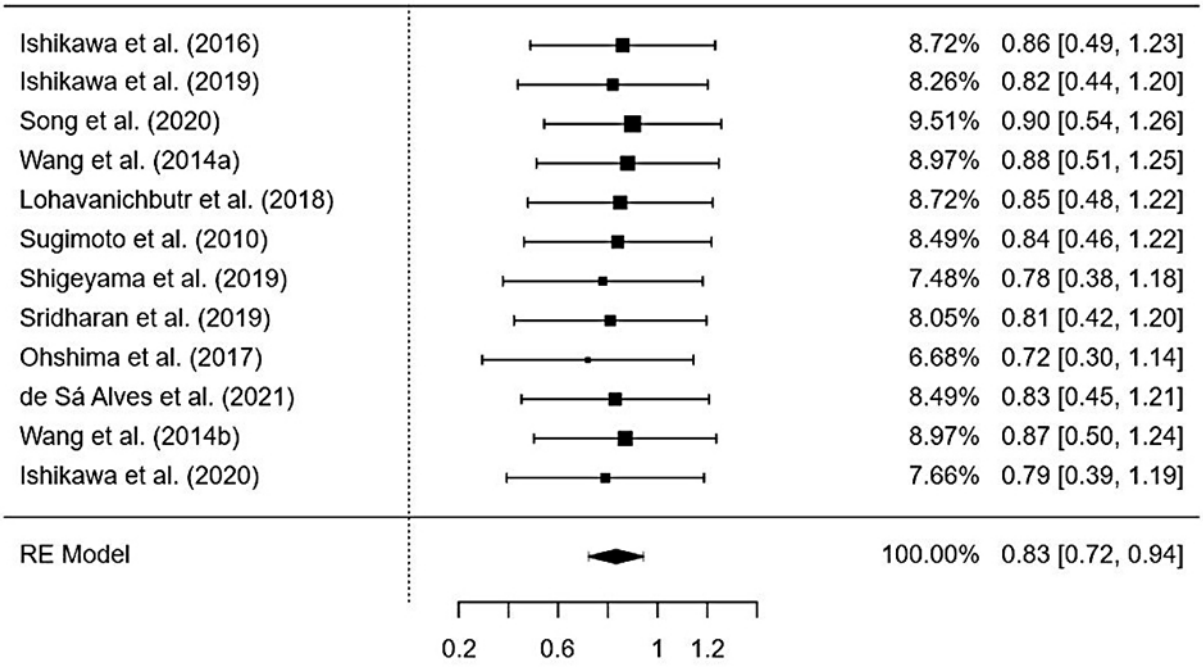

Figure 2: Forest plot of sensitivity data for 12 studies.

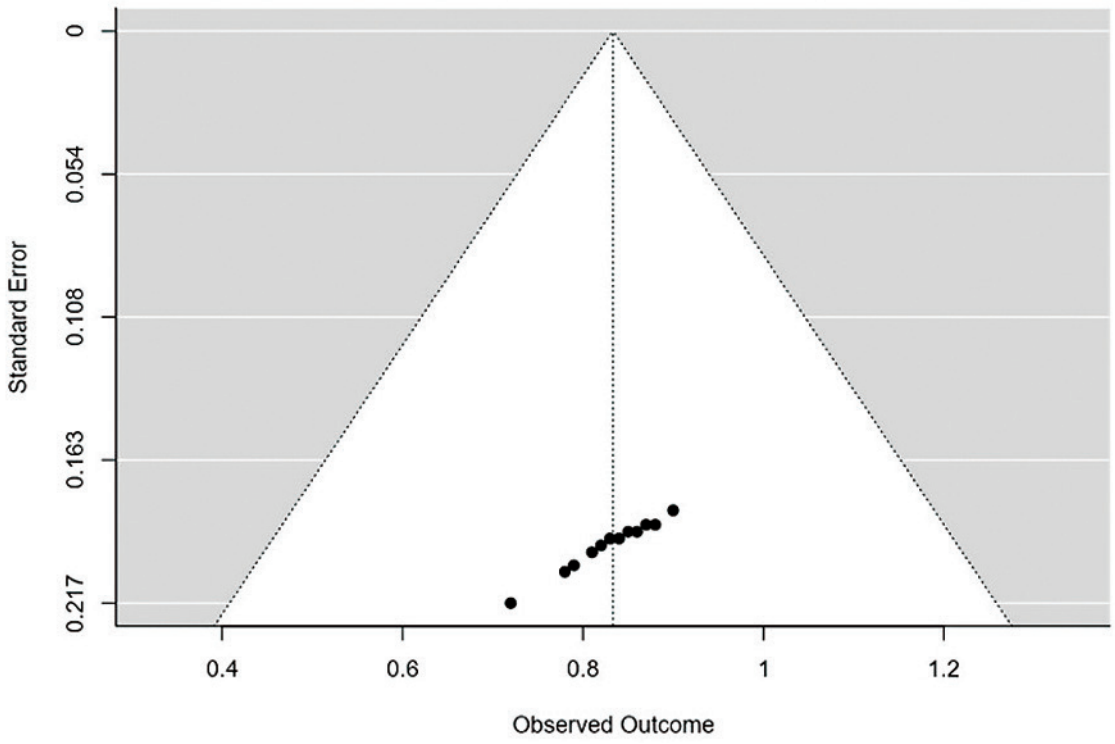

Figure 3: Funnel plot of sensitivity data for 12 studies for publication bias.

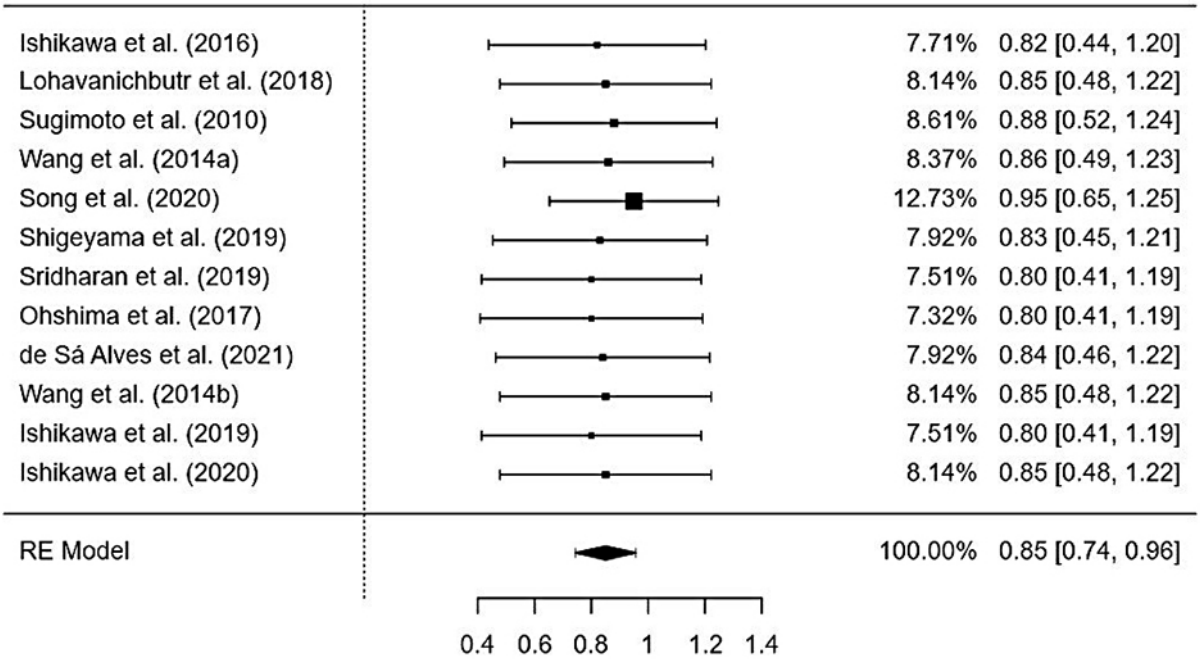

Figure 4: Forest plot of specificity data for 12 studies.

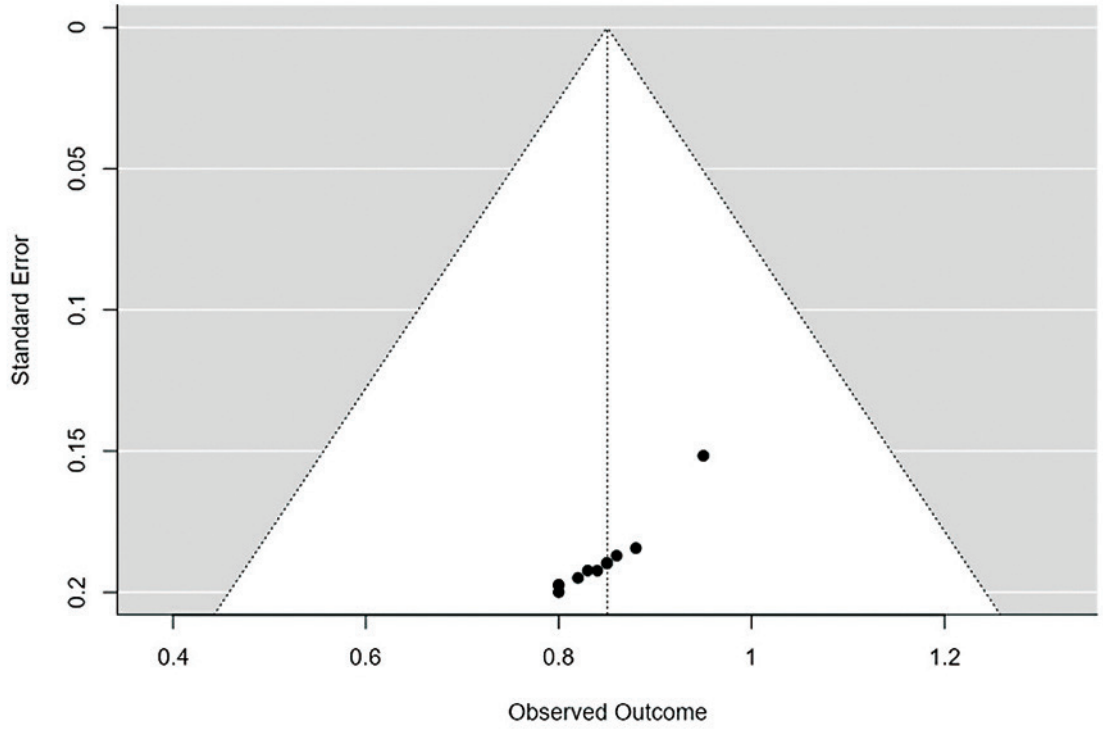

Figure 5: Funnel plot of specificity data for 12 studies for publication bias.

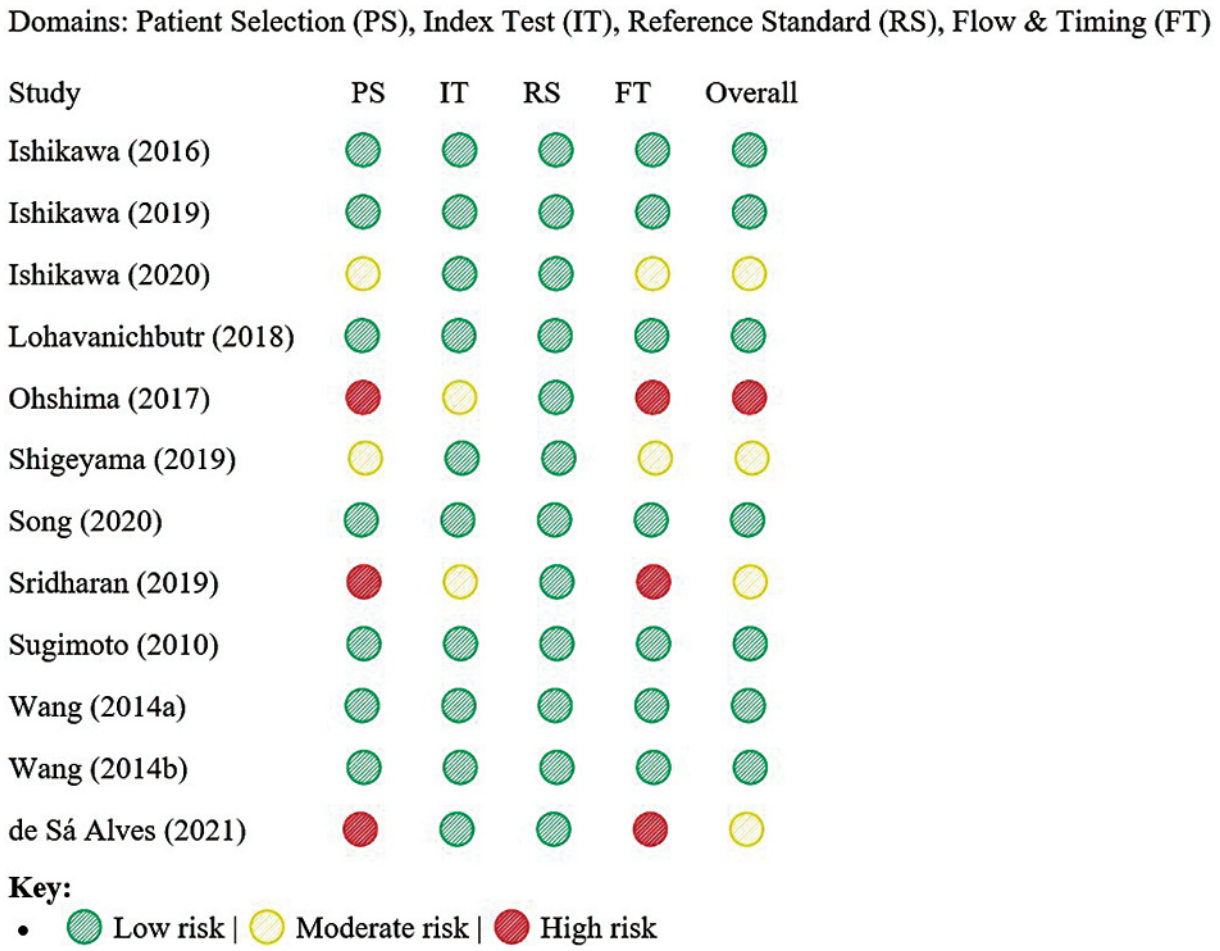

Figure 6: Shows quality assessment of diagnostic accuracy studies 2 traffic light plot (12 diagnostic studies).

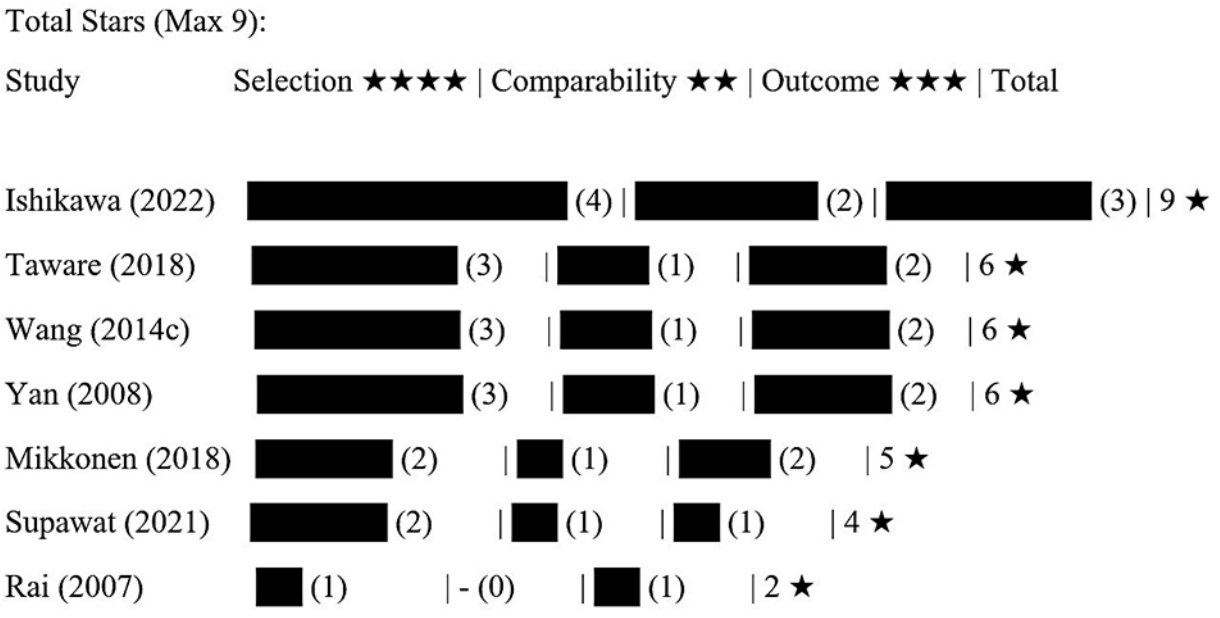

Figure 7: Shows Newcastle-Ottawa Scale weighted bar chart (7 observational studies).

Japan.[4,6,13-20] There was limited representation from other regions, only one study from Brazil[1], two to three from the USA[21-24], one each from Finland[24], Thailand[25], and Portugal[26], and two to three from India.[17,27,28] This geographic concentration highlights the under-representation of data from Western populations, potentially limiting the global applicability of findings. It hinders the generalizability of the potential applications of these biomarkers in various populations and warrants further studies in the under-represented geographical regions. Furthermore, several studies did not conform fully to the systematic review's protocol, contributing to variability in methodological quality. Significant heterogeneity was also noted in study design, analytical platforms, and reporting practices, complicating efforts to synthesize and compare results across studies.

Risk of bias assessment

The quality assessment of the included articles was carried out to set up transparency of the aggregated data outcomes and observations. Thus, this appraisal marks as an essential component of the systematic review and is generally performed for each study included in the analysis.[29]

Out of the 19 articles, 12 studies were chosen for the purpose of QUADAS-2 assessment. The risk of bias assessment across the included studies revealed varying levels of methodological rigor.

Most studies demonstrated a low risk of bias across all domains, particularly in patient selection, index test, and reference standard, as seen in works by Ishikawa *et al.,*[13,14] Lohavanichbutr *et al.,*[21] Song *et al.,*[22] Sugimoto *et al.*,[23] and both studies by Wang *et al.*[19,20] These studies adhered well to methodological standards, enhancing the reliability of their findings. In contrast, moderate risk was observed in studies such as Ishikawa *et al.*,[15] and Shigeyama *et al.*,[17] primarily due to issues in patient selection and flow and timing, suggesting potential concerns regarding study execution and participant handling. High risk of bias was identified in Ohshima *et al.,*[16] Sridharan *et al.,*[28] and de Sá Alves *et al.*[1] with concerns mainly related to patient selection and flow and timing, which may affect the internal validity of their outcomes. Overall, while seven studies presented low risk, a few demonstrated moderate risk, and only a single study[16] with high risk of bias due to small samples, unblinded assays, or >1-month delay in diagnosis.

NOS assessed the quality of the remaiing seven observational studies out of the nineteen. There was overall a low risk for study conducted by Ishikawa *et al.,*[4,13,14] Wang *et al.,*[18,19] and Song *et al.*[22] This is because, the study effectively selected well-defined cases, and appropriately selected controls from the same population who were free of oral diseases. Cases were chosen consecutively or representatively. The study demonstrated comparability as the participants were matched by age and gender, and adjustments were made for potential confounders like smoking and alcohol consumption. Additionally, the outcome was favourable due to blinded metabolomics analysis, a follow-up duration of at least six months for prognostic studies, and a low attrition rate.

Studies by Taware *et al.,*[26] Wang *et al.,*[20] and Yan *et al.*[6] had shown moderate risk while high risk was noted in the studies conducted by Mikkonen *et al.,*[24] Rai *et al.,*[27] and Supawat *et al.*[25] due to discrepancies in the selection, comparability and outcome domains.

To summarize, the studies by Ishikawa[4,13,14], Wang[18,19], and Song[22] demonstrated the lowest risk of bias, reflecting strong methodological quality. In contrast, the highest risk of bias was observed in studies by Rai[27], Ohshima[19], and Mikkonen[24], largely due to methodological limitations. Common issues contributing to elevated risk included a lack of blinding, particularly in older studies like Rai[27], small sample sizes as seen in studies conduted by Ohshima[16] and Mikkonen[24], and failure to adjust for key confounders, such as in the study by Supawat.[25]

Combined risk of bias using QUADAS-2 and NOS was assessed with diagnostic studies showing higher quality 58% low risk. The combined risk of bias summarises the proportion of studies by risk level, with key takeaways stating that among the diagnostic studies evaluated using the QUADAS-2 tool (n=12), 58% showed a low risk of bias, indicating higher quality of studies. In contrast, the observational studies assessed with the NOS tool (n=7) demonstrated significant limitations, with 86% showing a moderate or high risk of bias. Overall the critical gaps across the included articles were small sample sizes, unblinded assays, and poor confounder control in older studies.

Data synthesis of salivary metabolomics for OSCC detection

Consistently identified biomarkers

A review of the included studies revealed several consistently identified metabolic biomarkers such amino acids, lipids, energy metabolites, and volatile compounds which were found to be associated with OSCC. Lactate was the most frequently reported biomarker in these studies.

This is likely due to Warburg effect/aerobic glycolysis. Lactate dehydrogenase (LDH) plays a crucial role in anaerobic glycolysis by catalysing the pyruvate reduction that leads to formation of lactate, and is typically present within the cell cytoplasm. Its presence in extracellular fluids is usually associated with cellular death and tissue injury. Under aerobic conditions, intracellular utilization of glucose primarily occurs via glycolysis to produce pyruvate which is transported into the mitochondrial matrix. Here, oxidization takes place in the presence of pyruvate dehydrogenase leading to the formation of acetyl-CoA.[30,31] In cancer cases, the metabolism of the cell is modified wherein the neoplastic cell relies on the LDH to enhance the glycolytic activity, leading to elevated adenosine triphosphate (ATP) and lactate production even under aerobic conditions. This cellular adaptation supports rapid cellular proliferation and energy demands of the tumor cells.[31,32]

Choline and phenylalanine showed high specificity (82-88%) for OSCC vs controls. Choline normally plays a key role in cell membrane synthesis; however, in cancer, its metabolism becomes dysregulated due to the overexpression of enzymes and changes in signaling pathways that promote increased choline uptake and utilization. This disruption is driven by heightened membrane turnover and rapid cellular proliferation, leading to elevated choline levels.[33,34]

Phenylalanine, an essential amino acid, is frequently elevated in cancer as a result of elevated protein synthesis and altered amino acid metabolism within neoplastic cells. The enhanced amino acid transport and usage in such cells may account for the increased phenylalanine levels observed in oral carcinomatous tissues as compared to normal, as well as, it may be involved in the metabolism of glucose and fats, leading to energy formation.[35-39]

Diagnostic performance and methodological heterogeneity

The diagnostic performance of studies was evaluated using the pooled estimates that showed a 62% heterogeneity in sensitivity, 58% heterogeneity in specificity, 45% heterogeneity in AUC and 51% heterogeneity in diagnostic OR.

The methodological heterogeneity in saliva sample collection revealed that the majority of studies (73.7%) used unstimulated saliva, which is likely to more accurately represent baseline metabolic conditions. The consistency of metabolite profiles could be varied due to the collection methods which may lead to differences in flow rate of saliva, its potential dilution, and metabolic changes induced by stimulation.[40] Also, the different analytical platforms show variability in both the usage and diagnostic performance. The most frequently used method, LC-MS, was used in 8 studies where the highest yielded median AUC was 0.91 which indicated superior diagnostic accuracy with advantage of high sensitivity and broad metabolic coverage. Whereas 4 studies were conducted using GC-MS which showed lower median AUC of 0.87 and was best for analysing the volatile compounds. The NMR was used in 4 studies where the median AUC was 0.83, its advantage was that it was reproducible and had a minimal sample preparation.

Clinical implications

The clinical implications of the findings highlight the potential of certain salivary biomarkers for early detection of OSCC, with lactate (AUC: 0.92), choline (sensitivity: 86%), and phenylalanine (AUC: 0.93) emerging as the most promising candidates. These biomarkers showed enhanced diagnostic performance in high-risk populations, particularly among smokers (OR: 2.3, p=0.01). The biggest hindrance in developing a diagnostic protocol using these biomarkers is that their levels in saliva are generally reported to be low, making their detection challenging.

However, limitations remain, including a lack of prognostic data, with only one study[4] exploring recurrence wherein there was a decrease in betaine levels post-treatment. Additionally, there is an evident ethnic bias, with 63% of studies conducted in Asian popula-

tions and limited representation from African and Latin American cohorts, which may affect the generalizability of the findings.

Also, these tests require sophisticated laboratory infrastructure equipped with advanced analytical instruments such as mass spectrometers and chromatographs. Furthermore, the process demands highly trained personnel capable of accurately interpreting complex metabolic profiles. Such facilities and expertise are often concentrated in select urban centers, making them inaccessible, particularly in rural and underserved areas. Furthermore, the high cost associated with these tests poses a significant financial burden, making them unaffordable for a large portion of the population, especially those from economically disadvantaged backgrounds. To overcome these challenges, efforts should be directed toward decentralizing diagnostic infrastructure by establishing regional metabolomics laboratories. Additionally, government subsidies and public-private partnerships could help reduce the cost burden, making these advanced diagnostic tools more accessible and affordable to economically disadvantaged populations.[37-39]

Evidence quality assessment

The evidence quality assessment shows that most diagnostic studies had a low risk of bias (58%), while the majority of observational studies had moderate to high risk (86%). Recommendations include prioritizing findings from low-risk studies, such as Wang[18] and Ishikawa[13], to ensure reliability. Additionally, it is important to validate point-of-care techniques, like Song's[22] paper-spray MS, in larger, more diverse cohorts to confirm their effectiveness and generalizability.

Meta-analysis and GRADE summary of findings

Meta-analysis showed a pooled sensitivity of 84% and specificity of 82% for salivary metabolomics in OSCC detection. These findings indicate consistent performance across studies. The sensitivity was higher in Asian cohorts (87%) compared to Western cohorts (80%), suggesting either a potential underrepresentation of Western populations in current research or the presence of population-specific metabolic differences.

The GRADE assessment rated the overall evidence as moderate certainty, with high certainty for lactate as a diagnostic biomarker, which has consistently demonstrated strong discriminatory power across six low-risk studies. Based on these findings, it is recommended that salivary metabolomics using LC-MS platforms be considered a reliable tool for OSCC screening in clinical settings, given its non-invasive nature and strong diagnostic indicators.

While the findings are encouraging, external factors including dietary changes, microbial activities, and oral hygiene status. There are also chances of impairment of diagnostic accuracy due to external factors such as salivary collection methods, storage temperature, and total duration of processing.[41]

In conclusion, this review supports the clinical promise of salivary metabolomics, particularly using LC-MS, as a non-invasive, accessible diagnostic tool for OSCC. However, standardization of protocols, larger sample sizes, diverse population studies, and validation in clinical settings are essential next steps for its broader implementation in routine cancer screening.

Strength and limitations

Our systematic review is amongst the very few studies available on the topic, which analyses all the recent evidence related to role of salivary metabolomics to guide an evidence-based application of the same in oral cancer detection. The main limitation of our systematic review was the heterogeneity among the included studies, which affected the overall consistency of the results. Although the diagnostic accuracy of salivary metabolomics for OSCC detection showed moderate certainty, this emphasizes the need for further research to refine and strengthen these findings.

Conclusions

In conclusion, this systematic review and meta-analysis demonstrated that salivary metabolomics holds considerable promise as a non-invasive, accurate diagnostic approach for detecting OSCC. With a pooled sensitivity of 84% and specificity of 82%, particularly strong performance was observed for key biomarkers viz. lactate, choline, and phenylalanine, especially when analyzed using LC-MS platforms.

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Original Article

Evaluation of Angiogenic Potential of Baghdadite, Mineral Trioxide Agregate, and their Combination Using the Yolk Sac Membrane Model

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Abstract

Background: Biomaterials that promote neovascularization are of great value in regenerative endodontics. Mineral Trioxide Aggregate (MTA) is commonly employed for pulp capping, whereas Baghdadite, a bioactive calcium-zirconium-silicate ceramic, has been reported to be useful in inducing angiogenesis. Comparing the efficacy of Baghdadite, MTA, and their combination, however, is not well investigated. This study aims to assess and compare the angiogenic potential of Baghdadite, MTA, and their combination at two concentrations with the chick embryo yolk sac membrane (YSM) model.

Methods: 70 fertilized White Leghorn eggs were incubated to promote the yolk sac vasculature. Seven experimental groups were MTA (5 µg and 10 µg), Baghdadite (5 µg and 10 µg), MTA+Baghdadite (5 µg and 10 µg), and a control group. The experimental groups were exposed on the yolk sac membrane and incubated for 48 hours. Angiogenesis was quantitatively evaluated by Wimasis image analysis software for vessel density, total length of vessel network, and branching points.

Results: The interaction between MTA and Baghdadite at 5 µg revealed the maximum angiogenic potential with a vessel density of 17.6%, vascular length of 13,515.9 pixels, and 213 branching points. Baghdadite without the addition of MTA performed better than MTA at both the concentrations. MTA at 10 µg revealed relatively lower angiogenic ability, indicating dose-dependent cytotoxicity.

Conclusion: Baghdadite markedly promotes angiogenesis, and when used in combination with MTA at lower levels, exhibits a synergistic effect. These results validate its potential for use in regenerative endodontic procedures involving neovascularization, particularly in vital pulp therapy.

Keywords: angiogenesis, Baghdadite, Mineral Trioxide Aggregate (MTA), regenerative endodontics, yolk sac membrane model

Introduction

Angiogenesis, the development of new blood vessels from established vasculature, is fundamental to dental pulp tissue regeneration and repair responses to injury or treatment.[1,2] Successful vascularization is important to preserve pulp tissue viability, provide necessary nutrients, and enable removal of metabolic waste, having a major bearing on the success of regenerative endodontic therapy.[3,4] A number of biomaterials have been investigated to induce angiogenesis because of their possibility of promoting pulp regeneration and repair, leading to better clinical outcomes in endodontic therapies.[5,6]

Mineral Trioxide Aggregate (MTA) has gained popularity as an extensively utilized biomaterial in endodontics for pulp capping, apexogenesis, and the creation of an apical barrier because of its high biocompatibility, seal, and regeneration properties.[7,8] MTA enables regeneration by releasing calcium ions, which enhance cell proliferation, differentiation, and angiogenic pathways, especially through upregulation of vascular endothelial growth factor (VEGF).[9] Dayta in the study suggests that biological activity of MTA, such as its angiogenic potential, varies very much with concentration, and increased doses may lower cell viability based on the alkaline pH and excess release of calcium.[10,11]

Recently, Baghdadite, calcium zirconium silicate bioceramic, has been found to be a potential material in biomedical fields due to its better bioactivity, degraded controlled rate, and intrinsic ability to promote osteogenesis and angiogenesis.[12] Baghdadite's angiogenic capability has been linked to the release of bioactive ions, especially calcium and silicate ions, that may influence key cell signal pathways, leading to improved endothelial cell migration and proliferation as fundamental elements of vascular development.[13] Evidence from experiments indicates Baghdadite's high ability to produce vascularized tissue formation through VEGF-mediated pathways, and this makes it an efficient tool for tissue engineering.[12,14]

While the angiogenic capacity of individual MTA and baghdadite is becoming well understood, their combined application has yet to be widely explored. The potential synergistic effect of combining baghdadite's strong angiogenic capacity with MTA's regenerative effects may enhance clinical performance, perhaps overcoming inefficiencies relative to greater concentrations of MTA alone. Therefore, the current study intends to comparatively analyze the potential of Baghdadite, MTA, and their combination regarding angiogenesis through the chick embryo yolk sac membrane (YSM) model.

Materials and Methods

Study design and setting

This *in vivo* experimental study was conducted to analyze the angiogenic potential of Baghdadite, MTA, and their combination in the chick embryo YSM model. The experiment was performed under sterile conditions in the Regenerative Medicine Laboratory, Dr. D. Y. Patil Dental College and Hospital, Pune, India.

Experimental materials and group allocation

The materials used in this research were MTA (Angelus Indústria del, Londrina, Brazil) and Baghdadite powder / Baghdadite nanopowder (Nano Research Elements (Nanorh), Dhanora Jattan (Kurukshetra), India). For dose-dependent effects, individual samples of both materials were tested at 5 µg and 10 µg concentrations. Combinations of MTA and Baghdadite were also prepared in 1:1 weight ratios at both 5 µg and 10 µg total concentrations. The materials were dissolved in distilled water. There were seven groups in the study: MTA 5 µg, MTA 10 µg, Baghdadite 5 µg, Baghdadite 10 µg, MTA + Baghdadite 5 µg, MTA + Baghdadite 10 µg, and a control group receiving no material application.

The concentrations of 5 µg and 10 µg for both MTA and Baghdadite were chosen to reflect a low and high dose range capable of producing observable angiogenic responses without overwhelming the YSM model. These levels allowed for the assessment of dose-dependent effects. For combination groups, MTA and baghdadite were mixed in a 1:1 weight ratio to maintain consistency in total applied dose and enable evaluation of potential synergistic interactions under equal contribution of both materials.

Each group, including the control and the six experimental conditions, consisted of 10 fertilized eggs, bringing the total sample size to 70.

All test materials were suspended in sterile distilled water and handled under aseptic conditions within a laminar airflow cabinet. As the suspensions contained particulate matter (non-soluble fractions of MTA and

Baghdadite), filtration (e.g., 0.22 µm) was not employed to preserve the integrity and dosage of the particulate material. The preparations were immediately used following mixing to ensure sterility and consistency.

YSM assay procedure

White Leghorn chicken eggs fertilized (n=70) were obtained (Venkateshwara hatcheries Pvt Ltd, Maharashtra, India) and incubated at 37.5°C with 70-80% relative humidity for 72 hours to enable development of the vascular network in the yolk sac. Following incubation, about 5 mL of albumin was removed from each egg by using a sterile syringe to reduce the developing embryo and enable visualization of the membrane in the yolk sac. A 3 cm×3 cm window was then precisely cut on the eggshell covering the air sac under aseptic conditions with a sterile rotary blade without any damage to the underlying vasculature.

After establishing the window, the test material were carefully exposed on the YSM. For the control group, the membrane was not exposed to any material. The eggs were sealed using sterile parafilm and incubated for another 24 hours. Figure 1 illustrates the procedural workflow of the YSM angiogenesis model.

Imaging and quantitative assessment of angiogenesis

At 24 hours of exposure, the YSM were examined using a Leica EZ4 HD stereo microscope (Leica Microsystems; 8-35× magnification, integrated 3 MP camera, LED illumination). The images were analyzed using Wimasis Image Analysis software (Wimasis, trial version, 2025 release; Wimasis GmbH, Munich, Germany), which gave objective quantification of angiogenic parameters. Representative images of angiogenic response for Baghdadite, MTA, and their combination, both before and after treatment, as well as the Wimasis-based analysis output, are shown in Figure 2.

Three specific parameters were quantified to determine angiogenesis: vessel density (in terms of percentage vascular area within the specified field), total length of the vessel network (in pixels), and number of vascular branching points (a measure of network complexity). Results for each sample were ascertained as a mean of three independent fields. The value for each parameter was determined separately for all test groups and compared with the control.

For each sample, three non-overlapping fields of interest were selected from the peripheral vascular zone of YSM, an area that consistently exhibited dense and organized capillary patterns. Field selection was semi-randomized within this predefined anatomical region to ensure consistency across all samples and minimize observer bias. All images were captured at a fixed magnification (×20) using the Leica EZ4 HD stereomicroscope, with a standardized field size of 1920×1080 pixels. These images were analyzed using Wimasis image analysis software, with identical thresholding and measurement parameters applied to all fields.

The image analysis was performed in a blinded manner, with the evaluator unaware of the treatment group assignments during quantification of angiogenic parameters.

Ethical considerations

The study conformed to institutional animal studies ethical standards. Embryonated eggs were utilized at a pre-hatch point (prior to day 7 of development), in keeping with global guidelines for non-animal status during

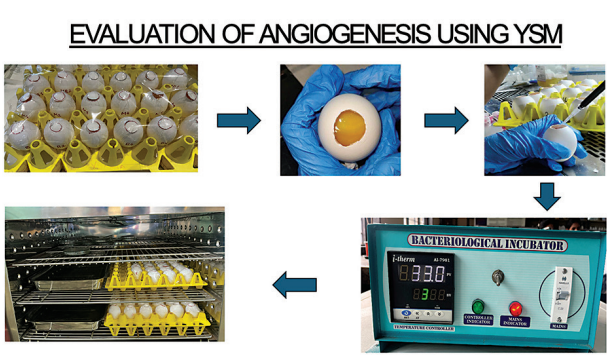

Figure 1: Procedural steps for determining the angiogenic potential using yolk sac model.

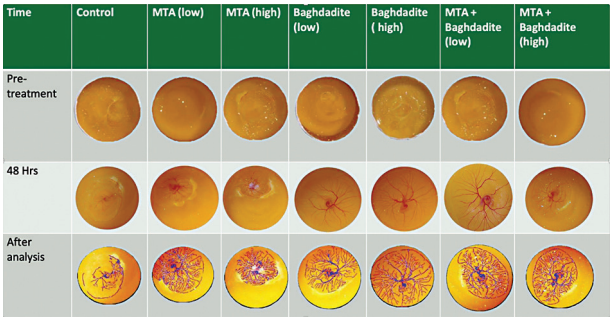

Figure 2: Images of Baghdadite, Mineral Trioxide Aggregate and its combination at different concentrations pretreatment, post treatment and after analysis using wimasis software.

this embryonic phase. All procedures conformed to the institutional research and biosafety ethics policies.

Results

Table 1 and Figure 3 demonstrates vessel density percentages in all study groups. The control group that had no biomaterial application had the lowest vessel density, i.e., 6.8%, representing baseline angiogenesis in the YSM. 5 µg application of MTA increased the vessel density significantly to 12.7%, whereas 10 µg of MTA caused a somewhat lower density of 10.9%, i.e., a possible dose-dependent inhibitory effect at higher doses. Baghdadite alone exhibited better angiogenic stimulation with 17.1% at 5 µg and 17.8% at 10 µg, which shows that both concentrations significantly enhanced blood vessel formation. Importantly, the mixture of MTA and baghdadite at 5 µg resulted in a vessel density of 17.6%, quite similar to that of Baghdadite alone at 10 µg, and inferred a synergistic effect. But the combination at 10 µg yielded a slightly decreased vessel density of 15.1%, showing that although synergistic effects are present at smaller concentrations, increased doses can cause a decline in angiogenic response.

The findings illustrated in Table 2 further support the vessel density results by measuring the overall vessel network length in pixels. Figure 4 shows the total vessels network length of all the experimental groups. In the control group, an average minimum vascular network length of 2757.4 pixels was observed, ensuring minimal baseline angiogenic activity. Conversely, MTA at 5 µg significantly lengthened network length to 10,909.1 pixels and MTA at 10 µg shortened it to 5099.9 pixels, again suggesting a probable cytotoxic or inhibitory effect at increased concentrations. Baghdadite at 5 µg yielded a network length of 12,900.1 pixels, further validating its robust angiogenic profile. Whereas Baghdadite at 10 µg exhibited the slight decrease to 10,460.1 pixels, it nevertheless far surpassed MTA alone. Significantly, the combination group at 5 µg exhibited the best overall vessel length of 13,515.9 pixels, as a measure of synergistic improvement of the angiogenic network formation. Yet, the combination at 10 µg came in with only 6289.3 pixels, showing a drastic fall and further emphasizing the finding that greater concentrations potentially inhibit angiogenic potential.

Table 1: Vessel density (%).

Group	Vessel Density (%)
Control	6.8
MTA 5 µg	12.7
MTA 10 µg	10.9
Baghdadite 5 µg	17.1
Baghdadite 10 µg	17.8
MTA + Baghdadite 5 µg	17.6
MTA + Baghdadite 10 µg	15.1

Table 2: Total vessel network length (pixels).

Group	Vessel Network Length (px)
Control	2757.4
MTA 5 µg	10909.1
MTA 10 µg	5099.9
Baghdadite 5 µg	12900.1
Baghdadite 10 µg	10460.1
MTA + baghdadite 5 µg	13515.9
MTA + baghdadite 10 µg	6289.3

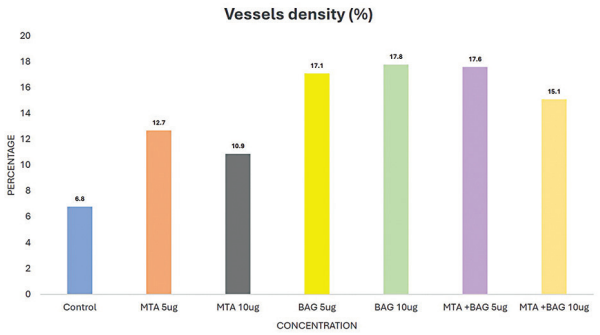

Figure 3: Vessels density of Baghdadite, Mineral Trioxide Aggregate and its combination at different concentrations.

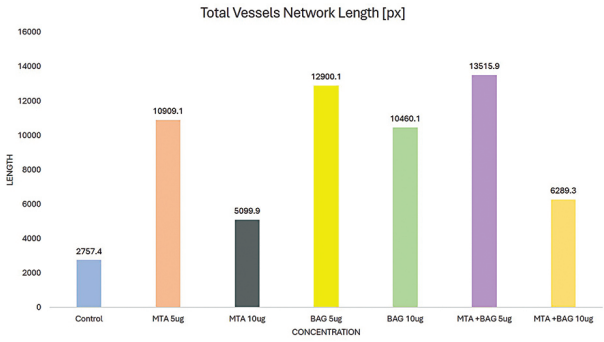

Figure 4: Total vessels network length of Baghdadite, Mineral Trioxide Aggregate and its combination at different concentrations.

Table 3 and Figure 5 summarizes the number of branching points in the vascular network as a parameter of network complexity and functional angiogenesis. The control group had 65 branching points, reflecting minimal vascular branching. MTA application at 5 µg and 10 µg resulted in comparable outcomes, with 151 and 153 branching points, respectively. Although both concentrations were better than the control, the reaction was moderate. Baghdadite at 5 µg produced 206 branching points, and 10 µg produced 179, both of which indicate a remarkable increase in vascular complexity. The MTA + Baghdadite combination at 5 µg performed the best in the number of branching points at 213, with its efficiency in promoting new vessel growth and increasing network connectivity. In contrast, the 10 µg combination group decreased to 170 branching points, again indicating that high concentrations can undermine pro-angiogenic activity, perhaps through high levels of ion release or shifting microenvironmental pH.

Discussion

The present study scientifically assessed and compared the angiogenic value of Baghdadite, MTA, and their mixture using the chick embryo YSM model. The results clearly indicate that Baghdadite alone and in combination with MTA significantly increased angiogenesis relative to MTA alone. Most significantly, the blend of Baghdadite and MTA at 5 µg concentration demonstrated a synergistic pro-angiogenic effect, as reflected in the highest vessel density (17.6%), longest vessel length (13,515.9 pixels), and highest number of branching points (213). Conversely, greater concentrations, especially MTA at 10 µg, had diminished angiogenic activity, arguably due to likely cytotoxicity by alkaline pH or over-release of calcium ions.

Table 3: Total branching points.

Group	Branching Points
Control	65
MTA 5 µg	151
MTA 10 µg	153
Baghdadite 5 µg	206
Baghdadite 10 µg	179
MTA + baghdadite 5 µg	213
MTA + baghdadite 10 µg	170

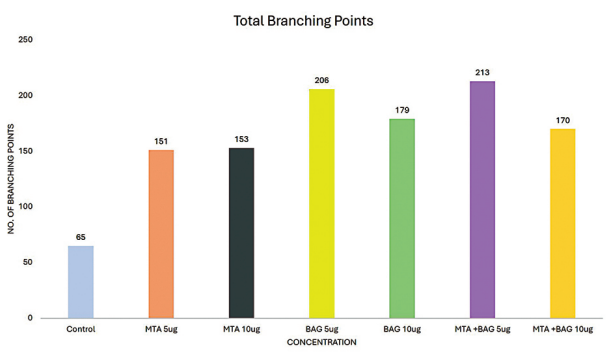

Figure 5: Total branching points of Baghdadite, Mineral Trioxide Aggregate and its combination at different concentrations.

The findings of this study highlight baghdadite's pronounced pro-angiogenic potential, consistent with its established bioactivity as a calcium–zirconium–silicate ceramic The resultant vessel density, network length, and branching points with Baghdadite alone (5 µg and 10 µg) confirm the hypothesis that its ionic dissolution products, predominantly calcium and silicate ions, are largely responsible for promoting angiogenesis. These ions have also been known to evoke endothelial cell signaling pathways, including those involving VEGF, that stimulate migration, proliferation, and vascular sprouting. Furthermore, the gradual and long-term release of ions by Baghdadite is capable of establishing a more favorable microenvironment for endothelial activity compared to that of MTA, which exhibits cytotoxicity at higher concentrations due to excessive release of ions and high pH.[12,13] The consistency of angiogenic response at both concentrations of Baghdadite validates its application in regenerative endodontic treatment where vascular support is crucial for repair and regeneration of the tissue.

The results of this study agree with the general trend in regenerative dentistry, in which improved vascularization is essential for the successful repair of pulp-dentin complexes following therapeutic treatments.[15,16] Proper vascularization ensures optimal delivery of nutrients and oxygen, thus stimulating cellular growth, differentiation, and tissue development.[17,18] The ability of Baghdadite to cause a vigorous angiogenic response seen in our research is especially encouraging because proper vascularization is an important step in pulp tissue regeneration and for successful clinical results in endodontics.

Baghdadite, a calcium zirconium silicate bioceramic, has gained significant interest based on its biocompatibility, sustained degradation, and bioactivity.[5] The angiogenic activity of Baghdadite is mainly due to its

dissolution products in ionic form, specifically calcium, zirconium, and silicate ions, known to promote angiogenesis by stimulating endothelial cell growth, migration, and differentiation.[6,19] Sadeghzade *et al.,* elaborately reviewed the biomedical potential of Baghdadite with a focus on its osteoinductive and pro-angiogenic biomaterial potential due mainly to controlled ionic release, especially calcium, zirconium, and silicate ions, regulating cellular responses critical for tissue regeneration.[12] The ions were shown to enhance cellular migration, proliferation, and differentiation, most notably in endothelial cells, playing a critical role in increased angiogenesis.[5,20] In addition, the ion-induced angiogenesis that occurred with Baghdadite in the present work also increases its likelihood of involvement in dental pulp regeneration, considering the life-sustaining role of vascularization in pulp vitality and function.[6]

On the other hand, whereas MTA is well-rooted as an endodontic biomaterial with strong evidence supporting its clinical effectiveness in vital pulp treatments like apexogenesis and direct pulp capping, its angiogenic activity, though desirable, seems extremely concentration-dependent.[21,22] Our findings indicated decreased angiogenic activity with higher doses (10 µg), which is consistent with the results from earlier studies showing that an increased release of calcium ions and consequent increase in pH can cause cytotoxicity on cells and inhibit the formation of blood vessels.[4,23] Parirokh and Torabinejad comprehensively discussed biocompatibility of MTA, stating that though it has desirable regenerative properties, high doses and extended exposure could diminish cell viability and affect therapeutic outcomes, highlighting the importance of optimal dosing in its clinical application.[10]

The synergistic action of the Baghdadite-MTA mixture at lower concentrations (5 µg) in this study presents a great clinical relevance. Combining Baghdadite's pro-angiogenic properties with MTA's sealing and regenerative capabilities could potentially optimize pulp regeneration outcomes. This synergism might be attributable to the complementary ionic profile released from the mixture, balancing angiogenic signaling without inducing excessive alkalinity or cytotoxicity. This result corresponds with emerging trends in regenerative endodontics, advocating the combination of different biomaterials to achieve comprehensive tissue regeneration.[12,24,25]

Notably, the idea of using blends of biomaterials to promote their biological activity has been advocated for by past research using different biomaterials in regenerative dentistry. As an example, Salehi *et al*., illustrated that the combination of bioactive ceramics and polymers enhanced angiogenesis and osteogenesis compared to single material in bone regeneration models.[14] The authors used a composite scaffold made of polylactic acid-Baghdadite and chitosan loaded with VEGF and achieved much enhanced angiogenesis and bone regeneration relative to monolithic scaffolds, thereby stressing the advantage of biomaterial composites in accelerating tissue healing through combined biological processes.[14] Our study carries this principle forward to regenerative endodontics specifically, demarcating the significance of well-optimized combinations leading to the desired therapeutic effects.

The chick embryo chorioallantoic membrane (CAM) model is an established and widely used *in vivo* model system for the study of angiogenesis. It has the benefits of being ethically acceptable, cost-effective, and permitting direct observation and measurement of angiogenic activity. The CAM model has been used in many studies to evaluate the angiogenic capacity of biomaterials and drugs.[26-28] In line with earlier reports, our approach allowed for objective, accurate quantification of angiogenic parameters with Wimasis software, and thus ensured reproducibility and less observer bias.[29]

From a clinical perspective, Baghdadite's physical properties, including porosity, structural integrity, and degradation profile, hold significant relevance in determining its suitability for regenerative endodontic procedures and vital pulp therapy. Baghdadite has a moderately porous structure, which is conducive to ionic exchange and angiogenesis while still being mechanically stable enough for intracanal use.[12,19] In contrast to certain traditional bioceramics that sacrifice strength in favor of greater porosity, Baghdadite has an ideal balance between compressive strength and bioactivity, and it is structurally sound even at physiological conditions.[12,20] This is a significant aspect especially in applications like VPT, wherein the material is exposed to occlusal forces and fluids. Moreover, although MTA is well established due to its excellent sealing property for endodontic applications[10], the addition of Baghdadite may improve the biological profile without compromising the seal especially at optimized low concentrations. Despite the fact

that there is limited data regarding Baghdadite's sealing potential in clinical situations, its inclusion into hybrid bioceramic formulations is promising for imminent use. Furthermore, having a minimum depth of placement is necessary to ensure proper sealing and regenerative response, such factors should be studied further in preclinical models so as to determine clinical guidelines.[21,22]

It should be noted that the used concentrations in this research (5 µg and 10 µg) were chosen to allow sensitive detection of angiogenic activity in the YSM model but prevent vascular occlusion or structural damage. Although these values are lower than those found in clinical MTA use, the purpose of the current investigation was not to mimic clinical dosing but to screen comparative pro-angiogenic activity of the materials alone and in combination. Preparation of set cement forms or extracts according to ISO 10993-12 would be critical in later cytocompatibility investigations assessing clinical-grade formulations. Further research using MTA–Baghdadite composite cements at clinically appropriate doses is justified to verify these results under translationally relevant conditions.

Future studies would also aim to further identify the molecular and cellular processes involved in Baghdadite-induced angiogenesis. In-depth analysis of particular signaling pathways invoked by Baghdadite, e.g., VEGF receptor pathways, hypoxia-inducible factor (HIF-1α), and subsequent angiogenic cascades, would be essential to provide key information in maximizing clinical applications.[30,31] Moreover, additional confirmation of the resultant synergistic effects in more clinically applicable models, for instance, rodent pulpal regeneration models or human-derived dental pulp stem cell (DPSC) cultures, would add to the translational value of our results.

Although the YSM assay used in this study provides a speedy and affordable platform to assess early angiogenic reactions, it mainly records short-term neovascularization patterns within a 24-hour time frame. While increasing the observation time to 48 hours might permit additional measurement of vessel remodeling and maturation, preserving the yolk sac and keeping embryo disturbance low beyond 24 hours proves to be practically difficult. Notably, YSM model differs from the better-studied CAM assay, which is based on a different extra-embryonic membrane architecture and developmental schedule. The differences have been resolved to prevent conceptual duplication.

It should further be noted that the YSM model, although appropriate for high-throughput screening of angiogenic capacity, does not mimic the intricate cellular and matrix structure of the dental pulp. Thus, the present findings, although promising, need to be ascertained in clinically relevant *in vivo* models, e.g., regenerative endodontic treatment of immature necrotic teeth. These models would assist in assessing the impact of baghdadite and its combination with MTA on long-term vascularization, pulp-dentin complex formation, and functional regeneration results. Future investigations combining histological and molecular studies will prove invaluable in bringing these results towards clinical practice.

One of the significant considerations for clinical translation is determining long-term biocompatibility, mechanical stability, and degradation behavior of Baghdadite-MTA composites. Determining the nature and biological response to degradation products is critical to be able to predict long-term clinical behavior and to confirm safe and effective use in regenerative endodontics. In addition, determining the inflammatory response and immunogenicity potential of such biomaterials over long durations is important to define clinical safety and efficacy.

While this study centered on the biological function of Baghdadite, it is noted that commercial feasibility is a significant factor in clinical implementation. MTA, despite broad usage, is costly for its relative production and proprietary mix requirements. Baghdadite, as a synthetic silicate-based ceramic with scalable production capabilities, could provide a more cost-efficient option when standardized dental-grade compositions are commercially available. Nevertheless, as yet, Baghdadite is mainly reserved for experimental and biomedical research environments, and comparative costs of use in clinical contexts are as yet unavailable. Further research into cost–benefit ratios, especially when combined with MTA or other bio ceramics, would be informative in gauging its translational value in resource-constrained environments.

Conclusions

The present investigation makes a significant addition to the field by illustrating the increased angiogenic ability of Baghdadite and its synergistic effect when used in combination with MTA at optimal levels. The strong quantitative data generated through the use of the YSM assay illustrates the potential of these biomaterials

for application in clinical regenerative therapies with a focus on dosage and mixtures to achieve maximal therapeutic effect. Future research should aim at clarifying underlying biological processes, verifying these in more advanced pre-clinical and clinical models, and maximizing material properties for clinical application in regenerative endodontic treatments.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Article

The Effects of Solutions and Surface Treatments on the Shear Bond Strength Between Aged 3D-Printed Provisional Restorations and Repair Materials

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Abstract

Objectives: To evaluate the effects of different solutions, surface treatments, and repair materials on the shear bond strength (SBS) between aged 3D-printed provisional materials and repair materials.

Methods: This study printed samples with 3D-printed resin and divided them into 16 groups (n=10): non-immersion and three solutions (40% ethanol, heptane, and cola) × two surface treatments (no abrasion [NA] and sandpaper and sandblasting [SP&SB]) × two repair materials (Poly(methyl methacrylate) [PMMA] and bis-acryl). The samples were immersed in the solutions for six days and then surface-treated. Nine samples were randomly selected for surface examination, including surface roughness, surface characteristics, and contact angle. Then, they were bonded with the repair materials, followed by 2,500 cycles of thermocycling. SBS was determined using a universal testing machine, and failure modes were determined by stereomicroscope. A three-way ANOVA was conducted to evaluate the effects of the solution, surface treatment, and repair material on SBS. SBS, surface roughness, and contact angle were compared among groups using one-way ANOVA, followed by post-hoc Tukey's tests.

Results: The three-way ANOVA analysis revealed a significant interaction among solutions, surface treatments, and repair materials ($p<0.05$). The mean SBS did not differ significantly between immersed and non-immersed groups. Among the solutions, surface treatments, and repair materials, SBS significantly increased after SP&SB surface treatment ($p<0.05$) and was significantly higher in the bis-acryl group than in the PMMA group ($p<0.05$). Cohesive failure was primarily observed when SP&SB and/or bis-acryl was applied. The mean Ra and contact angle were significant different after applied SP&SB ($p<0.05$). Additionally, SP&SB groups exhibited an irregular surface with multiple porosities.

Conclusions: The solutions alone did not significantly affect the SBS between aged 3D-printed provisional materials and two repair materials. However, SBS differed significantly after SP&SB treatment of the aged 3D-printed provisional materials and/or when they were repaired with bis-acryl.

Keywords: bis-acryl, food-stimulating agents, PMMA, provisional crown, sandblasting

Introduction

The fabrication of the provisional restoration is one of the essential procedures in fixed prosthodontics treatment. For patients requiring full-mouth rehabilitation, especially those with parafunctional habits, long-term provisional restorations are crucial for comprehensively evaluating occlusion, function, and aesthetics over several months.[1,2] Provisional restorations must be durable but easily repairable since they may be worn or broken down due to prolonged use and functional forces.[3] Previously, provisional restorations were fabricated using conventional techniques, which had several drawbacks, including residual monomer, heat from exothermic reactions, and shrinkage after polymerization.[4] Currently, provisional restorations are commonly fabricated using computer-aided design (CAD) and manufacturing (CAM) technology. The CAD/CAM approach offers various advantages, including reduced material and manufacturing errors, shorter processing times, and decreased material usage.[5,6]

With CAD/CAM technology, provisional prostheses are fabricated using two techniques: subtractive manufacturing (SM) and additive manufacturing (AM) or 3D-printed technology.[7] In SM, a prefabricated block is milled using computer-controlled milling tools. However, this technique has drawbacks, such as challenges with recycling waste materials and addressing inaccessible areas due to the limited radius of the milling tool.[1,7,8] In contrast, AM involves building the material layer by layer, allowing more complex shapes to be produced, eliminating the wear of milling burs, and reducing material consumption.[1] Previous studies have found that the mechanical properties of 3D-printed provisional material were greater than those of poly(methyl methacrylate) (PMMA) and milled PMMA.[9-12] The accuracy of the 3D-printed provisional crown was comparable to SM and superior to the conventional technique.[13,14] Furthermore, Jain *et al*.,[15] demonstrated that 3D-printed provisional material was an alternative to long-term provisional material.

Long-term provisional restorations are subjected to biodegradation due to various contributing factors, including exposure to saliva, chemical components in the diet, and diverse mechanical and thermal stresses.[16-18] *In vitro* studies apply food-stimulating agents (FSAs) according to the US Food and Drug Administration standards to simulate the effects of food and beverages.[19] FSAs function as plasticizers by penetrating the polymer matrix and increasing intermolecular spacing, contributing to the enlargement and dissolution of polymer chains, thereby degrading the mechanical and physical properties of provisional materials.[20-23] Additionally, a previous study has found that acidic beverages can deteriorate the fracture resistance of bis-acryl composite crowns.[24] The biodegradation of provisional materials may also be initiated by fatigue caused by the relatively low but repetitive forces generated during normal chewing.[16] Physiologic occlusal forces in natural dentition and parafunctional habits, such as bruxism and nocturnal forces, typically range from 200 to 900 N; which is sufficient to deteriorate provisional crowns.[25,26] Furthermore, Luthardt *et al*.,[27] evaluated the clinical outcomes of 64 bis-acrylic provisional restorations in 32 patients, reporting fractures in four cases after a mean duration of 37.5 days. Therefore, chairside repairs remain necessary in clinical practice.

When repairing provisional materials, surface preparation prior to bonding has been recommended to enhance bond strength, as aged restorations exhibit fewer free radicals, fewer free carbon double bonds, and greater water absorption.[28,29] Mechanical surface treatments, such as sandblasting with aluminum oxide and grinding with a carbide bur, have been shown to increase the bond strength between 3D-printed materials and repair materials.[30-33] However, there is controversy regarding different repair materials. Monomethacrylates and dimethacrylates have frequently been used for the chairside repair of conventional provisional crowns.[34] Previous studies have reported that 3D-printed provisional materials had greater shear bond strength (SBS) with bis-acryl than PMMA.[31,35] However, Palavicini *et al*., reported the opposite.[36] Nonetheless, the effects of surface treatments and repair materials on SBS between aged 3D-printed provisional material and repair material have not been well established. Therefore, this study aimed to investigate the SBS between aged 3D-printed provisional material and repair materials, considering the effects of solutions, surface treatments, and repair materials. The first hypothesis was there was no significant different in SBS between repair materials and aged 3D-printed provisional after immersed in different solutions. The second was there was no significant difference in SBS between repair materials and aged 3D-printed provisional

restorations, regardless of whether surface treatment is applied. The third hypothesis was there was no significant difference in SBS among different types of repair materials.

Materials and Methods

This study used digital light processing (DLP) 3D-printed provisional material and two repair materials. Their details are provided in Table 1, and the experimental design is illustrated in Figure 1.

Sample size calculation

G*power software (version 3.1.9.7; Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) was used to determine the sample size. The effect size was determined from the pilot study, with a value of α=0.05 and a power of 0.95. The sample size was calculated to be 8, but in order to account for potential variability, this study elects to include 10 samples.

Sample preparation

The cylindrical shaped specimens were digitally

Table 1: Description of the 3D-printed provisional material and repair materials.

Material	Manufacturer	Composition	Type	Lot. number
NextDent C&B MFH	NextDent B.V., Soesterberg, Netherlands	Not applicable.	UV-light cured resin	WT162N03
UNIFAST™ Trad (PMMA)	GC America, Inc.	Powder: poly((ethyl methacrylate)-co-(methyl methacrylate)) poly(methyl methacrylate), dibenzoyl peroxide, titanium dioxide, iron(III) oxide, cellulose acetate Liquid: Methyl methacrylate, N,N dimethyl-p-toluidine.[37]	Self-cure resin	2212271
Protemp™ 4 (Bis-acryl)	3M ESPE, St. Paul, MN, USA	Catalyst Paste: ethanol, 2,2'-((1-methylethylidene) bis(4,1-phenyleneoxy)) bis-diacetate, benzyl-phenyl-barbituric acid, silane treated silica, tert-butyl peroxy- 3,5,5-trimethylhexanoate Base Paste: dimethacrylate (Bis-EMA 6), silane-treated amorphous silica, reaction products of 1,6-diisocyanatohexane with 2-((2-methacryloyl) ethyl) 6-hydroxyhexanoate and 2-hydroxyethyl methacrylate, silane-treated silica.[37]	Self-cure resin	9169729

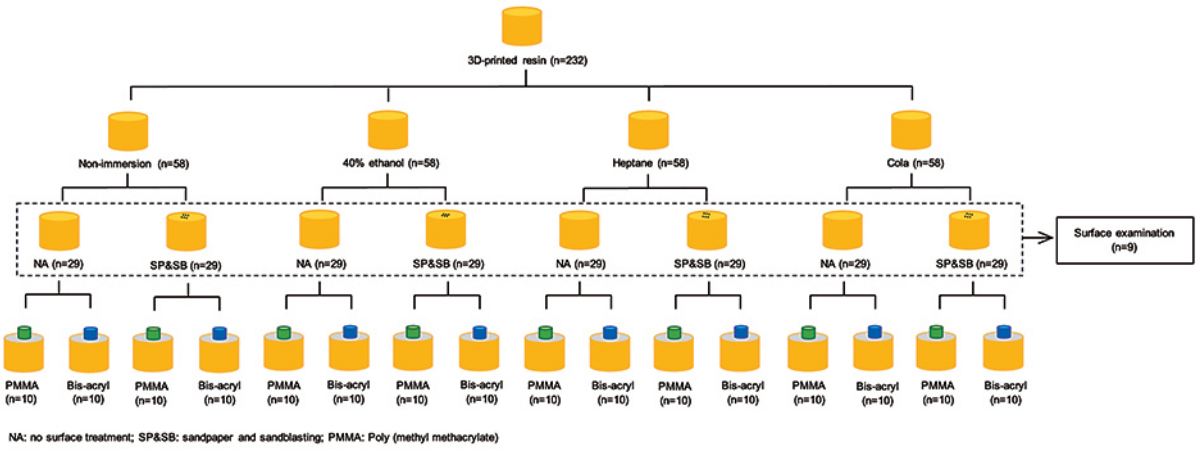

Figure 1: The experimental design.

designed using CAD software (Thinkercad, version 2024; Autodesk, Inc., San Francisco, CA, USA) with a diameter of 8 mm and thickness of 12 mm and exported in the Standard Tessellation Language (STL) format. Next, the STL files were transferred into the CAM software (3D Sprint; 3D Systems, Rock Hill, SC, USA). Then, 232 samples were printed in 50-µm layers with a 0° angle using photopolymerized resin (NextDent C&B MFH; NextDent B.V., Soesterberg, Netherlands) and a DLP 3D Printer (5100 3D Printer; NextDent B.V., Soesterberg, Netherlands). Next, the support structures were removed, and the specimens were cleaned with 91% isopropanol. Then, they were post-polymerized in a UV oven (LC-3DPrint Box; NextDent B.V., Soesterberg, Netherlands) for 40 minutes to promote the completion of the curing process. Finally, the samples were immersed in glycerin to complete the reaction of the remaining monomer. After fabrication, each sample's average surface roughness (Ra) was assessed using a contact profilometer (Surftest SJ-310; Mitutoyo, Kanagawa, Japan) to achieve a consistent surface roughness.

Aging processes of 3D-printed provisional materials

The 232 samples were categorized into four solution treatment groups (n=58/group): no immersion (None) and immersion in 40% ethanol (Eth), heptane (Hep), or cola. Here, 40% ethanol represented alcoholic beverages; heptane represented vegetable oils, butter, and meat-based oils; and cola represented acidic drinks. The samples were immersed in 200 mL of the solution for six days, corresponding to six months of intraoral use[38], in an incubator at 37°C to mimic oral cavity conditions. The pH was measured before and after immersion. The solutions were changed daily to avoid contamination. After six days, the samples were rinsed with distilled water and dried.

Surface treatments

The samples in each solution treatment group were divided into two surface treatment subgroups (n=29/group): no abrasion (NA) and grinding with 220 grit sandpaper (DCC waterproof abrasive paper; TOA, Thailand) followed by sandblasting with 50 µm aluminum oxide (SP&SB) using an airborne-particle abrasion device (Basic Classic Fine Sandblasting Unit; Renfert, Germany) for 10 seconds at a pressure of 0.2 MPa, maintaining 10 mm from the sample's surface. Then, the samples were subjected to ultrasonic cleaning in distilled water for five minutes to remove debris generated during the surface treatment process. Then, nine samples from each group were randomly selected for surface examination by scanning electron microscopy (SEM; fourth-generation VEGA; TESCAN, Czech Republic), surface roughness measurement using a contact profilometer (Surftest SJ-310; Mitutoyo, Kanagawa, Japan), and contact angle measurement using a contact angle meter (KINO, Boston, MA, USA).

Repair procedures

The remaining 20 samples in each surface treatment subgroup were further divided into two repair material subgroups (n=10/group): PMMA and bis-acryl. According to the ISO 29022:2013 standards, the procedure used a stainless steel (SS) mold measuring 3 mm in diameter and height, with the unexposed areas protected by masking tape. PMMA and bis-acryl were prepared according to the manufacturer's instructions. Next, the material was injected into the SS mold until it was completely filled and covered with a microscope glass slide. Polymerization was allowed to proceed for five minutes. Then, the SS mold and masking tape were removed and wiped with gauzed-soak alcohol to eliminate the smear layer formed by atmospheric oxygen. All specimens were immersed in distilled water at 37°C for a duration of 24 hours before thermocycling.

Thermocycling

Artificial aging was performed using a thermocycling system (HWB332R, CWB332R, TC301) designed to replicate the temperature fluctuations experienced in the oral cavity. Thermocycling was conducted for 2,500 cycles, alternating the temperature between 5°C and 55°C, with a dwell time of 30 seconds at each temperature. This process simulates the thermal conditions that occur in the oral cavity over three months.[39] The samples were immersed in distilled water at 37°C for 24 hours before measuring the SBS.

SBS and failure mode analysis

The samples were mounted into a testing jig compatible with a universal testing machine (model 5566; Instron, Norwood, MA, USA). Next, they were exposed to shear forces using a flat blade at a crosshead speed of 1.0 mm/min until failure occurred. The bond strength values (MPa) were determined by dividing the failure load (N) by the surface area of the cylindrical specimen (mm^2), as shown in the formula below. Then, the specimens were analyzed by optical microscope (Eclipse 50i; Nikon,

Tokyo, Japan) to determine the failure mode, which was classified into adhesive, mixed, and cohesive failure. Adhesive failure was defined when less than 10% of the repair resin remained. When over 50% of the temporary base material fragmented, cohesive failure was identified. Mixed failure served as the intermediary between the two failure modes.[31] 5 samples of each failure modes were randomly selected for analysis the surface characteristic by scanning electron microscope analysis (TESCAN VEGA's 4th, TESCAN, Czech Republic)

σ (MPa) = F / A, where F is the load at failure (N), and A is the repaired surface area (mm^2).

Statistical analysis

Each group's SBS (MPa), surface roughness (Ra), and contact angle were calculated and are reported as the mean±standard deviation. The data were analyzed using SPSS Statistics (version 25.0; IBM, Corp., Armonk, NY, USA). The effects of the solution, surface treatment, and repair material on SBS were examined using a three-way analysis of variance (ANOVA). The SBS, surface roughness, and contact angle were compared between groups using one-way ANOVA with post-hoc Tukey's tests. A $p<0.05$ was considered statistically significant.

Results

The three-way ANOVA analysis showed a significant interaction among the three factors—solution, surface treatment, and repaired material ($p<0.05$; Table 2). Additionally, there were significant interactions between solution and surface treatment ($p<0.001$; Table 2), and between solution and repaired material ($p<0.05$; Table 2). The interaction between surface treatment and repaired material was also significant ($p<0.001$; Table 2).

SBS

The mean and standard deviation of the SBS (MPa) between aged 3D-printed provisional material and repair materials, along with the p-values, were demonstrated in Table 3.

The mean SBS was greatest for aged 3D-printed provisional material immersed in 40% ethanol, followed by SP&SB surface treatment and repair with bis-acryl (Eth/SP&SB/Bis-acryl; 49.08±2.40 MPa) and lowest for aged 3D-printed provisional material immersed in 40% ethanol, followed by NA surface treatment and repaired with PMMA (Eth/NA/PMMA; 9.99±2.11 MPa).

The mean SBS did not differ significantly between the None/NA, Eth/NA, Hep/NA, and Cola/NA groups, regardless of the repair material. However, the mean SBS was significantly higher in the SP&SB group than in the NA group, regardless of the repair material and solution. In addition, the mean SBS was significantly higher in the NA/Bis-acryl group than in the NA/PMMA group. Similarly, the mean SBS was significantly greater in the SP&SB/Bis-acryl group than in the SP&SB/PMMA group.

Following SP&SB, the mean SBS differed significantly between the Eth/SP&SB/Bis-acryl group and the None/SP&SB/Bis-acryl group. Similarly, the mean SBS

Table 2: The three-way ANOVA analysis the effect of 3 factors: solutions, surface treatment and, repaired materials on SBS (MPa) with significant level of $p<0.05$.

Tests of between-subjects effects					
Dependent variable: shear bond strength					
source	**Type III Sum of Squares**	**df**	**Mean square**	**F**	**Sig.**
Corrected model	21132.659	15	1408.844	131.399	.0000
intercept	100560.784	1	100560.784	9379.033	.0000
Surface treatment	13684.081	1	13684.081	1276.277	.0000
Repair material	6025.789	1	6025.789	562.010	.0000
solution	116.018	3	38.673	3.607	.015
Surface treatment*repair material	482.122	1	482.122	44.966	.000
Surface treatment*solution	642.803	3	214.268	19.984	.000
Repair material*solution	87.107	3	29.036	2.708	.047
Surface treatment*repair material*solution	94.731	3	31.577	2.945	.035

Table 3: The mean and standard deviation of SBS (MPa) by solution, surface treatment, and repair material.

Solution	PMMA		Bis-acryl	
	NA	SP&SB	NA	SP&SB
None	12.70±2.62^{a}	24.05±4.01^{c,d,e}	22.89±2.77^{b,c,d}	38.00±1.82^{f}
Eth	9.99±2.11^{a}	28.41±4.30^{e}	18.64±1.40^{b}	49.08±2.40^{g}
Hep	10.91±2.36^{a}	26.83±4.85^{d,e}	19.57±2.67^{b,c}	41.58±3.41^{f}
Cola	12.11±3.35^{a}	26.49±4.52^{d,e}	19.78±4.46^{b,c}	40.10±2.76^{f}

Each same superscript letter indicates no significant difference between groups at $p>0.05$.; None: no immersion; Eth: 40% ethanol; Hep : heptane; PMMA: Poly(methyl methacrylate); NA: no surface treatment; SP&SB: sandpaper and sandblasting.

was greatest in the Eth/SP&SB/PMMA group, followed by the Hep, Cola, and None groups, regardless of the repair material.

Failure modes

The failure modes observed in each experimental group are illustrated in Figure 2. Regardless of the solution applied. The SP&SB/Bis-acryl groups predominantly exhibited cohesive failure, whereas the NA/PMMA and NA/Bis-acryl groups primarily demonstrated adhesive failure. However, the None/NA/Bis-acryl group predominantly showed mixed failure and the SP&SB/PMMA groups exhibited a predominance of mixed failure as well. SEM images of the failure modes are presented in Figure 3.

SEM micrographs

SEM images of the 3D-printed provisional materials are presented in Figure 4. The surface showed a smooth layer of printed polymer in the None group (Figure 4A). However, the surface exhibited a more irregular, visibly dissolved morphology and distinct polymer beads in the Eth, Hep, and Cola groups (Figure 4 B-D). Figure 5 shows visible crazing lines (arrows) on the surface in the Eth groups. The surface showed homogenous multiple porosities and irregularities in the SP&SB groups (Figure 4E-H). The surface in the Eth/SP&SB groups showed greater morphological variation, characterized by varied sizes and shapes, numerous porosities, and a distinctly roughened surface (Figure 4F).

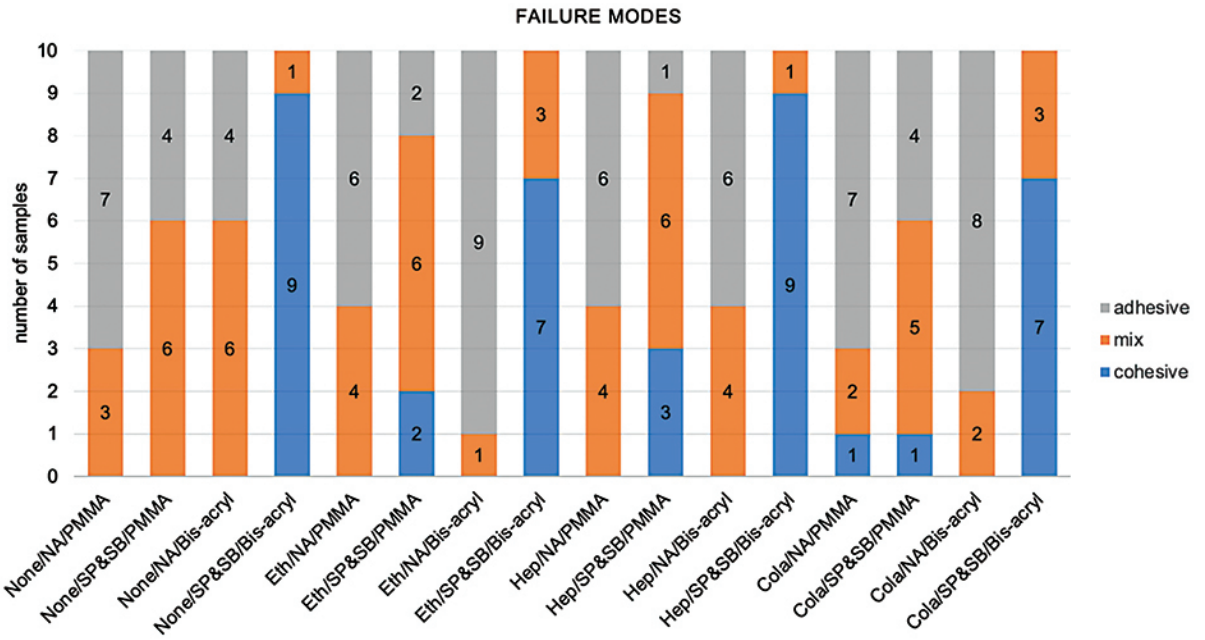

Figure 2: Distribution of failure modes in each experimental group.

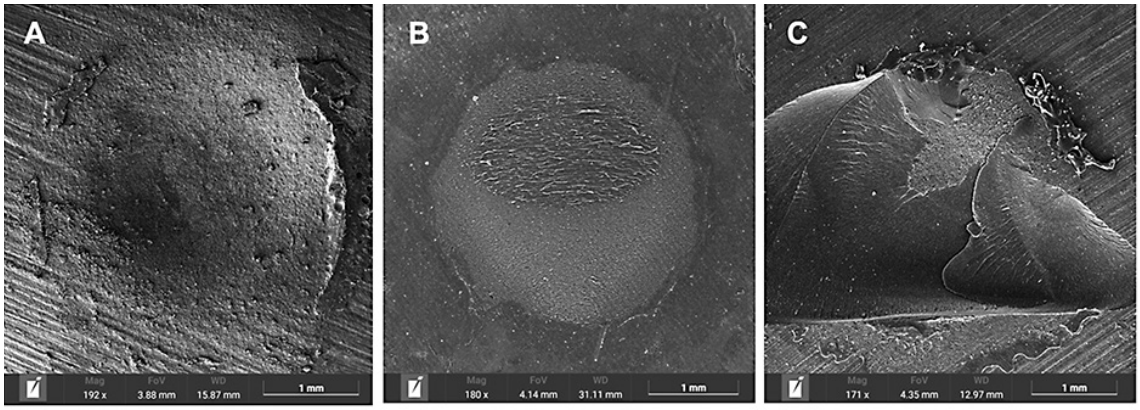

Figure 3: SEM micrographs of each failure mode. (A) Adhesive failure; (B) mixed failure; (C) cohesive failure.

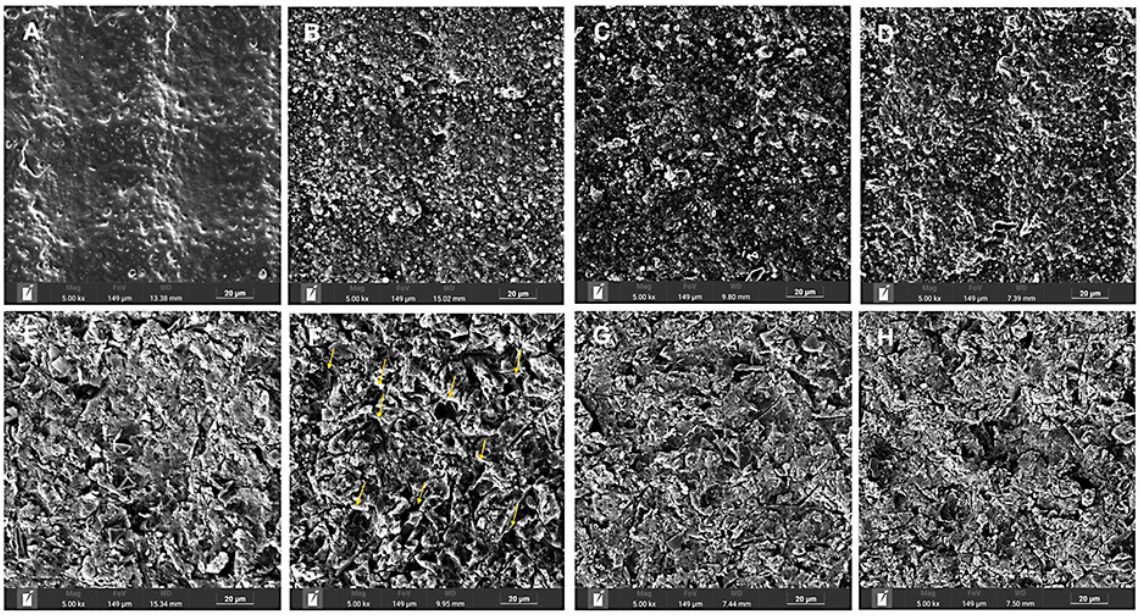

Figure 4: SEM micrographs of specimens treated with each solution and surface treatment (magnification: 5.00 kx). (A) None/NA; (B) Eth/NA; (C) Hep/NA; (D) Cola/NA; (E) None/SP&SB; (F) Eth/SP&SB (arrows indicate numerous porosities); (G) Hep/SP&SB; (H) Cola/SP&SB.

Surface roughness

The mean and standard deviation of surface roughness (μm) are presented in Table 4. The mean Ra was highest in the Eth/SP&SB group (3.48±0.30 μm) and lowest in the None/NA group (0.96±0.06 μm). Comparing the mean Ra between surface treatments, it was significantly higher in the SP&SB groups than in the NA groups. However, mean Ra showed no significant difference between the NA groups among solutions.

Contact angle

The mean and standard deviation of the contact angle are presented in Table 4. In all solutions, the contact angle was significantly lower in the SP&SB subgroup

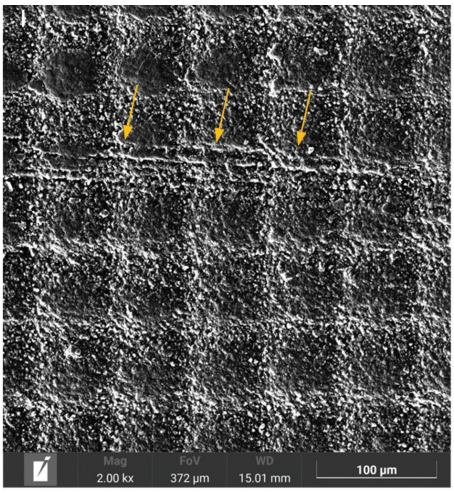

Figure 5: An SEM micrograph of a specimen in the Eth group (magnification: 2.00 kx). The arrows indicate crazing line.

compared to the NA subgroup. Among the SP&SB groups, the contact angle was the lowest in the Eth/SP&SB group (82.26±2.36), differing significantly from the None/SP&SB group. However, the contact angle did not differ significantly between solutions among the NA groups.

Discussion

Our study investigated the effects of solutions, surface treatments, and repair materials on the SBS between 3D-printed provisional materials and repair materials. According to the result. The first hypothesis was accepted, while the second and third one were rejected.

It demonstrated that solutions alone did not significantly impact the SBS between the 3D-printed provisional material and repair materials. In contrast, previous studies have reported that FSAs affected the hardness, flexural strength, and surface roughness of conventional provisional materials.[22,40,41] Additionally, FSAs were found to decrease the SBS between the conventional denture base and hard reline material.[20] Our study found that the surface roughness and contact angle of aged 3D-printed provisional materials did not differ significantly between the Eth, Hap, and Cola groups and the None group. Moreover, previous studies showed that the surface morphology of 3D-printed denture base materials demonstrated superior chemical stability compared to conventional and milled denture base materials.[32,42,43] Furthermore, the uppermost part of the 3D-printed provisional material showed greater polymerization than its foundation.[44]

In our study, SBS did not differ significantly between the Cola and None groups, consistent with previous studies.[20,21] The corrosive effects of acids are determined by their pH and pKa; a lower pKa signifies a stronger acid with an enhanced ability to donate protons.[45] Fatemi *et al*.,[20] found that acetic acid did not significantly affect the SBS between a conventional denture base and hard reline material. Similarly, citric acid is a weak acid with a relatively high pKa. Therefore, it was expected to have a negligible effect on the SBS of the materials.[20] In our study, phosphoric acid, the main acid in cola, is also a weak acid. Therefore, it might only slightly affect the surface roughness and integrity of 3D-printed provisional materials.

Whether repaired with PMMA or bis-acryl, the SBS and surface roughness differed significantly between the SP&SB and NA groups, consistent with previous studies.[31,35,43,46,47] SEM images also demonstrated a roughened surface with porosities. Grinding the surface with 220-grit sandpaper resembled using a carbide bur to eliminate contaminated surfaces that contain fewer free radicals.[48] Sandblasting promoted an irregular surface and increased the surface bonding area, enhancing micromechanical retention.[30] Furthermore, the contact angle significantly decreased after SP&SB, resulting in increased surface wettability, which facilitates the penetration of repair materials into the substrate. Moreover, the failure mode after SP&SB was predominantly cohesive failure. These results demonstrated that SP&SB was a reliable and effective technique for enhancing the SBS of aged 3D-printed provisional materials.

In our study, the mean SBS was highest in the Eth/SP&SB group among solutions, whether repaired with PMMA or bis-acryl. Moreover, SBS differed significantly between the Eth/SP&SB/Bis-acryl group and the None/SP&SB/Bis-acryl group. Previous studies have reported that ethanol caused a greater reduction in the mechanical properties of the provisional material than heptane since it exhibited a stronger plasticizing effect.[20,22,40] Solutions penetrated the interface layer due to the printed structure, where the interlayer bonding was weaker than the intralayer bonding, leading to the dissolution of the polymer chains.[44,49,50] As shown in the SEM images (Figure 5),

Table 4: Mean and standard deviation of surface roughness (Ra) and contact angle by solution and surface treatment.

Solution	Surface roughness (Ra)		Contact angle	
	NA	SP&SB	NA	SP&SB
None	0.96±0.06^{a}	2.01±0.23^{b}	96.04±1.22^{g}	82.26±2.36^{e,f}
Eth	1.17±0.15^{a}	3.48±0.30^{c}	92.84±2.56^{g}	73.70±3.21^{d}
Hep	1.45±0.08^{a}	2.36±0.04^{b}	90.74±1.03^{g}	78.18±1.06^{d,e}
Cola	1.29±0.03^{a}	2.1±0.23^{b}	89.10±4.31^{g}	80.06±2.86^{d,e}

Each same superscript letter indicates no significant difference between groups at $p>0.05$.; None: no immersion; Eth: 40% ethanol; Hep: heptane; PMMA: Poly(methyl methacrylate); NA: no surface treatment; SP&SB: sandpaper and sandblasting.

crazing lines were observed at the interface layer due to ethanol's plasticizing effect, compromising the structural integrity and potentially increasing susceptibility to pressure from sandblasting. Furthermore, previous studies have indicated that ethanol is a more effective polymer solvent for conventional provisional materials than heptane, as their solubility parameters are closer.[15,23,24] However, the solubility of 3D-printed materials has been insufficiently studied.

The SBS was significantly higher in the bis-acryl group than in the PMMA group within the same solutions and surface treatments, consistent with previous studies.[31,51] Jeong *et al*.,[31] reported that bis-acryl composites could achieve complex polymerization with 3D-printed resins. In contrast, 3D-printed resins are composed of photopolymerized resins with bifunctional monomers, which do not copolymerize with methyl methacrylate monomers.[52] Moreover, bis-acryl composites use an automix system that ensures a consistent mixture and minimizes the risk of air bubbles, resulting in fewer flaws and porosities.[51,53] The optimal SBS was also achieved when the repair materials had comparable chemical composition to the substrates.[54] The 3D-printed resin was primarily composed of ethoxylated bisphenol A dimethacrylate, urethan dimethacrylate, and bisphenol A-glycidyl methacrylate (Bis-GMA).[55,56] In addition, bis-acryl and Bis-GMA composites share similar chemical structures.[57] Therefore, the chemical composition of 3D-printed provisional resins might be more similar to bis-acryl composites than to conventional PMMA. similarity was further supported by the predominantly cohesive failures found in bis-acryl groups.

Based on the three-way ANOVA, an interaction was observed between solutions, surface treatments, and repair materials. The SBS was greatest in the Eth/SP&SB/Bis-acryl group and lowest in the Eth/NA/PMMA group. These findings suggest that SP&SB and/or bis-acryl significantly enhance the SBS of aged 3D-printed provisional materials, since SP&SB improved micromechanical retention by increasing surface roughness and wettability, the vinyl group in the Bis-acryl composite facilitated chemical bonding. A cohesive failure was mainly observed when bis-acryl and/or SP&SB were applied. In contrast, the solutions did not affect the SBS. However, 40% ethanol appeared to have a more pronounced effect on the structural integrity of the 3D-printed provisional materials than heptane and cola. According to the findings from our study, aged 3D-printed provisional materials should be ground and sandblasted before repairing, and bis-acryl was recommended as the repairing material for greater bond strength. However, using sandpaper might not be practical in a clinical setting, hence a previous study suggested grinding PMMA with a carbide bur before repairing PMMA for comparable results.[48]

Our study had several limitations. Firstly, it used an *in vitro* experimental design that tested only one type of 3D-printed provisional resin and two types of repair materials. Secondly, provisional crowns are not continuously immersed in solutions in the oral environment; they are exposed intermittently when the patient consumes foods and beverages. Thirdly, it did not simulate occlusal force or the presence of saliva. Therefore, *in vivo* studies should be conducted to evaluate the effects of the oral environment on 3D-printed provisional crowns. Furthermore, future studies should investigate other types of provisional crowns and repair materials.

Conclusions

Based on the findings of this *in vitro* study, solutions alone did not significantly impact the SBS between the 3D-printed provisional material and repair materials. However, the SBS differed significantly when the aged 3D-printed provisional material underwent SP&SB and/or was repaired with bis-acryl.

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Conflict of Interest

The authors declare no conflicts of interest.

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Effect of Various Intracanal Calcium Hydroxide Dressing Materials on pH Changes in Simulated External Root Resorption Defects

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Abstract

Objectives: To evaluate the effect of placement of different CH dressing materials in the root canals on pH changes in simulated external root resorption defects.

Methods: Seventy-five extracted single rooted teeth were decoronated to the length of 14mm. Root canals were prepared with peeso reamers. An external defect of 0.7mm depth and 1.4mm in diameter were made on the root surface, 5 mm from the apex and then assigned into 5 groups with 15 teeth in each group. Group 1: CH+distilled water, Group 2: CH paste, Group 3: Biodentine, Group 4: CH+2% chlorhexidine gel+Zinc oxide, Group 5: Distilled water. The materials were placed in the assigned groups and pH was measured with a microelectrode at 30 minutes, 24 hours, 7 days, 14 days, 21 days, 28 days and 3 months respectively.

Results: Results revealed significant differences in the pH between the groups at all time intervals ($p \leq 0.05$). Group 1 samples showed highest pH at all time intervals when compared with other groups.

Conclusions: Calcium hydroxide and distilled water group maintained high pH at different time intervals in comparison with other groups followed by Biodentine group, calcium hydroxide paste and 2% CHX gel+CH+ZnO in the descending order and the pH could not be sustained by any of the material at the end of 3 months time interval.

Keywords: biodentine, calcium hydroxide, chlorhexidine, external root resorption

Introduction

Calcium hydroxide (CH) a commonly used dental material dissociates into calcium ions (Ca^{2+}) and hydroxyl ions (OH^-) in aqueous medium. The ability of OH^- to pass across the dentinal tubules helps to prevent the activity of clastic cells on the root surface from functioning and to provide an environment that encourages the action of hard tissue repair.[1] A number of factors could affect how quickly OH^- passes from dentin like the interplay of ions and dentin, thickness of dentin, shape of dentinal tubule and properties of the solute.[2] Aqueous, viscous, and oily vehicles are different delivery methods employed to provide intracanal calcium hydroxide. Previous research has shown that the type of vehicle used affects ion diffusion.[1] Following placement of CH inside the canals, continued depletion of OH^- is possible by several pathways, including the apical region, auxiliary canals, dentinal tubules, abnormalities brought on by resorption. The depletion of OH^- has also been associated with capacity of dentin to act as a buffer for CH and its ability to dissolve organic tissue.[3] It is necessary to keep changing calcium hydroxide from canals, depending on the degree of root resorption. According to Abbott, medication can be kept inside the canals upto 1 year while replacement needs to be done in every 90 days.[4] After four weeks, the pH significantly lowers, hence Chamberlain advised replacing CH pastes more frequently. Even though this kind of therapy is commonly acknowledged, frequent alterations of CH paste pose a clinical drawback.[5] Furthermore, repeated contact with CH over time changes characteristics of dentine, leading to fracture.[6]

In previous studies it has been proposed that the use of Mineral Trioxide Aggregate (MTA) may minimize the negative effects of long-term CH therapy.[7-11] Gilles and Olivier (Septodont, Saint-Maur-des-Fossés, France) introduced Biodentine (BD), a novel calcium silicate-based substance, in 2010. Biodentine results in production of CH along with calcium silicate hydrate, when mixed and can retain pH in the canal as well on the exterior of the canal. Additionally, biodentine helps reduce the adverse effects of prolonged CH therapy.[12]

In 2014, Zinc oxide (ZnO), CH and 2% chlorhexidine (CHX) gel mixture was also suggested in treating resorption on the exterior of the root.[13] When compared to medicaments without ZnO, this medication demonstrated better radiopacity and long-term stability in the canals without getting frequent replacement. None of the research have investigated the effect of duration of this medication's high pH in the management of external root resorption.[6] Therefore, the objective of this study was to compare the effect of CH alone, Biodentine and combination of 2% CHX gel, ZnO and CH on the pH changes in simulated external root resorption defects, the present study was undertaken.

Materials and Method

Study design and ethical approval

The institutional ethics committee received the study protocol and gave its approval vide Ref. No. TMDCRC/IEC/20-21/PPD1 dated 19/02/2021.The sample size was estimated following power analysis which was more than 80% along with confidence interval of 95%. Since there are 5 groups, the total sample size estimated was 15*5=75.

A total of 115 single rooted, extracted teeth were collected from the Department of Oral and Maxillofacial Surgery. Immediately after extraction all the teeth were subjected to thorough washing to get rid of blood & the adherent tissues and debridement of surface was done with hand scaler, followed by ultrasonic scaler and rubber cup with applied slurry pumice. The samples were subsequently preserved in distilled water with 0.1 % thymol crystals were added to it at 4°C for a maximum of 7 days. Thereafter samples were stored using distilled water grade 3 ISO 3696 in a refrigerator and the distilled water was replaced weekly to limit the deterioration of samples. Single rooted human teeth with intact root extracted due to orthodontic/periodontal reasons were included. Teeth with enamel cracks, fractures or fracture lines, developmental malformations, carious lesions and/or restorations and with erosions were excluded.

A total number of 95 single rooted extracted teeth from collected specimens were distributed randomly in 5 groups. Group 1: Calcium hydroxide powder + distilled water; Group 2: Commercially available Calcium hydroxide paste; Group 3: Biodentine; Group 4: Calcium hydroxide powder+ Zinc oxide powder+ 2% Chlorhexidine gel; Group 5: Distilled water

All the teeth were decoronated at cementoenamel junction (CEJ) to equal root length of 14mm by using a diamond disk (Figure 1). An external defect of 0.7 mm in depth, diameter 1.4 mm on the buccal surface of the

root surface, 5 mm from apical end was made by using a round bur. The external cavities were washed with 3 ml of 17% ethylene diamine tetra acetic acid (EDTA) for one minute and again washed with 5ml of distilled water. Then the root canals were accessed, and pulp tissue was removed. Initially the canals were instrumented with 'k' files followed by enlargement upto peeso reamer size '3'. All the canals were irrigated with 5.25% sodium hypochlorite and rinsed with distilled water followed by drying the canals with paper points. The distribution of collected tooth specimens to respective study groups after processing is shown in Flow chart 1 as per CRIS guidelines (Checklist for Reporting *In vitro* Studies).

Group 1 (n=15): A manually prepared mixture of Calcium hydroxide powder (Prodent, Ratnagiri, Maharashtra, India) and distilled water in the ratio of 1gm:1ml was filled in all the root specimens using 18-gauge needle.

Group 2: (n=15): Commercially available calcium hydroxide pastes (Ammdent, Mohali, Punjab, India) was placed into the prepared canals.

Group 3: (n=15): Biodentine (Septodont,Saint-Maur-des-Fossés, France) was mixed as per manufacturer's instructions and inserted into the canals of prepared samples using 'k' files.

Group 4: (n=15): In this group, calcium hydroxide powder (Prodent, Ratnagiri, Maharashtra, India), 2% chlorhexidine gel (Prevest Denpro, Jammu, Jammu and Kashmir, India) and zinc oxide powder (Deepak enterprises, Mulund, Mumbai, India) were mixed in 2:1:2 ratio as described by Soares *et al.*[13] All three materials were placed on a glass slab and then mixed with mixing spatula until a creamy consistency is achieved. The mixed paste was then placed into the canals using a 'k' file.

Group 5: (n=15): Control group where the root canals were filled with distilled water.

Once materials were placed into the canals, these were evaluated radiographically for any voids (Figure 1). If voids are detected, then canals were refilled with respective material in all the groups except group 5. The surfaces of all root specimens were painted with nail varnish, leaving the external defect area. The apical and coronal ends of the root specimens were sealed with sticky wax to ensure proper placement of the materials. Nail varnish and sticky wax ensured that the OH^- can only diffuse into the medium through the external root resorption defect that has been prepared. Each root specimen was stored in separate screw capped vials which were filled with distilled water up to the root tip (Figure 2). The root specimens were attached to the inside of the lid of the vial using sticky wax. This is to ensure better handling of the specimens while measuring the pH. After measuring the pH, the root specimens were kept in the vials with distilled water and stored at 37° Celsius and regularly changed at different time intervals after pH measurements.

Measurements of pH were done using a pH meter with microelectrode (WTW Sentix, Xylem Analytics, Germany) (Figure 3). The pH of the specimens with external root defects stored in distilled water was determined at following time intervals: 30 minutes, 24 hrs, 7 days, 14 days, 21 days, 28 days and 3 months. Along with the pH meter and microelectrode, the equipment had 3 buffer solutions (pH 4.01, pH 7.00 and pH 10.01) and a potassium chloride (KCl) solution (Figure 3). Before

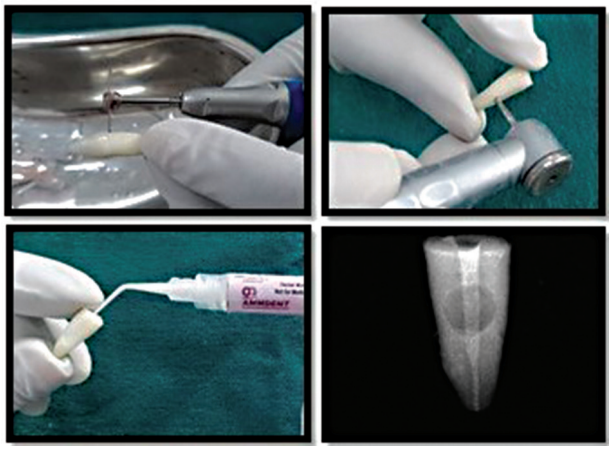
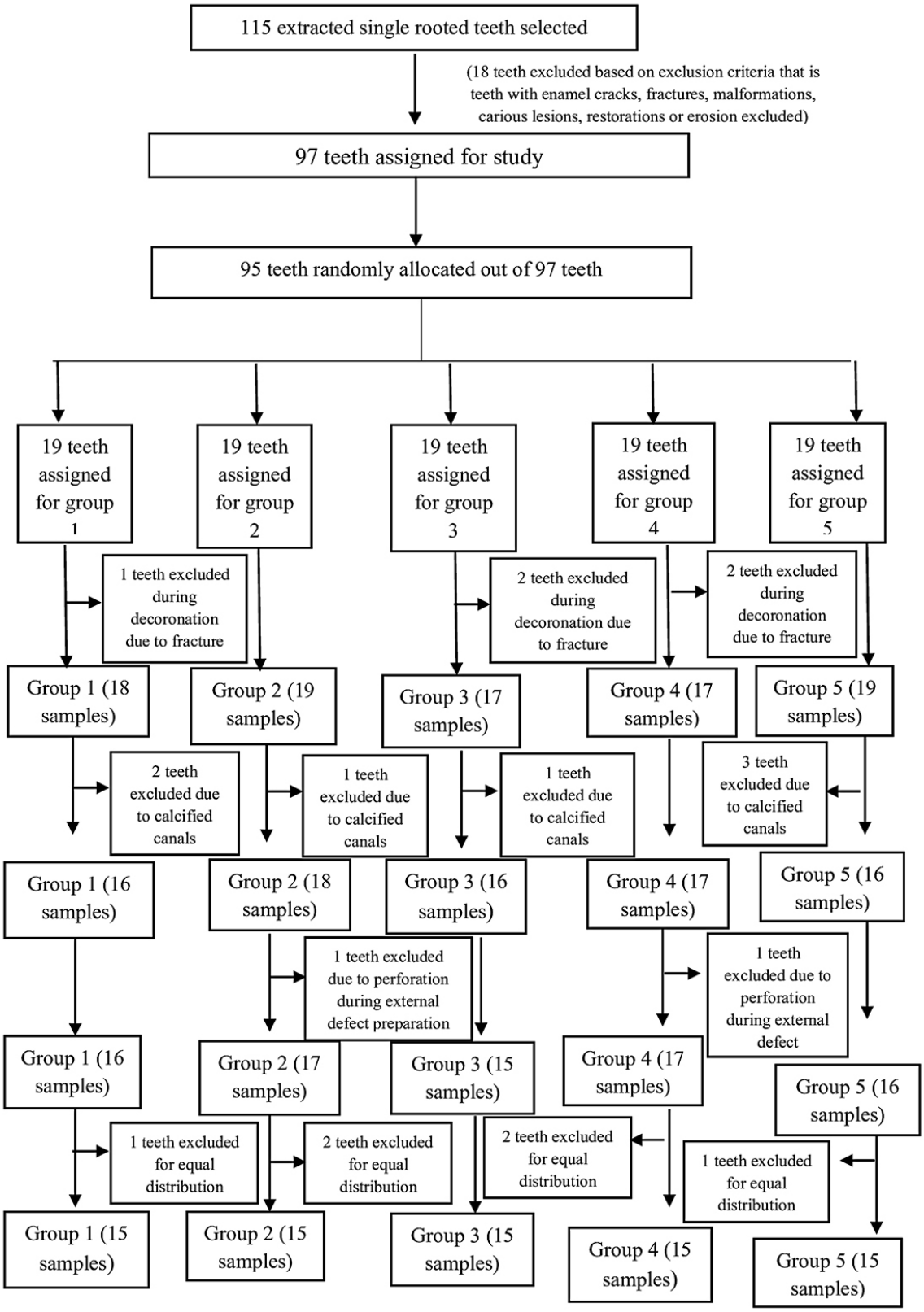


Figure 1: Preparation of samples followed by placement of CAOH based material and radiographic confirmation.

Figure 2: Prepared samples of all the 5 groups.



Flow chart 1: Distribution of processed samples according to CRIS guidelines. (Checklist for Reporting *In vitro* studies)

initiating the pH measurements of the samples, calibration of the pH electrode was done to ensure high measurement accuracy (Figure 4).

Figure 3: pH meter with microelectrode.

Figure 4: Microelectrode being rinsed with distilled water and dabbed dried with tissue paper.

For measuring the pH, the microelectrode tip was dipped at least or beyond its platinum junction in the solution/ medium to be tested (Figure 5). Therefore, the vials in which the samples were stored in distilled water were brought below the microelectrode tip and dipped until the level of the distilled water crossed the platinum junction of the microelectrode. The AR (automatic reading) display on the pH meter display will blink with automatic upward or downward adjustment of pH value until the exact pH value is displayed and blinking of AR on the pH meter stops. Then the final pH value that was displayed on the pH meter will be recorded as pH of that sample. In between each reading, the pH microelectrode washed using distilled water and was dried with the help of tissue paper. The same procedure was repeated for all the 15 samples in each group. If the level of reference electrolyte (KCL) inside the microelectrode becomes low with time, refilling was done through the opening using the KCl bottle supplied by the manufacturer. The mean of 3 readings was recorded as pH for each root specimen.

The data thus obtained was inserted in Microsoft excel 2009 spread sheet and analyzed using SPSS software version 20.0 for windows (statistical package for social sciences, IBM SPSS statistics, IBM corp., 2011). Analysis of variance was utilized to compare pH among the groups at different time intervals with post hoc Bonferroni for inter group comparison. A repeated measure ANOVA was applied to compare the pH of different time intervals within the group with post hoc Bonferroni for comparison between subsequent time intervals. The level of significance was pre-determined at $p \leq 0.05$.

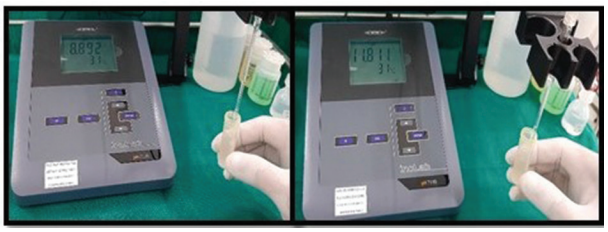


Figure 5: Evaluation of pH of samples in various groups.

Results

Table 1 shows the pH of various materials used in this study. Table 2 shows the comparison of pH levels across five groups over different time intervals using the ANOVA test. Group 1 shows a significant change in pH over time, with a consistently decreasing trend from a mean of 9.9 at 30 minutes to 7.74 at 3 months (p=0.001 for all intervals). The other groups (2-5) show minimal fluctuations in pH, with their mean values remaining relatively stable across all time intervals. Group 5 consistently maintains the lowest pH levels, ranging from 7.06 at 30 minutes to 6.55 at 3 months, while Groups 2, 3, and 4 show only minor variations. The significant p-value for Group 1 suggests a notable impact over time, while the other groups do not show statistically significant changes. This indicates that only Group 1 experienced a meaningful shift in pH, while the rest remained largely stable.

Table 3 shows the intergroup comparison of pH using post hoc Bonferroni test. Table 3 presents the inter-group comparison of pH levels using the Post hoc Bonferroni test at different time intervals. Group 1 consistently shows

Table 1: pH values of materials used in the study.

S.No.	Material	pH
1.	Distilled water (fresh)	6.124
2.	Distilled water (3 months storage)	5.701
3.	KCl solution (microelectrode under hydration)	7.583
4.	CAOH paste+ Distilled water (after mixing)	12.622
5.	CAOH paste (commercial product)	11.607
6.	Biodentine (commercial product)	12.107
7.	Chlorhexidine gel	8.112
8.	CAOH powder+ Zinc oxide powder+ CHX gel (after mixing)	12.326

Table 2: Comparison of the pH among the groups at different time intervals using ANOVA test.

Time interval	Groups	Minimum	Maximum	Mean	S.D	*p* value
30 min	Group 1	7.56	12.28	9.9	1.2	0.001*
	Group 2	7.44	8.89	8.06	0.38	
	Group 3	8.16	9.82	8.8	0.54	
	Group 4	8.14	8.89	8.4	0.2	
	Group 5	6.94	7.15	7.06	0.06	
24 hrs	Group 1	7.45	11.1	9.39	1.27	0.001*
	Group 2	6.85	10.77	8.06	0.88	
	Group 3	7.82	9.54	8.36	0.37	
	Group 4	7.94	10.46	8.45	0.62	
	Group 5	6.87	7.21	7.01	0.09	
7 days	Group 1	7.38	11.57	9.35	1.73	0.001*
	Group 2	7.56	9.51	8.07	0.46	
	Group 3	7.55	8.25	7.99	0.17	
	Group 4	7.55	8.71	8	0.33	
	Group 5	6.59	7.01	6.86	0.12	
14 days	Group 1	8.02	11.55	9.58	1.38	0.001*
	Group 2	7.96	8.69	8.24	0.23	
	Group 3	7.72	8.29	7.98	0.2	
	Group 4	7.36	8.09	7.81	0.21	
	Group 5	6.47	7.06	6.79	0.16	
21 days	Group 1	7.85	11.78	9.25	1.52	0.001*
	Group 2	7.78	8.26	8.07	0.15	
	Group 3	7.61	8.3	7.95	0.17	
	Group 4	7.11	7.95	7.48	0.26	
	Group 5	6.63	6.99	6.78	0.1	
28 days	Group 1	7.86	11.76	8.95	1.24	0.001*
	Group 2	7.05	7.4	7.21	0.12	
	Group 3	7.65	8.39	8.02	0.21	
	Group 4	7.22	8.14	7.68	0.28	
	Group 5	6.65	6.82	6.71	0.06	
3 months	Group 1	6.93	10.59	7.74	0.83	0.001*
	Group 2	6.69	7.76	7.41	0.33	
	Group 3	6.86	8.01	7.42	0.28	
	Group 4	6.02	6.97	6.65	0.31	
	Group 5	6	7.13	6.55	0.32	

*significant

significant differences (p<0.001) when compared to Groups 2, 3, 4, and 5 across all time intervals, indicating a distinct change in pH over time. The mean differences between Group 1 and other groups gradually decrease over time, reflecting a declining trend in pH for Group 1. Comparisons between Groups 2, 3, 4, and 5 mostly show non-significant differences (p>0.05), suggesting relatively stable pH levels among these groups. However, at 3 months, Group 1 vs. Groups 3 and 4 remain significantly different (p<0.001), while comparisons with Group 2 become non-significant, indicating that Group 1's pH approaches other groups over time.

Table 4 and Figure 6 represents the comparison of change in pH within the groups at different time intervals using Repeated measures ANOVA. The intra-group comparison between various time intervals was also statistically significant at p≤0.05. Calcium hydroxides mixed with distilled water showed sustain high release of hydroxyl ions at all tested time intervals when compared with other groups. Table 4 shows the comparison of pH changes within each group over different time intervals using repeated measures ANOVA. Group 1 showed a significant pH decline over time (p<0.001), starting with the highest mean pH at 30 minutes (9.90) and dropping to 7.74 at 3 months. This suggests a substantial decrease in pH over time. Group 2 maintained a relatively stable pH, with minor fluctuations from 8.06 at 30 minutes to 7.41 at 3 months. Similarly, Group 3 and Group 4 experienced gradual declines, with pH values staying close over time but showing statistically significant changes (p<0.001). Group 5 had the lowest and most stable pH values, ranging from 7.06 at 30 minutes to 6.55 at 3 months. The graph illustrates the comparative pH changes within different groups over time. Group 1 (blue line) starts with the highest pH (9.9 at 30 minutes) and shows a steady decline, reaching 7.74 at 3 months, indicating a significant drop over time. Groups 2, 3, and 4 (yellow and orange lines) show moderate declines, with pH values remaining relatively stable until around 14 days before gradually decreasing further. Group 5 (brown line) consistently maintains the lowest pH values, starting at 7.06 and dropping slightly to 6.55 at 3 months, showing the least variation among all groups. Group 3 exhibits minor fluctuations, initially increasing slightly before following a downward trend. The overall trend suggests that Group 1 experiences the most substantial pH reduction over time, while Group 5 remains the most stable. The significant differences across time intervals highlight the impact of prolonged exposure on pH levels.

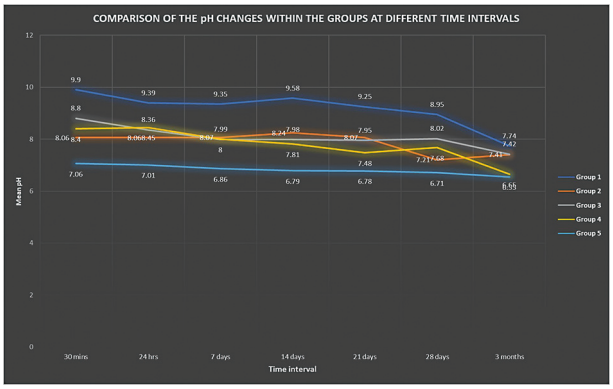


Figure 6: Graphical representation of comparative pH changes within the groups at different time interval.

Discussion

There are multiple factors that affects the tissue response to trauma but all these factors lead to clastic activity which eventually results in the resorptive mechanism.[2] Since odontoclasts need an acidic environment to dissolve minerals, the alkaline environment that is formed as a result of placement of calcium hydroxide in the root canals might inhibit their activity.[14] Previous studies have made attempts in showing the impact of calcium hydroxide in repairing periodontium and external root resorption.[2,15] It was observed that CH based materials when placed inside the canal of the specimens showed varying pH changes at all the time intervals.[16] There was a gradual decrease in the pH of group 1 at 30 mins, 24 hrs and 7 days then rise in pH was observed at 14th day followed by gradual decrease at 21 days, 28 months and 3 months. In group 2 pH showed slight increase from 8.06±0.38 to 8.07±0.46 at 7th day and 8.24 at 14th day followed by decrease in pH at 21st day, 28th day and 3 months. Group 3 showed gradual decrease in pH after 30 mins, 24 hrs, 7 days, 14 days, 21 days then a slight increase in pH at 28 days followed by decrease in pH at 3 months. Group 4 results showed increase in pH after 30 mins at 24 hrs followed by decrease in pH at 7 day, 14 day, 21 day, 28 day and 3 months. Group 5 showed decrease in pH during all the time intervals from 7.06±0.06 to 6.55±0.32 since the specimens had distilled water inside the canals. And distilled water has the tendency to gradually turn acidic with time.

Table 3: Inter group comparison of pH using Post hoc Bonferroni test.

		Group 1 V/s				Group 2 V/s			Group 3 V/s		Group 4 V/s
		Group 2	Group 3	Group 4	Group 5	Group 3	Group 4	Group 5	Group 4	Group 5	Group 5
30 min	Mean diff	1.84	1.10	1.50	2.85	-0.74	-0.34	1.01	0.40	1.75	1.35
	p value	0.001*	0.001*	0.001*	0.001*	0.017*	1.00	0.001*	0.82	0.001*	0.001*
24 hrs	Mean diff	1.33	1.03	0.94	2.38	-0.30	-0.40	1.05	-0.10	1.35	1.44
	p value	0.001*	0.001*	0.012*	0.001*	1.00	1.00	0.001*	1.00	0.001*	0.001*
7 days	Mean diff	1.28	1.36	1.34	2.49	0.08	0.06	1.21	-0.02	1.13	1.14
	p value	0.001*	0.001*	0.001*	0.001*	1.00	1.00	0.001*	1.00	0.001*	0.001*
14 days	Mean diff	1.33	1.59	1.76	2.78	0.26	0.43	1.45	0.17	1.19	1.02
	p value	0.001*	0.001*	0.001*	0.001*	1.00	0.74	0.001*	1.00	0.001*	0.001*
21 days	Mean diff	1.18	1.30	1.77	2.47	0.12	0.59	1.29	0.47	1.17	0.70
	p value	0.001*	0.001*	0.001*	0.001*	1.00	0.23	0.001*	0.69	0.001*	0.07
28 days	Mean diff	1.74	0.93	1.27	2.24	-0.81	-0.47	0.50	0.34	1.31	0.97
	p value	0.001*	0.001*	0.001*	0.001*	0.001*	0.31	0.21	1.00	0.001*	0.001*
3 months	Mean diff	0.33	0.32	1.09	1.19	-0.01	0.77	0.86	0.77	0.87	0.10
	p value	0.56	0.60	0.001*	0.001*	1.00	0.001*	0.001*	0.001*	0.001*	1.00

*significant

Table 4: Comparison of the pH changes within the groups at different time intervals using repeated measures ANOVA.

Groups	Time interval	N	Minimum	Maximum	Mean	S.D	*p* value
Group 1	30 mins	15	7.56	12.28	9.90	1.20	0.001*
	24 hrs	15	7.45	11.10	9.39	1.27	
	7 days	15	7.38	11.57	9.35	1.73	
	14 days	15	8.02	11.55	9.58	1.38	
	21 days	15	7.85	11.78	9.25	1.52	
	28 days	15	7.86	11.76	8.95	1.24	
	3 months	15	6.93	10.59	7.74	0.83	
Group 2	30 mins	15	7.44	8.89	8.06	0.38	0.001*
	24 hrs	15	6.85	10.77	8.06	0.88	
	7 days	15	7.56	9.51	8.07	0.46	
	14 days	15	7.96	8.69	8.24	0.23	
	21 days	15	7.78	8.26	8.07	0.15	
	28 days	15	7.05	7.40	7.21	0.12	
	3 months	15	6.69	7.76	7.41	0.33	
Group 3	30 mins	15	8.16	9.82	8.80	0.54	0.001*
	24 hrs	15	7.82	9.54	8.36	0.37	
	7 days	15	7.55	8.25	7.99	0.17	
	14 days	15	7.72	8.29	7.98	0.20	
	21 days	15	7.61	8.30	7.95	0.17	
	28 days	15	7.65	8.39	8.02	0.21	
	3 months	15	6.86	8.01	7.42	0.28	
Group 4	30 mins	15	8.14	8.89	8.40	0.20	0.001*
	24 hrs	15	7.94	10.46	8.45	0.62	
	7 days	15	7.55	8.71	8.00	0.33	
	14 days	15	7.36	8.09	7.81	0.21	
	21 days	15	7.11	7.95	7.48	0.26	
	28 days	15	7.22	8.14	7.68	0.28	
	3 months	15	6.02	6.97	6.65	0.31	
Group 5	30 mins	15	6.94	7.15	7.06	0.06	0.001*
	24 hrs	15	6.87	7.21	7.01	0.09	
	7 days	15	6.59	7.01	6.86	0.12	
	14 days	15	6.47	7.06	6.79	0.16	
	21 days	15	6.63	6.99	6.78	0.10	
	28 days	15	6.65	6.82	6.71	0.06	
	3 months	15	6.00	7.13	6.55	0.32	

*significant

The diffusion of OH^- into the external root resorption defect was shown in several studies.[5,17] Aguiar *et al.,* in their study demonstrated that CH within the canal increases pH of the external root surface.[18] Therapeutic effect of calcium hydroxide relies on dissociation of Ca^{2+} and OH^- and the availability of OH^- to alter the pH of medium. The pH raises as amount of OH^- increase.[19] Not only OH^- even Ca^{2+} might play an important role. But how the presence of Ca^{2+} affects the pH measurements is unknown. Hence, Ca^{2+} and OH^- dissociation and the interplay between these ions need to be addressed.

The pH level in the exterior defect on the surface was shown by Tsesis *et al.,*[20] to be inversely related to the thickness of the dentin, and difficult to control in investigations since different individuals can have different thickness of dentin.

According to Chamberlain *et al.,*[5] the pH of the experimental group increased quickly over the first 14 days compared to the control group, and then gradually decreased to the control group's average pH level at 21 and

28 days. Hence their results showed that the pH dropped after 14 days similar to the present study. Heward *et al.,*[7] also observed significantly higher pH at 4 weeks in CH group and MTA group. Soares *et al.,*[13] used a new combination of CH+ 2% CHX gel +ZnO and this paste demonstrated antimicrobial properties. Lima *et al.,*[6] in their study assessed effect of CHX in gel and liquid form and ZnO in CH paste on root pH in resorption defects and showed that despite having higher viscosity than other pastes, the combination of 2% CHX gel, CH, and ZnO allowed the diffusion of OH^- through dentin tubules and maintained alkaline pH. There are no studies available in the literature that had investigated the root dentin pH using this combination material along with other CH based materials.

The effect of intra oral temperature *in-vivo* on the dissociation curve of CH has not been explored in this study. The initial pH values of materials and the values of the solution as a result of OH^- diffusion from the external defects showed significant difference. Therefore, there is some delay of several days when rise in pH could be observed. At each time point after the pH of solution is measured, the solution was replaced to avoid the saturation of the solution. Based on the observations of this study, Group 1 showed highest alkaline pH followed by other test groups but long-term sustainability of the same needs to be further explored. The difference between the present study and the previous ones could be attributed to various compositions of dressing materials and observation for a longer time for pH measurements. This study included CH powder, CH pastes, Biodentine, CH with CHX gel and ZnO.

Further studies on the rate of dissociation of Ca^{2+} and OH^- need to be explored more extensively since this study focused only on the dissociation of OH^-, but Ca^{2+} also have a role to play. Fracture resistance of CH treated teeth/roots is another aspect that requires attention since use of CH as intracanal has a drawback of weakening the dentin structure eventually leading to tooth fracture. *In vivo* comparison of CH based materials in reimplanted cases/external root resorption cases also needs to be explored. Future histologic/biochemical studies are also required to evaluate the interaction of clastic cells involved in root resorption mechanism with Ca^{2+} and OH^-.

Conclusions

• OH^- diffuse through the dentine and potentially enabling them to reach a site of simulated resorption defect.

• Placement of different calcium hydroxide dressing materials resulted in varying pH changes on the external root surface at different time intervals.

• Calcium hydroxide and distilled water group maintained high pH at different time intervals in comparison with other groups followed by Biodentine group, CH paste and 2% CHX gel+CH+ZnO in the descending order and the pH could not be sustained by any of the material at the end of 3 months' time interval.

Declarations

Ethics approval

Institutional ethics committee gave its approval with Ref. No. TMDCRC/IEC/20-21/PPD1 dated 19/02/2021.

Competing interests

The authors declare no competing interests.

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Original Article

Correlation Between a Simple Tool for Evaluating Masticatory Function Value with Masticatory Performance by Sieve Method and Masticatory Ability by Questionnaire Among Older Adults

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Abstract

Objectives: This study aimed to find the correlation between a simple tool for evaluating masti-catory function value with masticatory performance and masticatory ability among older adults and using masticatory function value for evaluating subjects with different mastication groups.

Methods: The sample was 100 older adults in the dental clinic of Lee Hospital, Lamphun, Thailand. The samples were interviewed with general information, dental examination, tooth number, and occlusal pair. The wax biting test was given to samples, compared with the masticatory performance by sieve method and the masticatory ability by questionnaire. Data were analyzed using descriptive and analytical statistics.

Results: A high positive correlation between a simple tool for evaluating masticatory function value with masticatory performance by sieve method (correlation coefficient=0.54) and masticatory ability by questionnaire (correlation coefficient=0.64). The difference in the number of teeth, Occlusal pairs, and Eichner index classification have different masticatory function values (p<0.05). The number of teeth, occlusal pair, and masticatory ability can predict masticatory function values (R=0.83; R2=0.68; F=68.12; p<0.05). Masticatory groups divided by masticatory function value are related to masticatory groups divided by masticatory performance. (Chi-Square=8.24, p<0.05)

Conclusions: The simple tool created for evaluating masticatory function value by wax biting test can used to measure masticatory function. It is a reliable tool, easy to use in the clinic, convenient, and not complicated. It can differentiate masticatory function values in people with different mastication.

Keywords: masticatory ability, masticatory function value, masticatory performance, oral hypofunction

Introduction

The aging process involves various degenerative changes across multiple systems. In the oral cavity, this manifests as a decline in function[1] known as oral hypofunction. This condition can lead to decreased food intake, resulting in inadequate nutrient intake which affects the digestive system and overall health, ultimately leading to malnutrition and declining quality of life. Diagnosis at an early stage, with appropriate treatment and care, can prevent the progression of oral dysfunction in older adults. Reduced masticatory function, a component of oral hypofunction, can be assessed using various methods. These include counting the number of occlusal pairs[2], measuring particle size, utilizing the sieve method[3] or colorimetric analysis[4], applying the mixing ability index[5] to evaluate masticatory performance, measuring bite force[6], and administering questionnaires to evaluate masticatory ability.[7] Each method requires specialized equipment, which can be complicated, time consuming, and expensive.[7] Consequently, these methods are often unsuitable for clinical use in rural areas. Given these limitations, we aimed to develop a simple method which can be applied in hospital or dental clinic settings. Previous studies have not evaluated masticatory function using the wax biting test, despite the fact that dental wax sheets are readily available at dental clinics, inexpensive, and easy to use for creating bite marks. Therefore, we are interested in developing this method for assessing masticatory function. In this study, we define the masticatory function value as the measurement of wax thickness resulting from biting in the maximum habitual intercuspation position.

This study aimed to examine the correlation between a simplified assessment tool for evaluating masticatory function and established measures of masticatory performance as determined by the sieve method. Self-reported masticatory ability was assessed through a standardized questionnaire among older adults. Additionally, this study sought to use masticatory function values to evaluate older adult participants across different mastication groups and classify masticatory groups based on masticatory function values.

Materials and Methods

The study sample comprised 100 older adults who attended the dental clinic at Lee Hospital in Lamphun, Thailand. The sample size was calculated using the formula: $n=\left[\frac{z_{\alpha}+z_{\beta}}{z}\right]+3$. Data were collected between October 2022 and September 2023. The inclusion criteria were: (i) age 60 years or older, (ii) at least one occlusal pair, (iii) occlusion stability (stable contact on all teeth with equal intensity in the centric relationship), and (iv) the ability to understand written and spoken Thai and respond to the point range used in questionnaires. The exclusion criteria were as follows: (i) systemic disease which could influence jaw movement, for example Parkinson's disease, (ii) symptomatic temporomandibular disorders (pain and effect on chewing ability), (iii) severe pain effect of mastication, for example toothache and trigeminal neuralgia, and (iv) hyposalivation. All participants provided written informed consent after receiving a full explanation of the study's objectives and procedures. The study protocol was independently reviewed and approved by the Ethics Committee of the Faculty of Dentistry, Chiang Mai University (No.18/2022).

The mean participant age was 66.19 years (range: 60-81; standard deviation: 4.66). All participants underwent a structured interview and a standardized oral examination which included the following components: 1. Counting the number of teeth and occlusal pairs, 2. Determining masticatory function values using the wax biting test, 3. Measuring masticatory performance using the sieve method, and 4. Evaluating masticatory ability by questionnaire.

Number of teeth and occlusal pairs

The functional remaining teeth, including dental prostheses, were counted. The number of occlusal pairs was clinically determined by counting the antagonist teeth in occlusion and categorizing them according to Eichner's index of tooth loss, with particular emphasis on the molars and premolars. The three classifications are Class A: four occlusal support zones; Class B: one to three occlusal support zones; and Class C: no occlusal support zones.[8] Figure 1 shows four occlusal pairs of antagonist teeth in occlusion.

Masticatory function values by the wax biting test

The masticatory function value was determined using a wax bite test. Pink dental wax sheets (CAVEX, Cavex Holland BV Co., the Netherlands) with dimensions of 60×90×1.5 mm were kept at 23±2°C. Participants were instructed to chew a 5 mm cotton between both jaws for

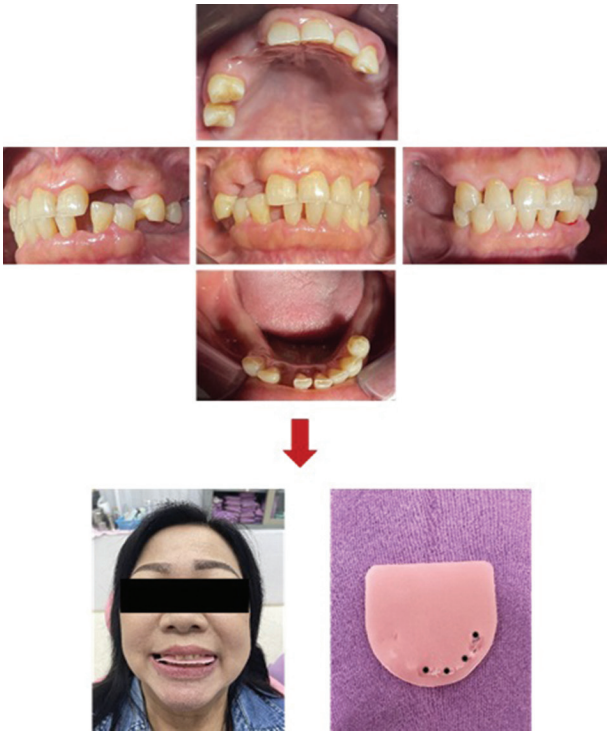

Figure 1: Wax biting test by a participant.

30 seconds to develop familiarity with the procedure, then bite each wax sheet using the habitual intercuspation bite pattern. The intercuspation bite patterns were replicated thrice to calculate the average change in wax thickness. Wax sheets were applied with the curved top facing upward to identify each occlusal pair. A dental wax gauge caliper was used to assess the thickness at the thinnest point of each occlusal pair, ensuring that the caliper was held perpendicular to the wax surface at the measurement point. The average thickness (C) was calculated from these values.

The change in wax thickness (D) can be calculated using the formula (Figure 2): $D = N - C$

Where: N = 1.5 mm (initial thickness of the wax sheet before biting)

C = Average thinnest thickness of the wax sheet at all occlusal points (mm)

Thus, the change in wax thickness (D) (mm) is: $D = 1.5 - C$

The change in wax thickness (D) was used to calculate the masticatory function value. The masticatory function value formula is based on the concepts of Hooke's law and Young's modulus, in which force (F) varies directly with displacement (x). The calculation of the chewing function was as follows:

Masticatory function value = Number of occlusal pairs (B) × Changed wax thickness (D)

The masticatory function value (Figure 1) calculated from the thickness of the wax sheets after biting on all three sheets, was then averaged to obtain the final result (Table 1).

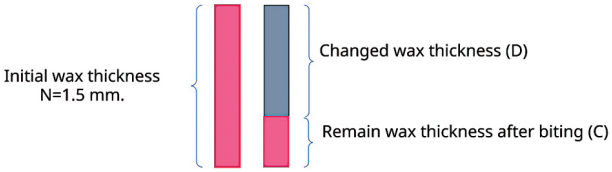

Figure 2: The calculation of the wax thickness change (D).

Masticatory performance by the sieve method[3]

Each participant chewed 3 g portions of fresh, whole carrot (1×2×0.5 mm) 30 times. After chewing, carrots were expectorated into a container. The expectorated carrot particles were poured onto stacked US Standard No. 5-mesh (0.157-inch openings) and No. 100-mesh (0.0059-inch openings) sieves. Approximately 250 ml of water was used to wash the carrot through each sieve. The portions of carrot remaining on the No. 5-mesh and No. 100-mesh sieves were transferred to Whatmann No.1 filter paper. Each filter paper was allowed to dry for 24 hours at room temperature. After drying, the filter papers were weighed.

Table 1: The calculation of the masticatory function value from participants.

Wax	(A) Thickness at the thinnest point of each occlusal pair (mm)	(B) Occlusal pair	(C) Average thickness	(D) Changed wax thickness (D=1.5 - C)	Masticatory function value (B X D)
No. 1	0.10, 0.10, 0.20, 0.00	4	0.10	1.40	5.60
No. 2	0.20, 0.10, 0.20, 0.10	4	0.15	1.35	5.40
No. 3	0.10, 0.20, 0.20, 0.10	4	0.15	1.35	5.40
Average masticatory function value of 3 sheets					5.47

Masticatory performance was calculated by dividing the weight of the carrot passing through the No.5 mesh sieve by the total weight of the separated feed (particles collected from the No.5 and No.100 mesh sieves), then multiplying this fraction by 100 and expressing it as a percentage.

Masticatory ability by questionnaire

Masticatory ability was measured using a food intake questionnaire consisting of 14 frequently consumed food items adapted from a previously validated study.[9] Participants were asked to rate their chewing ability for each food type on a 4 point scale, ranging from "could not chew at all" (0 points) to "could chew well" (3 points). The total score for the 14 food items, ranging from 0-42, was calculated as each participant's "perceived chewing ability score." Higher scores indicated better chewing ability.

Statistical analyses

Data analysis was conducted using SPSS Version 29.0 for Windows. The correlation between a simple tool for evaluating masticatory function value with masticatory performance by the sieve method, and masticatory ability by questionnaire was analyzed using the Pearson correlation coefficient. Different mastication groups were compared using an independent t-test. Logistic regression analysis was used to predict the masticatory function values. The relationships between masticatory groups divided by masticatory function value and those divided by masticatory performance were analyzed using the chi-squared test. A p-value of less than 0.05 was considered statistically significant.

Results

Of the participants, 41% had fewer than 20 teeth, and 59% had 20 or more teeth. The means and standard deviations of the numbers of residual teeth, occlusal pairs, and posterior occlusal pairs were 20.56±6.17, 8.10±3.91, and 3.56±2.72, respectively. In total, 57% of participants had fewer than 10 occlusal pairs, while 43% had 10 or more occlusal pairs. Notably, 93% of participants did not wear dentures (Table 2).

The distribution of participants according to Eichner's index of tooth loss was as follows: 74% in Group A, 26% in Group B, and 0% in Group C. The means and standard deviations for masticatory performance, masticatory ability, and the masticatory function value were 37.61±36.23, 27.46±8.40, and 7.55±3.94, respectively.

The intraclass reliability of the measurement was assessed using Cronbach's Alpha Coefficient (α), which yielded a value of α=0.97, indicating high reliability. The Pearson Product-Moment Correlation was employed, along with the test–retest reliability method, resulting in a coefficient of stability of 0.96, further demonstrating high reliability.

The results showed a strong positive correlation[10] between a simple tool for evaluating the masticatory function value with masticatory performance by the sieve method (correlation coefficient=0.54) and masticatory ability by questionnaire (correlation coefficient=0.64) (Table 3).

The difference in the number of teeth (groups with fewer than 20 teeth and groups with 20 or more teeth), occlusal pairs (groups with fewer than 10 occlusal pairs and groups with 10 or more occlusal pairs), and Eichner index classification were associated with significantly different masticatory function values (p<0.05) (Table 4). The results of the logistic regression analysis revealed that the number of teeth, occlusal pairs, and masticatory ability can predict masticatory function values (R=0.83; R2=0.68; F=68.13; p<0.05) (Table 5). Prediction of the masticatory function value was calculated by the following formula (Equation 1):

Masticatory function value
= -1.92+(0.15) number of teeth
+(0.50) occlusal pairs+(0.09) masticatory ability

There was a significant association between masticatory groups divided by masticatory function value and masticatory groups divided by masticatory performance (chi-square=8.24, p<0.05) (Table 6).

Discussion

In this study, a simple tool for evaluating the masticatory function value showed a strong positive correlation with masticatory performance as measured by the sieve method and masticatory ability as assessed by questionnaire, both of which are standard methods with high reliability in objective testing and subjective measurement.[11,12] The masticatory function value obtained through the wax biting test can be used to measure and differentiate masticatory function among participants belonging to different masticatory groups. Analysis of

Table 2: Demographics data.

Characteristics	Quantity	Percent
Sex		
Male	32	32
Female	68	68
Age		
Mean (S.D.)	66.19 (4.66)	
Min – Max	60-81	
Underlying disease		
No	65	65
Yes	35	35
Number of teeth		
Mean (S.D.)	20.56 (6.17)	
Min - Max	6-32	
Teeth number group		
< 20 teeth group	41	41
≥ 20 teeth group	59	59
Occlusal pairs		
Mean (S.D.)	8.10 (3.91)	
Min – Max	1-16	
Occlusal pairs group		
< 10 occlusal pairs group	57	57
≥ 10 occlusal pairs group	43	43
Denture		
No	93	93
Yes	7	7
Eichner classification		
A	26	26
B	74	74
C	0	0
Masticatory performance by sieve method	37.84 (36.32)	
Mean (S.D.)	0.01-99.91	
Min – Max		
Masticatory ability by questionnaire		
Mean (S.D.)	27.46 (8.40)	
Min – Max	5-42	
Masticatory function value by wax biting test		
Mean (S.D.)	7.55 (3.94)	
Min – Max	0.43- 16.91	

Table 3: The correlation between masticatory function value by the wax biting test with masticatory performance by sieve method and masticatory ability by questionnaire.

	Spearman correlation coefficient	*p*-value
Masticatory function value by wax biting test Masticatory performance by sieve method	0.54	<0.05**
Masticatory function value by wax biting test Masticatory ability by questionnaire	0.64	<0.05**

** Correlation is significant at the 0.05 level (2-tailed).

Table 4: Comparing of the masticatory function values for evaluating subjects in different mastication groups.

	Masticatory function value by wax biting test		t	x
	$\bar{x}$	S.D.		
Tooth number				
<20 teeth group	4.46	2.5	-9.07	<0.05*
≥ 20 teeth group	9.7	3.28		
Occlusal pairs				
<10 occlusal pairs group	4.8	2.38	0.07	<0.05*
≥10 occlusal pairs group	10.2	3.27		
Eichner classification				
Eichner A	10.75	3.61	5.49	<0.05*
Eichner B	6.42	3.1		

* Significant at the 0.05 level

Table 5: The Logistic regression analysis of the number of teeth, occlusal pairs, and masticatory ability by questionnaire for prediction masticatory function value.

	b	SEb	β	t	*p*-value
Number of teeth	0.15	0.06	0.23	2.47	<0.05*
Number of occlusal pairs	0.50	0.11	0.49	4.45	<0.05*
Masticatory ability by questionnaire	0.09	0.04	0.19	2.36	<0.05*
β_0 -1.92; SE_{est} = ±2.26 R=0.83; R^2=0.68; F=68.12; p<0.05					

* Significant at the 0.05 level

Table 6: The relationships between masticatory groups divided by masticatory function value and mastication group by masticatory performance by sieve method.

	Normal mastication divided by Masticatory function value (%)	Low mastication divided by Masticatory function value (%)	Total	Chi-square	*p*-value
Normal mastication divided by masticatory performance	12 (36.4%)	8 (11.9%)	20 (20%)	8.24	<0.05*
Low mastication divided by masticatory performance	21 (63.6%)	59 (88.1%)	80 (80%)		

* Significant at the 0.05 level

groups with varying numbers of teeth revealed that participants with 20 or more teeth exhibited higher masticatory function values than those with fewer than 20 teeth. The findings of this study are consistent with those of Miyaura *et al.,*[13] who observed a marked increase in biting pressure among individuals with at least 20 teeth. Similarly, Bates *et al.,*[14] found that a reduction in occlusal surface area may require individuals to chew for a longer duration in order to reach the swallowing threshold for food consistency.

This study found that participants with 10 or more pairs of occluding teeth had higher masticatory function values than those with fewer than 10 pairs. This is consistent with the findings of Witter *et al.,*[15] who reported that masticatory ability becomes impaired when fewer than 10 occluding pairs of teeth are represented.

This study found that Eichner Group A had higher masticatory function values than Eichner Group B, consistent with Ikebe *et al.,*[8] who found that participants in Eichner Group A had the highest masticatory performance of the three groups.

The masticatory function value by wax biting test

among older adults can be used to measure masticatory function. A reliability tool, it is easy and convenient for use in clinics. The findings of this study revealed a strong positive correlation between this simple assessment tool and both masticatory performance and masticatory ability. However, when conducting a regression analysis to predict masticatory function values using the wax biting test along with all other factors, it was found that the number of teeth, the total number of occluding pairs, and masticatory ability assessed by questionnaires had a very strong correlation with masticatory function. The multiple correlation coefficient was 0.83, and the masticatory function could be predicted with 68% accuracy.

The grouping method for masticatory function (Table 6) was based on the cut-off point from previous studies which reported that biting pressure increased significantly in subjects with fewer than 20 teeth[13] and that having 10 occluding pairs, specifically arranged from premolar to premolar, should satisfy function at a suboptimal but acceptable level for older adults.[16,17] Mastication ability scores below 80% were considered low; scores above 80% were classified as normal.[18]

Based on these findings, masticatory function values obtained from the wax biting test were grouped by substituting numerical values into Equation 1. Values below 8.94 were classified as low, while values greater than 8.94 were considered normal. Masticatory performance was categorized according to previous studies, in which individuals with levels below 80% were identified as having low performance.[19]

While the finding that the total number of occluding pairs significantly impacts masticatory function values is consistent with previous studies[16,17], it is not consistent with the emphasis on the number of posterior occluding pairs, as referenced in the study by Sarita *et al.,*[2] They found that individuals with fewer than four posterior occluding pairs experienced chewing problems such as being unable to chew all types of food or needing to consume specially prepared meals. The results of the present study support the theory that the total number of occluding pairs has a greater impact on masticatory function than the number of posterior occluding pairs.

Of the group with dentures, six older adults wore removable dentures. The removable dentures were slightly worn, with good retention and effective chewing; however, their masticatory function values were lower than those of fixed dentures. This finding is consistent with the study by Liedberg *et al*.,[20] who reported that individuals with removable dentures have a reduced ability to chew hard foods compared with those with fixed dentures.

In this study, the recording of occlusion was performed at the position of maximum habitual intercuspation, which is the position where the opposing teeth achieve the greatest occlusal contact, depending on each individual's natural bite. The masticatory function value was measured at the position most frequently used by older adults, which reflects stable occlusion; if this position involved mobility of teeth or caused discomfort while chewing, older adults would avoid using those teeth for the occlusion. A previous study investigating the relationship between periodontal conditions and masticatory ability found that the average clinical attachment level was negatively correlated with bite force and occlusal contact area. This is because the loss of clinical attachment of periodontal structures can lead to tooth mobility, which affects the occlusal contact area.[21] Therefore, it is important to consider periodontal status when evaluating mastication.

A limitation of this study is the method used to measure the thickness of the wax bite, as only vertical force was evaluated. This method does not measure dynamic chewing efficiency or reflect neuromuscular adaptation in individuals with missing teeth, and lacks correlation with muscle activity. It is therefore suitable in local dental clinical settings to assess masticatory function and support research data collection.

Conclusions

The results of this study demonstrate that the wax biting test is a reliable and accurate method of measuring masticatory function, and can therefore be effectively utilized in dental clinics to evaluate masticatory function. It is a simple, precise, and dependable technique which aligns well with standard procedures. However, it should be noted that this study was limited in that it did not reflect dynamic chewing efficiency or muscle activity. The wax biting test can be used to evaluate masticatory function and identify oral hypofunction, thereby ensuring proper eating, adequate nutrition, and the maintenance of a healthy digestive system and overall well-being, leading to an improved quality of life.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this article.

Consent

Written informed consent was obtained from the participants to publish this article.

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Effect of Dentine Sealing after Abutment Scanning on the Marginal and Internal Gaps of Zirconia Crowns: An *In Vitro* Study

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Abstract

Objectives: This study aimed to assess the effects of applying dentine sealing material after abutment scanning on the marginal and internal gaps of zirconia crowns and the thickness of different adhesive systems.

Methods: Forty extracted human molars were milled into uniform abutments and randomly chosen for scanning and fabricating a zirconia crown using CAD/CAM. Specimens were divided into four groups according to the dentine sealing technique: the control (C), total-etch (TE), self-etch (SE), and universal (U) adhesive groups. A silicone replica was made and longitudinally sectioned with the abutment for measuring adhesive thickness and gaps at different points (EF: external finish line, IF: internal finish line, AW: axial wall, CT: cusp tip, OI: occlusal incline plane, and CO: center of occlusal surface) under a stereomicroscope. Data were analyzed using a two-way ANOVA and multiple comparisons test.

Results: Regarding adhesive thickness, adhesive types and measuring points showed significant interaction. The TE group had a significantly higher adhesive thickness, especially in concave areas (IF and CO). The TE group also showed significantly wider gaps at the CT, OI, and CO and a significantly narrower gap at the EF, IF, and AW. The AW had the narrowest internal gap in all groups. The marginal gap increased in all experiment groups compared with the control but remained clinically acceptable (<120 µm).

Conclusions: The marginal gap increased significantly when the adhesive was applied after the final impression. High-viscosity adhesive produced a thicker adhesive layer, especially at the IF, causing marginal and internal gap increases.

Keywords: dentine sealing, digital impression, internal gap, marginal gap, zirconia crown

Introduction

Postoperative sensitivity after final cementation is one of the most common complications of full coverage fixed restorations.[1] As a large area of dentine is exposed during the tooth preparation process, failure to cover it or a gap present under the crown after cementation may cause this problem. In 1992, Pashley *et al.,*[2] introduced a technique involving bonding agent application after tooth preparation to establish a fully cured hybrid layer for sealing dentinal tubules to reduce post-operative sensitivity and increase bond strength of restoration, known as the immediate dentine sealing (IDS) technique.[2,3] This technique also has a positive impact on the longevity and retention of the fixed restoration.[3-5] Dentine bonding agents enhance bond strength at the dentine–resin interface[4], thereby improving adhesion to resin cement[3-5], which is commonly used for cementing zirconia crowns due to its ability to provide both micromechanical retention and chemical bonding when used with appropriate surface treatments and primers.[6] Furthermore, IDS also offers advantages for patient satisfaction by decreasing post-operative sensitivity during the provisional period and reducing the need for anesthesia during permanent cementation in the final visit.[7,8]

The ideal time to seal dentine is immediately after tooth preparation; however, this approach is not widely employed in routine procedures, as the abutment for a crown typically already provides sufficient resistance and retention form, thus leading to tooth sensitivity during provisionalization or after permanent cementation.[9] Dentine sealing after impression-taking is sometimes delayed since sensitivity occurs in later visits.[3] Furthermore, dentine sealing causes a fully cured adhesive layer to form a complete hybrid layer, preventing enormous pressure from being exerted on the dental pulp during final cementation.[10,11]

Various dentine bonding systems available in the market can be used to seal dentine.[12] They vary in the composition of the adhesive material, application procedures, and indications for use.[12] Many studies recommended three-step total-etch and two-step self-etch for dentine sealing due to their clinical effectiveness in achieving high bond strength.[13] Moreover, universal adhesives, a recent development, have versatile multimodal and multipurpose uses in clinical practice. Recent studies suggested that using universal adhesives as dentine sealing materials improved bond strength and reduced dentine permeability.[14,15] Some adhesive systems produce a relatively thicker adhesive layer than others (approximately 60-80 µm on smooth and convex surfaces and 200-300 µm on concave surfaces).[4,16]

In the fabrication of indirect restorations, space for the cement layer is intentionally prepared.[17] For zirconia crowns fabricated using CAD/CAM technology, cement space can be set in the software[18] based on the model obtained from the impression. When adhesive applied after taking the final impression, various dental adhesive systems may yield different thicknesses of adhesive layers, potentially interfering with the seating of the restoration and resulting in an unacceptable marginal gap.[19,20]

The recommended clinically acceptable marginal gap for long-term success is less than 120 µm.[21-23] Excessive marginal discrepancy causes the exposure of luting cement to the oral environment, leading to cement dissolution, roughness, microleakage, secondary caries, pulpal lesions, and periodontal problems.[24]

Nevertheless, if dentine sealing material must be applied after taking the final impression, the marginal gap should remain within the clinically acceptable range. Therefore, this study aimed to investigate the effect of applying dentine sealing material after taking the final impression on the marginal and internal gaps of zirconia crowns and the thickness of three dental adhesives *in vitro*.

Materials and Methods

Specimen preparation

The use of human tissue was approved by the Human Experimentation Committee (No. 1/2024), Faculty of Dentistry, Chiang Mai University. Forty human caries- and restoration-free molars were extracted and stored in a 1% chloramine T trihydrate solution for a week and then placed in grade 3 distilled water until use.

The cusps on the occlusal surface of the tooth samples were flattened using a carborundum disc on the trimmer until no grooves were visible. The tooth was then embedded in the center of a silicone mold with the occlusal surface placed downward, as the designed abutment was located at the center of the block. The silicone mold was fabricated from a high-viscosity polyvinyl siloxane (PVS) impression material (Express XT Putty Body; 3M ESPE, Neuss, Germany) using the Celtra Duo ZLS block (Dentsply Sirona, Hanau-Wolfgang, Germany) as the

template. The dimension of the block was 18x14x12 mm. A mixture of low-viscosity epoxy resin (Chem Builder Co. Ltd., Chonburi, Thailand) was poured into the silicone mold to embed the tooth sample. The epoxy resin was left to set for 24 hours. The shaft of a used Celtra Duo ZLS block was attached to the base of the epoxy resin block using epoxy glue (Quick Epoxy Steel; Alteco Chemical Pte. Ltd., Tuas Avenue, Singapore) (Figure 1).

The tooth specimen was prepared for abutment of the zirconia crown using a dental milling machine (CEREC Primemill MCXL; Dentsply Sirona, Bensheim, Germany). The 3D abutment design created in 3D design software (SolidWorks 2023 SP3.0; Dassault Systèmes SolidWorks Corp., Waltham, MA, USA) on the computer was modified from the work of Fernández-Estevan *et al.*[25] The abutment preparation design comprised a rounded rectangular shape with a height of 4 mm, a convergence angle of 6°, and a shoulder with a rounded internal line angle finish line with a depth of 1 mm (Figure 2). The dimensions of the abutment were designed according to the tooth preparation guidelines recommended by Goodacre *et al.,*[26] to ensure proper resistance and retention form. The specimen blocks were milled using a step bur 12S (Dentsply Sirona, Bensheim, Germany) and 12S cylinder-pointed bur (Dentsply Sirona, Bensheim, Germany). Prior to milling, the machine detected and calibrated the block position. If the block dimensions were accurate, the software initiated the milling process, producing preparations with consistent positioning across specimens. To reduce errors in the size of the abutment preparation, the burs were replaced after milling 10 specimens (90% usage as displayed in the milling software). Only intact specimens were included in the study. Any samples exhibiting pulp exposure due to anatomical variations were excluded.

One abutment was randomly selected to represent all prepared abutments for digital impression-taking using an intraoral scanner (CEREC Primescan; Dentsply Sirona, Bensheim, Germany). A zirconia crown was designed in computer-aided design/computer-aided manufacturing (CAD/CAM) software with parameters set for a cement space of 80 µm and a crown thickness of 1 mm at the margin and 2 mm at the axial and occlusal surfaces. The zirconia crown was milled from a Cercon® ht disc (Dentsply Sirona, Hanau-Wolfgang, Germany) using a dental milling machine (CEREC InLab MC X5; Dentsply Sirona, Bensheim, Germany), then sintered and glazed according to the manufacturer's guidelines (Figure 3).

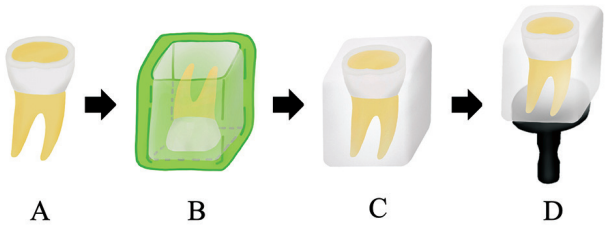

Figure 1: Embedding the tooth in epoxy resin procedure (A) The occlusal surface of the tooth was flattened; (B) A silicone mold was fabricated using putty PVS. The flattened tooth was positioned at the center of the mold base, since the designed abutment was centrally located, and epoxy resin was then poured into the mold; (C) The epoxy resin was left to set for 24 hours, then removed it from the mold; (D) The epoxy block was attached to the shaft of a used ceramic block.

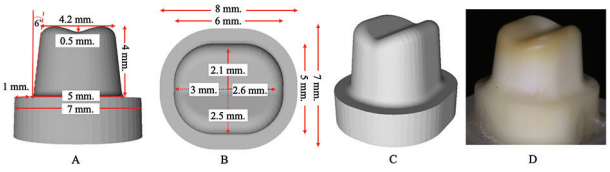

Figure 2: The abutment was designed on a computer using SolidWorks software. (A) Dimensions of the abutment in the proximal view; (B) Dimensions of the abutment in the occlusal view; (C) A 3D design of the abutment was created; (D) The abutment was milled from a 3D design using a milling machine.

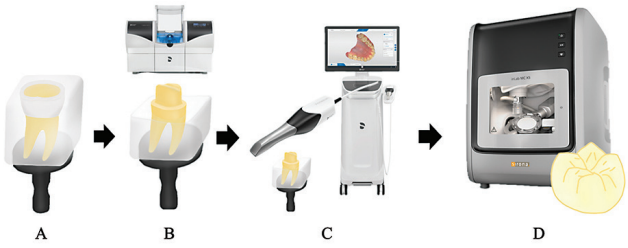

Figure 3: Specimen preparation procedure. (A) The occlusal surface of the tooth was flattened and embedded in epoxy resin, then attached to the shaft; (B) The machine calibrated the block position before milling, and the tooth block was milled into an abutment for a zirconia crown; (C) The abutment was scanned and designed for a zirconia crown; (D) The crown was fabricated by a milling machine.

Dentine sealing procedure

Forty abutments were randomly and equally allocated into four groups as follows:

- **Group 1: No dentine sealing (Control; C)** No dentine sealing material was applied on the prepared abutment surface.
- **Group 2: Total-etch adhesive (TE)** The prepared

surface was etched with 37.5% phosphoric acid for 15 seconds followed by rinsing with water for 20 seconds and gentle air-drying for 5 seconds. The primer (OptiBond FL Prime; Kerr Corporation, Orange, CA, USA) was applied with a light rubbing action for 15 seconds and dried with mild airflow for 5 seconds. The dental adhesive (OptiBond FL Adhesive; Kerr Corporation, Orange, CA, USA) was applied on the primed surface, avoiding the external finish line, followed by gentle air-blowing for 5 seconds, and then polymerized with a curing light for 20 seconds on each surface.

• **Group 3: Self-etch adhesive (SE)** The Primer (Clearfil™ SE Bond, Primer; Kuraray Noritake Dental Inc., Okayama, Japan) was applied on the prepared surface with a rubbing action for 20 seconds and dried with mild airflow, followed by dental adhesive application (Clearfil™ SE Bond, Bond; Kuraray Noritake Dental Inc., Okayama, Japan), avoiding the external finish line, gentle air-blowing for 5 seconds, and then polymerized with a curing light for 20 seconds on each surface.

• **Group 4: Universal adhesive (U)** The adhesive (3M™ Single Bond Universal adhesive; 3M ESPE, Neuss, Germany) was applied on the prepared surface with a rubbing action for 20 seconds, avoiding the external finish line, followed by gentle air-blowing for 5 seconds, and then polymerizied with a curing light for 20 seconds on each surface.

For Groups 2, 3, and 4, the air-blocking technique was performed after curing the adhesives by applying glycerin jelly (K-Y Lubricating Jelly Sterile; Doppel Farmaceutici SRL, Cortemaggiore, Italy) over the total preparation surface and light-curing for an additional 10 seconds to prevent oxygen-inhibited layer (OIL) formation, then rinsing and air-blowing with a triple syringe.

Adhesive thickness and marginal and internal gaps measurement

To measure the marginal and internal gaps for cement, the silicone replica was filled in this space and seated on the abutment. The thickness of the silicone replica represented the space for cement. To produce the replica, low-viscosity PVS (Express XT Light Body; 3M ESPE, Neuss, Germany) base and catalyst were mixed in a 1:1 ratio, filled into the zirconia crown, and seated onto the abutment under a 50 N load with a universal testing machine (Instron Corp., Canton, MA, USA) for 5 minutes. After the material was completely set, the crown was removed from the abutment, with the replica still attached to it. The high-viscosity PVS (Express XT Putty Body; 3M ESPE, Neuss, Germany) was mixed and loaded into an acrylic mold, and then applied on the abutment specimen to stabilize the thin film of the silicone replica. The abutment with the silicone replica was cut longitudinally in the buccolingual direction through the middle of the block using a low-speed cutting machine (Isomet®1000; Buehler, Lake Bluff, IL, USA). One half of the section was randomly selected to measure the thickness of the adhesive and silicone replica under a stereomicroscope equipped with a digital camera at 56X magnification (SZX7 & SZ-ILST LED illuminator stand & E-330 & Olympus, Tokyo, Japan); (Figure 4).

Seven digital images were captured from different areas of the abutment of each specimen for measuring the adhesive thickness (clear to dark brown color in micrographs) and gaps, including the marginal and internal gaps (purple color). Eleven points were selected for measurement: two points at the external finish line (EF), two points at the internal finish line (IF), two points at the center of the axial wall (AW), two points at the cusp tip (CT), two points at the occlusal incline plane (OI), and one point at the center of occlusal surface (CO); (Figure 5). Two values from the EF, IF, AW, CT, and OI of each specimen were averaged to represent the value for that point. ImageJ software (version 1.54 g, National Institutes of Health, Bethesda, MD, USA) was used to measure the adhesive thickness and gaps compared to a standard calibration slide. All measurements were performed by a single operator.

The data of adhesive thickness and gaps on the abutment from six measuring points and three adhesive types were statistically compared using a two-way ANOVA and Tukey's HSD multiple comparisons test with SPSS for MacOS, version 29 (SPSS Inc., Chicago, IL, USA). A *p*-value less than 0.05 was considered significant.

Results

Adhesive thickness

In general, the adhesive tended to accumulate in concave areas such as the internal finish line (IF) and the center of occlusal surface (CO), causing these two points to have a significantly greater thickness than the flat surfaces of AW, CT, and OI; (Figure 6). No adhesive thickness was found at the external finish line (EF) because no

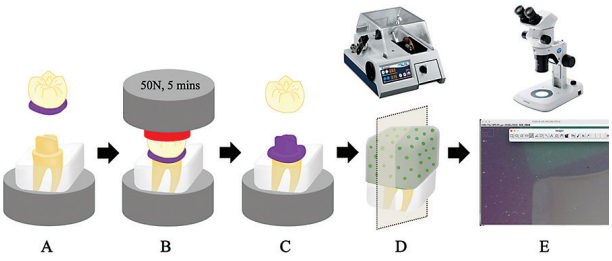

Figure 4: Adhesive thickness and gap measurement. (A) A silicone replica was made by loading light-body PVS into the crown; (B) The crown was seated under a 50 N load; (C) The crown was removed; (D) The putty PVS in an acrylic tray was placed to stabilize the silicone replica; (E) The specimen was sectioned in the buccolingual direction to measure the adhesive thickness and gaps under a stereomicroscope.

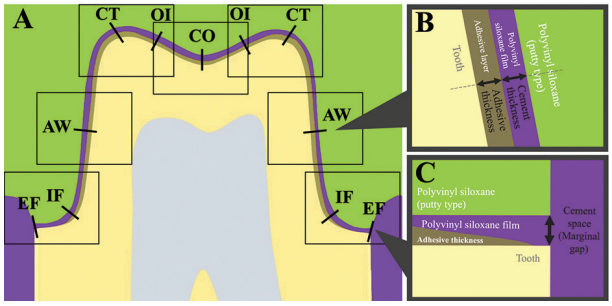

Figure 5: (A) Eleven measurement positions from seven images (rectangular frames) taken under a stereomicroscope (56X); external finish line: EF, internal finish line: IF, center of axial wall: AW, cusp tip: CT, occlusal incline plane: OI, and center of occlusal surface: CO; (B and C) Measurement of adhesive thickness and gaps, including the marginal and internal gaps.

adhesive was applied. Among the three adhesive systems, the TE group showed significantly greater adhesive thickness than the SE and U groups for all measurement points. However, there was no significant difference in adhesive thickness between the SE and U groups. The details of the thicknesses of the three adhesive systems at six measuring points are presented in Table 1. The statistical analysis suggested a significant interaction of adhesive types (TE, SE, and U) and measuring points (EF, IF, AW, CT, OI, and CO) regarding adhesive thickness ($p<0.001$).

The thickest adhesive was found at the internal finish line (IF) of the TE group, while the thinnest was present at the cusp tip (CT) of the U group. The second thickest point of the TE group was at the center of occlusal surface (CO), while the remaining points, including AW, CT, and OI, showed thinner adhesive with no significant differences among them. Similarly, the CO and IF points of the SE and U groups showed thicker adhesive than the AW, CT, and OI points.

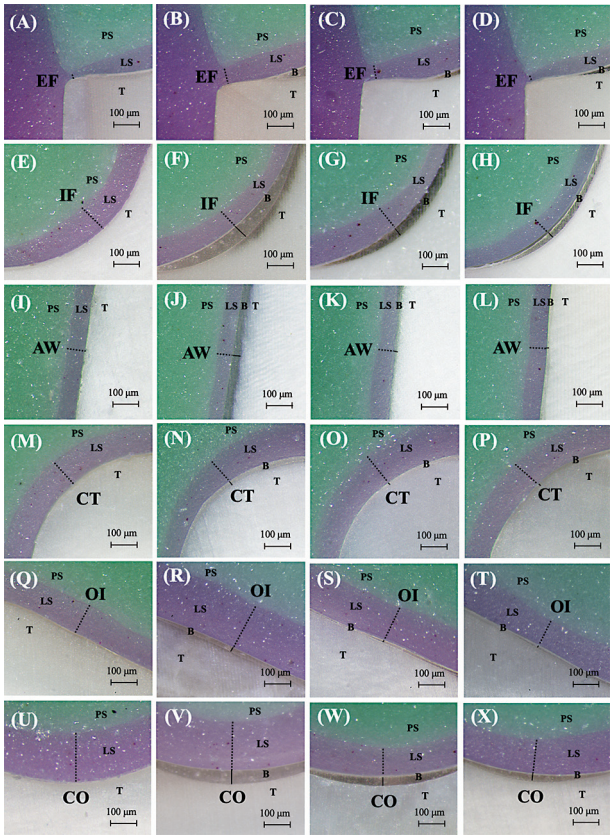

Figure 6: Examples of stereomicroscope micrographs (magnification 56x) of adhesive thicknesses (solid lines) and gaps (dashed lines) measured in each position of the C group (A, E, I, M, Q, and U), TE group (B, F, J, N, R, and V), SE group (C, G, K, O, S, and W) and U group (D, H, L, P, T, and X).
Abbreviations: PS: putty PVS, LS: light-body PVS, B: bonding, T: tooth, EF: external finish line, IF: internal finish line, AW: center of axial wall, CT: cusp tip, OI: occlusal incline plane, CO: center of occlusal surface.

Marginal gap and internal gap

The marginal gap at the external finish line (EF) of the TE group was significantly wider than that of the SE and U groups, followed by the control group, with no significant difference between the SE and U groups. At the IF and AW points, the gaps of the TE group were significantly thinner than those of the SE and U groups, and the control group yielded the widest gaps, with no significant difference between the SE and U groups. At the cusp tip (CT) and the occlusal incline plane (OI), the TE group showed significantly larger gaps than the other groups, while no significant difference at the center of

Table 1: Mean adhesive thickness and standard deviations (µm) of the experimental groups at different points.

Points	Mean ± standard deviation of adhesive thickness (µm)		
	Total-etch (TE)	Self-etch (SE)	Universal (U)
IF	105.26±14.26^{b,C}	39.54±9.06^{a,B}	37.41±7.65^{a,B}
AW	29.62±3.27^{b,A}	22.22±3.08^{a,A}	20.24±3.87^{a,A}
CT	20.90±3.37^{b,A}	17.98±1.87^{a,A}	17.46±2.23^{a,A}
OI	23.76±2.33^{b,A}	19.42±2.74^{a,A}	17.75±3.35^{a,A}
CO	77.09±13.43^{b,B}	42.32±10.65^{a,B}	35.25±11.39^{a,B}

^{a,b,c}Within a row, different superscript letters indicate statistically significant differences among the points ($p<0.05$) based on Tukey's HSD.
^{A,B,C,D}Within a column, different superscript letters indicate statistically significant differences among the groups ($p<0.05$) based on Tukey's HSD.

occlusal surface (CO) for all groups.

The two-way ANOVA showed a significant interaction between adhesive types (C, TE, SE, and U groups) and measuring points (EF, IF, AW, CT, OI, and CO points) regarding the gaps ($p<0.001$). The mean and standard deviation of the gaps are shown in Table 2. The multiple comparisons test suggested that the application of dentine sealing materials in this experiment significantly increased the gaps at some points compared to the control. For the control, the gaps were thinnest at the external finish line (EF) and widest at the center of occlusal surface (CO). The results of the SE and U groups were quite similar to those of the control group, while the TE group at the EF, IF, and AW points had significantly narrower gaps than at the CT, OI, and CO points.

Discussion

This study found that applying dentine adhesives after impression-taking significantly increased the marginal gap at the EF point, particularly in the total-etch (TE) group. While the maximum marginal gap exceeded 50 µm and could be detected with a dental explorer[27], it remained within the clinically acceptable threshold of 120 µm.[23] Despite this increase, the gaps are unlikely to compromise clinical outcomes, though long-term effects on restoration longevity should be further investigated.[28]

The adhesive layer was found to be thickest in concave regions (IF and CO), likely due to gravitational pooling during application.[29] This aligns with previous findings[4,16,30] showing that the adhesive tended to be thicker in the concave areas of the preparation. On the other hand, the adhesive thickness was not significantly different on flat surfaces (AW and OI) and convex surfaces (CT) across all adhesive groups. Among the adhesives, the TE group using Optibond FL showed the highest viscosity, attributable to its 48% filler content.[31] In contrast, SE and U adhesives (10-11% filler)[32,33] formed thinner layers. Since the detailed compositions of the adhesives were not published by the manufacturers, it was difficult to compare the viscosity of these adhesives. The different types of monomers and solvents contained in each adhesive system might also affect the viscosity.[34] The different viscosity

Table 2: Means and standard deviations of gaps, including the marginal and internal gaps (µm), of the control and experimental groups at different points.

Points	Mean ± standard deviation of gaps (µm)			
	Control (C)	Total-etch (TE)	Self-etch (SE)	Universal (U)
EF	17.94±1.76^{a,A}	61.69±7.89^{c,A}	27.45±3.92^{b,A}	23.82±3.60^{b,A}
IF	138.62±9.27^{c,C}	45.17±13.38^{a,A}	97.63±14.76^{b,C}	101.50±10.26^{b,C}
AW	91.08±2.97^{c,B}	53.18±8.45^{a,A}	65.02±8.00^{b,B}	66.71±10.07^{b,B}
CT	148.15±12.58^{a,CD}	196.30±28.22^{b,B}	133.42±13.31^{a,D}	130.16±16.56^{a,D}
OI	158.08±19.17^{a,D}	186.63±21.92^{b,B}	143.79±25.67^{a,DE}	149.33±22.22^{a,DE}
CO	195.89±15.85^{a,E}	185.65±47.62^{a,B}	164.74±36.70^{a,E}	157.03±35.47^{a,E}

^{a,b,c}Within a row, different superscript letters indicate statistically significant differences among the points ($p<0.05$) based on Tukey's HSD.
^{A,B,C}Within a column, different superscript letters indicate statistically significant differences among the groups ($p<0.05$) based on Tukey's HSD.

of the adhesives also affected their pooling, even when air-thinning was applied.[30]

Many factors affect the marginal fit of fixed restorations, including the finish line configuration and cement space. Regarding the finish line configuration, zirconia restorations require proper material thickness, with a recommended tooth reduction of 1 mm at the finish line.[35] A shoulder with a rounded internal line angle, which was the horizontal finish line chosen in this study, ensured sufficient thickness without causing over-contour of the restoration and provided better occlusal seating than a vertical finish line, feather edge, or shoulder-less preparations.[36,37] However, the horizontal finish line produced a wider marginal gap than a vertical finish line, even with the crown seated properly.[36] The narrower gaps at IF and AW could also affect crown seating since they reduced the venting of cement, resulting in a wider internal gap in the occlusal areas (CT, IO, and CO), especially in the TE group, where the crown would not be perfectly seated. When cement cannot escape from the inside, thicker cement can be found in the occlusal area, which affects the marginal gap.[38] In this case, the crown could have a hyper-occlusion and increased marginal gap after try-in, which can usually be seen in the clinic.

The cement space set in the software, including the radial spacer and occlusal spacer, would be filled with permanent cement during crown insertion. Many studies[39-41] have recommended a range of cement space in CAD/CAM from 30 to 200 μm to achieve a good marginal fit. In this study, the cement space for the zirconia crown was set at 80 μm, according to the manufacturer's recommendations.[42] It should not be too wide to prevent the excessive thickness of resin cement, which might cause polymerization shrinkage and a large number of voids, compromising the shear bond strength of the restoration.[43-46] However, insufficient cement space hinders the escape of cement, leading to poor crown seating.[47]

Ideally, the sum of the internal gap and adhesive thickness should be equal to the cement space set in the software. In the control group, even no adhesive was applied, the gaps were found to be wider than the set cement space in all areas. Furthermore, regarding the internal gap, the AW showed the narrowest gap in the control and all adhesive groups but was comparable to the IF in the TE group, while the occlusal area showed the widest gap in all groups. This difference was consistent with other studies.[47-49]

The difference in the internal gap in the AW and occlusal area could result from errors in the optical scanning process and the shrinkage of the pre-sintered zirconia block. During the processing of the 3D model after scanning, the sharp angle on the abutment was transformed into smoother and more continuous surfaces while the software generated the point cloud. This is one of the internal inaccuracies of the technique.[18] The machined zirconia block typically shrinks by approximately 25% during the sintering process.[50] However, the 3D model from the software estimation may not be sufficient to cover the actual shrinkage.[47] Increasing the cement space, particularly at the AW of the abutment (radial spacer in the software), might help solve this issue without further increasing the cement thickness at the occlusal region.

The sum of the measured adhesive thickness and the thickness of the silicone replica represented the total distance between the crown restoration and tooth surface or the sum of the adhesive and gap. Difference dental adhesives caused an increase in the sum of the adhesive and gap, as the TE group showed a greater sum of adhesive and gap than the SE, U, and C groups. The thickness of the adhesive in the TE group might have hindered the flow of cement more than other adhesives in the experiment, increasing the probability of incomplete crown seating, as evidenced by the larger marginal gap.[38] Spohr *et al.,*[16] also found that the sum of adhesive and gap in the occlusal region of crown preparation of the group in which adhesive was applied was greater than that of the group without adhesive.

Based on this study's results, dentine sealing after taking a digital impression with an intraoral scanner significantly increased the marginal gap within the limit of clinical acceptance (< 120 μm), together with significantly increased internal gaps. Especially in the TE group, the adhesive had high viscosity and less ability to flow and spread on the surface due to strong intermolecular forces;[51] thus, it tended to accumulate more in the concave areas, which enhanced molecular cohesion, than in the convex areas. In the case of the application of a high-viscosity adhesive, increasing the air-blowing or preheating process[52] might be helpful for thinning and spreading the adhesive by increasing molecular motion and enhancing fluidity.[53] However, it is challenging to control the thickness of the dental adhesive applied to

a scanned abutment. Therefore, although the marginal gaps observed in this study remain within clinically acceptable, the author does not recommend this approach. Furthermore, it may also affect occlusion due to incomplete seating of the crown.[54] If it must be performed, high-viscosity adhesives should be avoided.

Dentine sealing can be performed at various clinical stages: immediately after tooth preparation, after the provisional cementation of a temporary restoration, or after digital impression-taking but before the temporary restoration, as done in this experiment. The technique in which the dental adhesive is applied to freshly cut dentine before impression-taking or before fabricating a temporary restoration is called IDS, whereas in the delayed dentine sealing (DDS) technique, the dentine is sealed after it has been contaminated by the impression material or temporary restoration. IDS offers the advantages of stronger bond strength[3], while DDS helps to reduce tooth sensitivity.[55] However, the technique used in this experiment might not fully fit the criteria for IDS or DDS. The use of the silicone replica method in this study aimed to preserve the crown; as a result, permanent cementation of the crown was not needed, and sectioning the specimen was not necessary.[56-59] Although adhesive application was carefully controlled in this *in vitro* study, clinical conditions may differ. Challenges such as limited visibility, subgingival margins, or adhesive overextension beyond the finish line can lead to uneven application, which may affect crown seating and marginal adaptation.

Conclusions

The application of dentine sealing material after abutment scanning influenced the marginal and internal gaps of the zirconia crown. The marginal gap of all dentine sealing groups increased significantly but remained clinically acceptable. The internal gaps of the occlusal region were wider than those of the other areas. The high-viscosity bonding adhesive produced the thickest adhesive layer, especially at the internal finish line (IF), which caused the widest marginal gap, especially in the total-etch (TE) group.

Acknowledgment

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Conflicts of Interest

The authors declare no conflicts of interest.

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Biocompatibility Assessment of Blue Light-Activated Methacrylated Hyaluronic Acid Hydrogel with L929 Fibroblast Cell Line

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Abstract

Objectives: To evaluate the biocompatibility of blue light-activated methacrylated hyaluronic acid (BL-MeHA) hydrogel using the L929 cell line.

Methods: Biocompatibility was assessed using three different assays. In the indirect cytotoxicity assay, L929 cells were cultured in conditioned media that had been exposed to BL-MeHA for 24 hours, followed by an MTT assay to evaluate cell viability. In the 2D culture assay, L929 cells were seeded on top of the BL-MeHA hydrogel, and cell viability was measured on days 1, 3, 5, and 12 using the resazurin assay. For the encapsulation culture assay, L929 cells were embedded within the BL-MeHA hydrogel, and viability was similarly assessed on days 1, 3, 5, and 12 using the resazurin assay. Additionally, L929 cell morphology was examined using scanning electron microscopy (SEM).

Results: The indirect cytotoxicity assay demonstrated that L929 cells remained viable when cultured with the BL-MeHA extract. In both the 2D and encapsulation culture assays, L929 cells initially exhibited slower growth compared to the control group but reached comparable levels by day 12. Notably, there was no significant difference in cell viability between BL-MeHA samples cured for 60 and 90 seconds.

Conclusions: The BL-MeHA hydrogel exhibited no cytotoxic effects on L929 cells, indicating good biocompatibility. These findings support its potential use as a scaffold for future applications in cell encapsulation or drug delivery for soft tissue engineering.

Keywords: cell viability, hyaluronic acid, hydrogel, L929 cell

Introduction

The goal of tissue engineering is to regenerate new tissue at the site of injury to restore its original function.[1,2] Tissue loss may involve either soft tissue or hard tissue, such as bone, with the latter being particularly challenging to replace.[3-6] Tissue engineering relies on three fundamental components: cells, scaffolds, and biologically active molecules. The effectiveness of a scaffold depends on various factors, including its surface and bulk properties, as well as its clinical practicality. An ideal biomaterial should possess several key characteristics, it must be biocompatible, degrade into non-toxic by products, and avoid triggering an immune response. Poor biocompatibility can lead to immune activation, inflammation, or even tumour formation.[6] Biodegradability refers to the chemical or enzymatic breakdown of a material within the body over time. Ideally, these materials should break down into harmless substances that can be safely eliminated from the body.[1]

A wide range of biological scaffold materials has been developed for use in both soft and hard tissue engineering. Among these, hydrogels are among the most widely utilized due to their many advantageous properties. Their hydrophilic nature allows them to absorb water and swell efficiently, making them particularly suitable for drug delivery and tissue engineering applications.[7-9] Hydrogels can be synthesized from a variety of precursors, including polyethylene glycol, alginate, polyvinyl alcohol, chitosan, heparin, and hyaluronic acid. Hyaluronic acid-based hydrogels, in particular, offer excellent biocompatibility due to their similarity to components of the extracellular matrix. They can be enzymatically degraded within the body, and their structure can be modified to enhance physical characteristics, making them highly adaptable for a wide range of biomedical applications.[10-12]

In 2018, Trakiattikul *et al.,*[13] developed a methacrylated hyaluronic acid (MeHA) hydrogel incorporating mannitol and bovine serum albumin. Although the hydrogel demonstrated favourable physical properties, its gelation time of approximately 30 minutes was too long to be clinically practical. Subsequent studies showed that this MeHA hydrogels crosslinked with dithiothreitol (DTT) were biocompatible with human alveolar bone cells and human gingival fibroblasts.[14,15] However, the prolonged gelation time of 15-30 minutes remained a limitation for future clinical use. To address this issue, Chaopanitcharoen *et al.,*[16] in 2023 incorporated lithium trimethyl benzoyl phosphinate (LAP) into MeHA to develop a blue light- activated MeHA (BL-MeHA) hydrogel system, enabling better control over gelation time. Their results demonstrated that 90 to 120 seconds of light exposure produced the hydrogels with desirable physical properties, including uniform pore size and effective swelling behaviour, highlighting their potential as scaffolds for clinical application.

The objective of this study was to evaluate the biocompatibility of BL-MeHA hydrogel using the L929 mouse fibroblast cell line, in accordance with the International Standards Organization (ISO 10993-5) guideline for biomedical materials testing. The findings are intended to support the development of effective biological scaffolds for tissue engineering applications.

Materials and Methods

BL-MeHA hydrogel preparation and blue light activation

The BL-MeHA hydrogel was synthesized based on the protocol described by Chaopanitcharoen *et al.,*[16] Briefly, 1% (w/v) of 47-kDa hyaluronic acid (HA) was dissolved in a potassium phosphate buffer (pH 8). Methacrylic anhydride (Me, MW 154.16 g/mol) was then added dropwise at a 1:10 molar ratio relative to HA. The reaction was conducted at 4°C for 24 hours under continuous stirring with a magnetic stirrer. The resulting MeHA solution was transferred into dialysis tubing and dialyzed to remove unreacted substances. After purification, the solution was collected, flash-frozen, and lyophilized (Labconco lyophilizer, Missouri, USA) for 3 days. Multiple batches of the gel were synthesized, and samples were sent for analysis of the degree of modification using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy at the Scientific and Technological Research Equipment Center, Chulalongkorn University, Bangkok, Thailand. For the light curing unit, 3M™ Elipar™ S10 LED was used as the blue light source throughout this study.

Cell culture condition

L929 mouse fibroblast cells obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified eagle's medium (DMEM: Ward Medic) containing 10% fetal bovine serum

(Gibco™) and 1% penicillin–streptomycin (Invitrogen) (10%FBS-DMEM). The culture was kept in 5% CO_2 37°C incubator.

Indirect cytotoxicity assay

The BL-MeHA hydrogel was cast in a plastic mold to form a cylindrical shape of 20 mm in diameter and 5 mm in thickness. A blue light curing time of 90 seconds was used. Following curing, the hydrogel was soaked in 20 mL of serum free Dulbecco's modified eagle medium (SF-DMEM) for 24 hours. The extract conditioned medium was collected and subsequently diluted to concentrations of 50%, 25% and 12.5% in SF-DMEM, with the original undiluted medium representing 100%.

L929 fibroblasts were plated at a density of 10,000 cells per well in a 96-well plate and allowed to attach overnight. The following day, the cells were treated with 100 µL of BL-MeHA hydrogel conditioned media at concentrations of 100%, 50%, 25% and 12.5%. Additionally, 10% dimethyl sulfoxide (DMSO) and 10% FBS-DMEM were used as control groups to represent toxic and non-toxic conditions, respectively. After 24 hours of incubation, cell viability was assessed using the MTT assay. Three separate experiments were performed in triplicate.

2D culture assay

A volume of 80 µL of BL-MeHA hydrogel was formed at the bottom of each well in a 96-well plate using blue light irradiation for either 60 or 90 seconds. Then, 100 µL of serum-free DMEM (SF-DMEM) was added to each well and incubated for 24 hours. After incubation, L929 fibroblasts were seeded onto the hydrogel surface at a density of 5,000 cells per well. The cell culture medium (10% FBS-DMEM) was refreshed every two days. Cell viability was assessed using the resazurin reduction assay on days 1, 3, 5, and 12 after cell seeding. The resazurin solution (PrestoBlue™, Invitrogen; Thermo Fisher Scientific) was prepared according to the manufacturer's instructions. The control group consisted of L929 cells cultured directly on the surface of a 96-well plate. All experiments were performed in three independent replicates.

Encapsulation culture assay

Following lyophilization and sterilization with 99.99% ethanol, the prepared hydrogel was immersed in 10% FBS-DMEM, and LAP was added at a final concentration of 15 mg/L. A total of 100 µL of the BL-MeHA hydrogel mixture was combined with 2×104 L929 cells suspended in 100 µL of 10% FBS-DMEM. The mixture was gently pipetted up and down to ensure a homogeneous gel–cell suspension, which was then seeded into a 96-well plate. Polymerization was induced using blue light for either 60 or 90 seconds. After curing, 100 µL of 10% FBS-DMEM was added to each well and replaced every two days. Encapsulated cell viability was evaluated using the resazurin reduction assay on days 1, 3, 5, and 12. All experiments were performed in triplicate across three independent trials.

Evaluation of cell morphology

To evaluate L929 fibroblast cell morphology on BL-MeHA hydrogel, samples were collected on days 1, 3, 7, and 14 after cell seeding. Culture media were removed, and wells were washed twice with 1 mL of PBS. Cells were then fixed with 1 mL of 2.5% glutaraldehyde at 25°C for 4 hours, followed by two PBS washes (1 mL each, 5 minutes per wash). Samples were dehydrated sequentially with 500 µL of 25%, 50%, and 75% ethanol at 4°C for 5 minutes each. This was followed by three changes of 500 µL 100% ethanol at 4°C for 5 minutes each. Subsequently, 300 µL of hexamethyldisilazane (HMDS) was added to each well for 20 minutes, then removed. Samples were frozen at -20°C for 3 hours, then transferred to -80°C for 24 hours prior to lyophilization. After 24 hours of freeze-drying, the samples were examined using scanning electron microscopy (SEM) and analyzed using ImageJ software.

Data analysis

The results were presented as mean ± standard deviation (SD) and were subjected to statistical analysis using SPSS 25.0 software (SPSS Inc, Chicago, IL, USA). The absorbance values were assessed for normal distribution using the Shapiro-Wilk test. A Kruskal-Wallis and Dunn's multiple comparisons analysis of variance was conducted to assess the presence of significant differences between the groups. ($p \leq 0.05$)

Results

BL-MeHA synthesis

In this study, we synthesized 4 separate batches of MeHA to evaluate the degree of modifica-

tion using ^{1}H NMR spectroscopy. All 4 batches demonstrated a 100% degree of substitution, meeting the criteria established by Chaopanitcharoen *et al.*,[16] Figure 1 presented the spectra obtained from the ^{1}H NMR analysis.

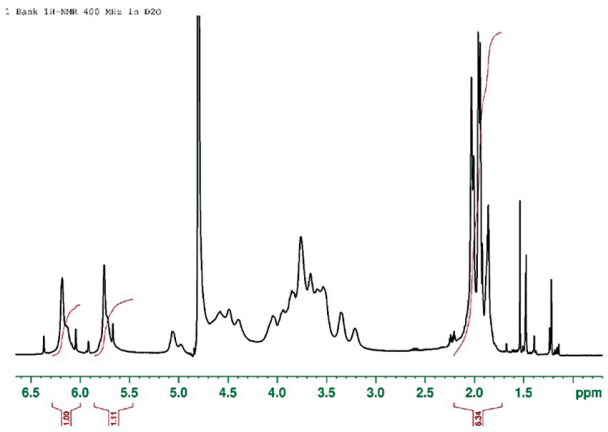

Figure 1: Degree of modification from proton nuclear magnetic resonance analysis.

Indirect cytotoxicity assay

After 24 hours of exposure to conditioned media derived from BL-MeHA hydrogel, the viability of L929 cells was assessed using the MTT assay as shown in Figure 2. The observed optical density (OD) values were 0.9371±0.0698, 0.9173±0.2137, 0.9208±0.0849, and 0.8736±0.0776 for the 100%, 50%, 25%, and 12.5% conditioned media groups, respectively. No statistically significant differences were found among these groups, indicating that the conditioned media was non-toxic to L929 cells. Furthermore, there was no significant difference when compared to 10% FBS-DMEM (0.9111±0.1015) control group. As expected, the 10% DMSO-treated group showed the lowest OD value at 0.4809±0.1143 which is significantly lower than all other groups.

2D culture assay

L929 cells were seeded onto the surface of the hydrogel and subsequently evaluated for viability using the PrestoBlue assay on days 1, 3, 5, and 12. The control group was conventional plastic cell culture plate. As shown in Figure 3, the control group exhibited the fastest growth starting from the 24-hour time point with the fluorescence value of 33,339.00±8,822.09, 37,684.33±11,611.77, 48,941.67±3,004.50 and 42,285.67±3,259.84 for day 1, 3, 5 and 12 respectively. In contrast, the L929 cells

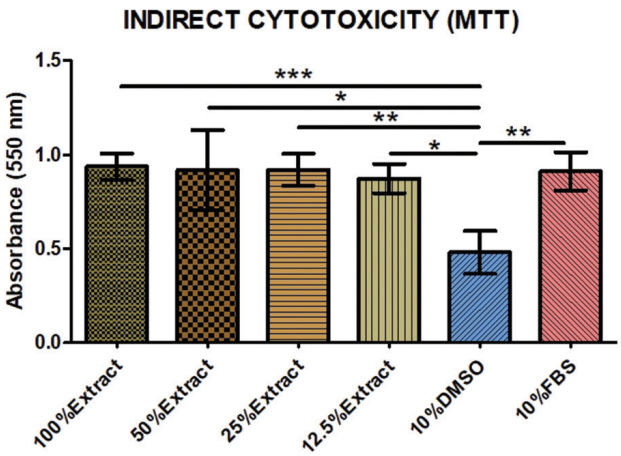

Figure 2: Indirect cytotoxicity assay (mean±SD, n=9, Kruskal-Wallis test and Dunn's multiple comparisons test, $p \leq 0.05$) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

growing on top of the gel showed slower initial growth but were able to catch up with the control group by day 12. The fluorescence value for day 1, 3, 5 and 12 was 10,544.33±4,544.26, 13,210.00±2,090.41, 26,749.33 ±6,002.81 and 43,603.67±1,594.29 for the 60s BL-MeHA and 7,607.33±3,440.67, 22,667.66±6,270.75, 27,623.33±9,056.13 and 44,008.67±5,609.76 for the 90s BL-MeHA, respectively. No statistically significant difference was found between the 60s and 90s groups. Hydrogel without cell and blank media were included as negative controls, both showing very low reading.

Encapsulation culture assay

It was found that L929 were able to grow when encapsulated within BL-MeHA although the growth rate was slow. As shown in Figure 4, the relative fluorescence values on day 1, 3, 5, and 12 were 1.00±0.00, 5.84±2.76, 8.29±4.15 and 42.30±21.92 times for 60s BL-MeHA

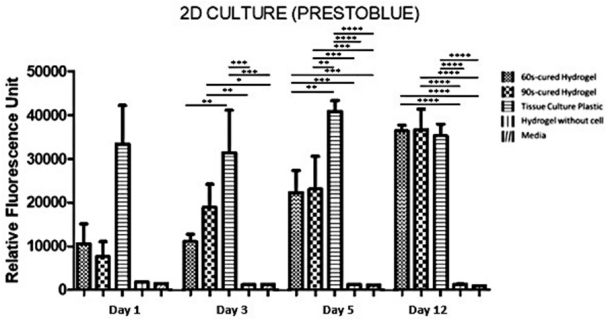

Figure 3: 2D culture assay (mean ± SD, n=9, One-way ANOVA and Tukey's multiple comparisons test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ***$p \leq 0.001$)

and 1.00±0.00, 3.67±1.71, 5.23±3.09, and 33.18±13.39 for 90s BL-MeHA, respectively. For all time points, no statistically significant difference was found between the 60s and 90s groups.

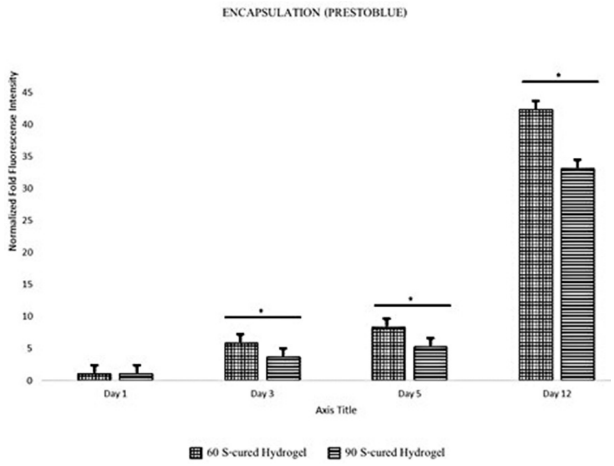

Figure 4: Encapsulation Culture Assay (mean±SD, n=9, Kruskal-Wallis test and Dunn's multiple comparisons test for intragroup analysis and Mann Whitney test for intergroup test, *$p \leq 0.05$).

Morphology assessment by scanning electron microscopy

On day 12 of the 2D culture assay, cell morphology was examined using scanning electron microscopy. As illustrated in Figure 5, both round and spindle-shaped L929 cells were observed, with some forming cellular clusters (A, B, C). Additionally, certain cells exhibited branching extensions and signs of cell division (I-arrow). These findings indicate that L929 cells were able to penetrate the hydrogel while maintaining their typical morphology and proliferative capacity within the hydrogel environment.

Discussion

Our group has been working on the development of hydrogel scaffolds for tissue engineering applications for some time. An earlier chemically crosslinked version showed promising physical and biological compatibility[13-15]; however, its prolonged setting time of approximately 30 minutes rendered it unsuitable for clinical use. The BL-MeHA hydrogel developed by Chaopanitcharoen *et al.,*[16] with significantly shorter curing times of 90 and 120 seconds, offers a practical solution to this limitation. In this study, we demonstrated that BL-MeHA not only possesses favorable physical properties but also exhibits excellent biocompatibility. The indirect cytotoxicity assay

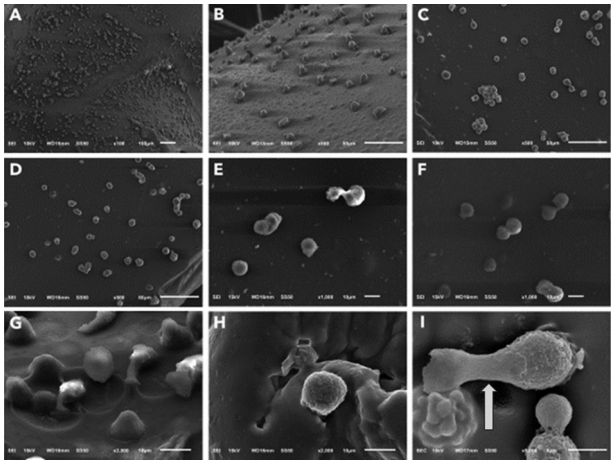

Figure 5: L929 morphology by scanning electron microscopy (2D culture assay day 12); (A) x100 and (B-D) 500X and 1000X(E-F) and 2000X (G-H) and 5000X (I) respectively.

demonstrated that L-929 cells remained morphologically normal after 24 hours of exposure to conditioned medium collected from BL-MeHA immediately after gel setting, even at the highest concentration of 100%, as well as at 50%, 25%, and 12.5%. This finding is promising for future applications, as the scaffold is intended to be in direct and prolonged contact with tissue. Interestingly, although the conditioned medium was serum-free, there was no statistically significant difference in L-929 cell growth compared to that observed with 10% FCS-DMEM. This may be attributed to the short 24-hour exposure period, during which the cells had limited time to proliferate. Nonetheless, the key finding is that L-929 cells remained viable, indicating that exposure to BL-MeHA conditioned medium is non-toxic.

L929 is an attachment-dependent cell line, requiring a surface for adherence in order to grow, survive and maintain its function.[17] In the 2D cell culture assay, L929 cells were able to grow on top of the BL-MeHA. Although cell numbers were significantly lower compared to those on a conventional cell culture plate at days 1, 3, and 5, the cells continued to proliferate and reached comparable levels by day 12. Additionally, there was no statistically significant difference in cell numbers between the 60-second and 90-second BL-MeHA groups, indicating that L929 cells were able to grow equally well on both gel types. Additionally, the encapsulation assay showed that L929 cells were able to survive and grow—albeit slowly—when embedded within the BL-MeHA hydrogel. This suggests that the gel permits sufficient diffusion of nutrients and

oxygen to support basic cellular viability. Once again, no significant difference was found between the 60s and 90s groups.

The use of blue-light activation to control the setting time has proven effective, offering a viable alternative to the chemically crosslinked method previously reported by Trakiattikul *et al.,*[13] While Chaopanitcharoen *et al.,*[16] demonstrated that curing durations of 90s and 120s produced optimal physical properties, the present study investigated shorter exposure times of 60s and 90s. These reduced durations were feasible due to the smaller volume of gel used, yet still yield satisfactory outcomes. Several factors can influence the required light-curing time, including the size and thickness of the gel, the intensity and quality of the light-curing units, and the presence of any materials that may obstruct light transmission.[18] It is therefore essential to determine appropriate curing time based on the specific conditions of each application. Nevertheless, the potential cytotoxic effects of direct LED light exposure should not be underestimated. Studies have demonstrated that intense violet or blue light can trigger photoreduction of flavins within mammalian cells, activating flavin-containing oxidases in mitochondria and peroxisomes, which in turn leads to the production of hydrogen peroxide (H_2O_2).[19] Additionally, LED curing lights have been shown to inhibit the proliferation of gingival epithelial cells and periodontal ligament fibroblasts, potentially causing damage to oral soft tissues.[20,21] Therefore, it is advisable to limit light exposure to the minimum duration necessary to achieve effective curing and reduce the risk of cytotoxicity.

In this study, the porous structure of the hydrogel is not visible in the SEM images due to the drying method used. The process involved gradually increasing ethanol concentrations to replace water in the sample, followed by final drying with hexamethyldisilazane (HMDS). While this method effectively preserves the cellular morphology close to its original state, it causes the hydrogel structure to collapse during drying, resulting in a less porous appearance under the electron microscope. This differs from the approach used by Chaopanitcharoen *et al.,*[16] where the hydrogel was dried before lyophilization, allowing better preservation of its porous architecture. However, lyophilization is not suitable for the current study, as it can damage animal cells, making it inappropriate for assessing cell-hydrogel interactions.

In conclusion, the results of this study demonstrate that the BL-MeHA hydrogel is biocompatible with the L929 cell line, as confirmed through indirect cytotoxicity, 2D culture, and cell encapsulation assays. These findings highlight the potential of BL-MeHA as a promising scaffold for the delivery of cells, growth factors, biomaterials, drugs, or other bioactive agents in future tissue engineering applications.

Conflicts of Interest

The authors declare no conflicts of interest.

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In Vitro Effects of Different Probiotic Mouthwash Formulations on the Growth of Oral Pathogens

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Abstract

Objectives: To investigate the effect of various probiotic mouthwash formulations on the quantity of oral pathogenic microorganisms.

Methods: This was a laboratory-based study testing four different mouthwash formulations: (1) 10% probiotic supernatant mouthwash, (2) 10% probiotic supernatant / 5% propolis mouthwash, (3) 10% probiotic supernatant / 1% cannabidiol mouthwash, and (4) 10% probiotic supernatant / 5% propolis / 1% cannabidiol mouthwash. A positive control (chlorhexidine mouthwash) and a negative control (0.9% saline solution) were also included. The inhibitory efficacy of these formulations was tested against two microorganisms: *Candida albicans* and *Streptococcus mutans*. Quantitative data were collected by counting colonies before and after treatment (CFU/mL). Descriptive statistics and One way ANOVA test were used for analysis ($p<0.05$).

Results: The results showed that each mouthwash formulation could inhibit both pathogenic microorganisms to varying degrees, with a greater effect observed on Streptococcus mutans than on *Candida albicans*. When the quantitative data from colony counts were subjected to statistical analysis, no statistically significant differences were found among the mouthwash formulations ($p>0.05$) in inhibiting both types of microorganisms. Notably, the mouthwash containing 10% probiotic supernatant, 5% propolis and 1% cannabidiol demonstrated a statistically significant inhibitory effect on *Streptococcus mutans* ($p<0.05$).

Conclusions: All four mouthwash formulations showed a tendency to inhibit both pathogenic microorganisms, although this was not statistically significant in all cases. The formulation containing 10% probiotic supernatant, 5% propolis, and 1% cannabidiol had the most favorable effect in inhibiting the cariogenic bacterium *Streptococcus mutans.*

Keywords: mouthwash, oral bacteria, probiotic

Introduction

Microorganisms, tiny living entities, have long been recognized as a cause of various human diseases. However, it's crucial to understand that not all strains of these microorganisms are problematic and solely responsible for illness. A familiar example is the human oral cavity, which harbors approximately 700 species of microorganisms[1], encompassing both pathogenic and non-pathogenic types. The unique environmental factors within the oral cavity create a distinct microbial community that interacts with one another, collectively known as the oral microbiota.

Under normal circumstances, even in the presence of potentially pathogenic strains, the oral microbiota does not cause disease in healthy individuals. In a healthy state (eubiosis), the oral microbiome is diverse and dominated by commensal species that contribute to homeostasis, maintain biofilm stability, and suppress the overgrowth of pathogens. Pathogenic microorganisms are present only in low numbers and are kept in check by microbial competition and host immune responses. In contrast, in disease-associated states (dysbiosis), microbial diversity often decreases, with a relative increase in pathogenic or opportunistic species, accompanied by metabolic changes that favor inflammation and tissue destruction.

Multiple factors—including the integrity of oral tissues, the composition of commensal microorganisms, dietary habits, oral hygiene practices, and systemic health—influence whether the oral microbiome remains in eubiosis or shifts toward dysbiosis. When conditions favor eubiosis, disease is unlikely to occur. However, significant alterations in these factors can disrupt microbial balance, leading to the proliferation of pathogenic microorganisms. Dysbiosis can initiate disease processes such as dental caries, periodontitis, cardiovascular disease, and diabetes.[2] While antibiotics are one way to manage these issues, their use can lead to other problems, including antibiotic resistance and a lack of specificity that may eliminate beneficial microorganisms. This has spurred the development of alternative approaches to combat pathogenic microorganisms by replacing them with non-pathogenic ones, a principle known as bacteriotherapy.[3] These non-pathogenic microorganisms, when consumed in sufficient quantities, confer health benefits and are termed probiotics.[4]

Probiotics have a wide range of applications in medicine, including dentistry, due to their antimicrobial and immunomodulatory properties. In the oral cavity, they can combat pathogens by interfering with biofilm formation, a key factor in the development of diseases such as dental caries and periodontitis, through competition for adhesion sites and nutrients.[5] Beyond their antimicrobial activity, probiotics also exert anti-inflammatory effects[6], which may help reduce undesirable post-surgical complications.[7,8] A derivative product, the cell-free supernatant containing bioactive compounds secreted by probiotics, namely bacteriocins, and has demonstrated benefits in reducing inflammation after oral surgery.[8] This supernatant also exhibits antibacterial effects[9] can inhibit *Streptococcus mutans (S. mutans)* by forming pores in the cell membrane, disrupting cell wall biosynthesis, and impairing biofilm development[10,11] through reduced glucan-mediated adhesion.

Another natural extract gaining significant attention for its medicinal properties is cannabis extract, classified as cannabinoids. These compounds can stimulate the endocannabinoid system (ECS) in the human body.[12] Plant-derived cannabinoids, introduced externally, are referred to as exogenous cannabinoids and include various types such as Tetrahydrocannabinol (THC), Cannabidiol (CBD), and Cannabigerol (CBG). These substances can act on cannabinoid receptors present in the human body, including immune cells. Their effects on the body are extensive, including pain reduction, anti-emetic properties, anticonvulsant effects, and anti-inflammatory actions through mechanisms that reduce the production of various pro-inflammatory cytokines.[13] In dentistry, they have been incorporated into various forms, such as tablets, capsules, topical oils, toothpastes, sprays, mouthwashes, chewing gums, and even dental filling materials.[13-15] This highlights the broad potential for developing these extracts for dental applications. With these reasons, Nisapa[16], incorporated CBD into mouthwash formulations and study how they can enhance anti-inflammatory property of the mouthwash by comparing the mouthwash containing 10% *Lactobacillus paracasei (L.paracasei)* supernatant with and without CBD at 0.25% ,0.5% and 1%. The result shows that the mouthwash with at least 0.5% CBD can significantly further reduce TNF-α production in human monocytic cell compared to the formular without CBD and the concentration that shows the most reduction is the one with 1% CBD.

Propolis, a resinous substance produced by bees for hive construction. It contains bioactive materials, flavonoids and phenolic acids, exhibits antibacterial, antiviral, antifungal, antiparasitic, and antioxidant activities. It also suppresses inflammation by inhibiting prostaglandin synthesis. These numerous properties have led to its long-standing use in medicine.[17] In dentistry, propolis has been investigated for use on post-surgical oral wounds in the form of mouthwash. Results indicate that it promotes epithelialization and possesses analgesic and anti-inflammatory effects.[18,19] As propolis proved to be medically beneficial, it was added to mouthwash and had been studied by Suetrongtrakool[20], which the study shows that with addition of propolis to the mouthwash formulars, they can further reduce TNF-α production in human monocytic cell compared to the mouthwash formulars without propolis.

Building on the demonstrated anti-inflammatory effects of *L. paracasei* supernatant, further research has focused on developing novel mouthwash formulations. An initial clinical study successfully reduced inflammation and postoperative complications following impacted third molar extraction using *L. paracasei* supernatant alone.[8] Subsequent development aimed to enhance this anti-inflammatory potential by incorporating additional bioactive components. For example, studies by Nisapa[16] and Suetrongtrakool[20] formulated mouthwashes containing varying concentrations of CBD and propolis. Both investigations reported promising laboratory findings, showing that addition of effectively inhibited TNF-α production. Among the tested combinations, the most promising was the formulation containing 10% *L. paracasei* supernatant, 1% CBD and 5% propolis.

However, beyond their anti-inflammatory properties, these developed mouthwash formulations likely possess another unexamined benefit, their ability to combat oral microorganisms. This is because all the components of the studied mouthwashes have demonstrated antibacterial, and potentially antifungal activity. If further research confirms their ability to reduce the number of oral pathogens, it will signify that these new mouthwash formulations possess both anti-inflammatory and antibacterial properties. This would make them an attractive alternative mouthwash for practical use in alleviating disease and improving patients' quality of life. The objective of this research was to investigate the effect of various probiotic mouthwash formulations on the quantity of oral pathogenic microorganisms.

Materials and Methods

This study was a laboratory-based investigation testing four mouthwash formulations. These formulations were referenced from previous research by our group, which demonstrated their efficacy in both laboratory and clinical settings. A key component of these formulations is 10% *L. paracasei* probiotic supernatant.

Independent variables

The independent variables in this study were the different types of mouthwash formulations, which included:

* Negative Control: 0.9% Sodium Chloride solution

* Probiotic Supernatant Mouthwash (10% *L. paracasei* supernatants)

* Probiotic Supernatant Mouthwash with Propolis (10% *L. paracasei* supernatants + 5% propolis)

* Probiotic Supernatant Mouthwash with CBD (10% *L. paracasei* supernatants + 1% cannabidiol)

* Probiotic Supernatant Mouthwash with CBD and Propolis (10% *L. paracasei* supernatants + 1% CBD + 5% propolis)

Dependent variable

The dependent variable was the colony count of oral pathogens observed after culturing with the various mouthwash formulations.

Population and sample

The microorganisms used in this experiment were *S. mutans* and *Candida albicans*. These two species were selected based on a literature review which indicates that *S. mutans* can produce insoluble polymers called glucans, a crucial substance for biofilm adherence to tooth surfaces. Thus, it plays a significant role in biofilm formation, which acts as a reservoir for pathogens and can lead to various oral diseases.[21] Similarly, *Candida albicans* is a commensal organism that, while normally non-pathogenic, contributes to biofilm formation in conjunction with other bacteria, leading to dysbiosis and various diseases.[22,23] Therefore, in this study, both species were used as representatives of an early colonizing bacterium and an oppor-

tunistic oral fungus, respectively, to evaluate the antimicrobial efficacy of the mouthwash formulations.

Research procedures

The research process was divided into four main parts:

Part I: Microbial isolation and standard growth curve determination

Candida albicans were obtained from Department of Microbiology, Faculty of Medicine, Srinakharinwirot University and *S. mutans* were obtained from Department of Stomatology, Faculty of Dentistry, Srinakharinwirot University. Both microbial species were isolated, and their standard growth curves were established to standardize microbial concentrations prior to testing by adjusting their optical density (OD) using a spectrophotometer. Initially, both strains were stored at -80°C and cultured on solid media: *S. mutans* was grown on BHI agar at 37°C in 5% CO_2 for 24 hours, while *Candida albicans* was cultured on SDA agar at 28°C for the same duration. Individual colonies from these solid media were then inoculated into liquid media, with *S. mutans* being transferred into BHI broth and *Candida albicans* into SDB. The optical density of the cultures was measured at a wavelength of 600 nm and recorded hourly over a 12-hour period. These OD readings over time were used to construct standard growth curves (OD vs. time), which were subsequently analyzed to determine the mid-logarithmic growth phase for use in downstream experiments.

Part II: Microbial exposure and viability assessment

Colonies from the initial solid media were inoculated into 50 mL Erlenmeyer flasks containing appropriate liquid media and cultured until *S. mutans* and *Candida albicans* reached their mid-logarithmic phase, as determined by previously established standard growth curves. The cultures were adjusted using a spectrophotometer at 600 nm to match McFarland standard 0.5 for *S. mutans* ($OD_{600} \approx 0.07$; ~1.5×10^8 CFU/mL) and McFarland standard 2.0 for *Candida albicans* ($OD_{600} \approx 0.5$; ~2×10^7 CFU/mL). For the direct contact test, 500 µL of each mouthwash formulation (Table 1), along with positive and negative controls, was transferred into 1 mL centrifuge tubes, followed by the addition of 500 µL of the adjusted microbial suspensions. This 1:1 mixing resulted in a final mouthwash concentration of 50% of the original formulation. The mixtures were vortexed thoroughly and incubated at 37°C for 1 minute to facilitate interaction. Following incubation, the mixtures were subjected to six 10-fold serial dilutions, reaching up to 10^{-6}. Aliquots of 100 µL from the 10^{-3}, 10^{-4}, and 10^{-5} dilutions for *Candida albicans* and from the 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions for *S. mutans* were spread onto solid culture media in duplicate and incubated for 24 hours. Colony counting was then performed to determine microbial viability under each condition.

Part III: Time-Dependent Microbial Exposure and Viability Assessment

This experiment further evaluated the antimicrobial effect of the selected mouthwash formulation containing 10% probiotic supernatant, 5% propolis, and 1% CBD against the pathogens that showed susceptibility in Part II. Microbial suspensions were prepared and adjusted to the same optical densities as described previously. 500 µL of mouthwash was mixed with 500 µL of microbial suspension in centrifuge tubes and incubated at 37°C for four exposure times: 0, 1, 2, and 3 minutes. Three replicate tubes per time point were prepared, and after each exposure, samples were serially diluted (up to 10^{-7}), plated in duplicate, and incubated for 24 hours. Colony-forming units were counted to assess microbial viability over time

Data collection and statistical analysis

Colony counting was performed, and the number of colonies in each sample was recorded. Counts with-

Table 1: Shows ingredients and volume of each mouthwash formular.

Ingredients	Volume (mL)				Function
	S	SC	SP	SCP	
0.9% Sodium Saccharin	0.33	0.33	0.33	0.33	Taste modifier
Lactobacillus paracasei supernatant	1	1	1	1	Active ingredient
Propolis	-	-	0.5	0.5	Active ingredient
Cannabidiol	-	0.1	-	0.1	Active ingredient
0.9% Normal saline solution	8.67	8.17	8.57	8.07	Solvent

in the readable range (30–300 colonies) were selected from all sets of duplicate plates prepared simultaneously. These values were compiled and averaged to represent the microbial load for each condition. Once all relevant data had been collected, statistical analysis was performed as described below:

Two preliminary assumptions for statistical analysis were tested:

* Normality of distribution using the Kolmogorov-Smirnov test.

* Homogeneity of variances using Levene's test.

If any of these tests yielded a *p*-value lower than the significance level (0.05), it indicated a violation of the assumption, necessitating the use of non-parametric statistics, specifically the Kruskal-Wallis test. However, if both tests yielded *p*-values higher than 0.05, then One-way ANOVA could be used. Statistical analysis was performed using GraphPad Prism version 10.4.2 (633)

Results

Based on experiments to establish the standard growth curves for both microbial species (Figure 1), *S. mutans* and *Candida albicans* reached their standard growth phases at 6 and 7 hours, respectively. This indicates that *Candida albicans* exhibits a slower growth rate compared to *S. mutans* bacteria.

The second part of the direct contact test involved evaluating the four mouthwash formulations against both microbial species, along with positive and negative control groups. Experiments were performed duplicate, and mouthwash concentrations were diluted 10-fold differently in each experimental set, as depicted in the laboratory testing framework image (Figure 2 and Figure 3).

The mouthwashes were mixed according to formular shown in Table 1. Then, pH of each formular were measured and recorded as shown in Table 2

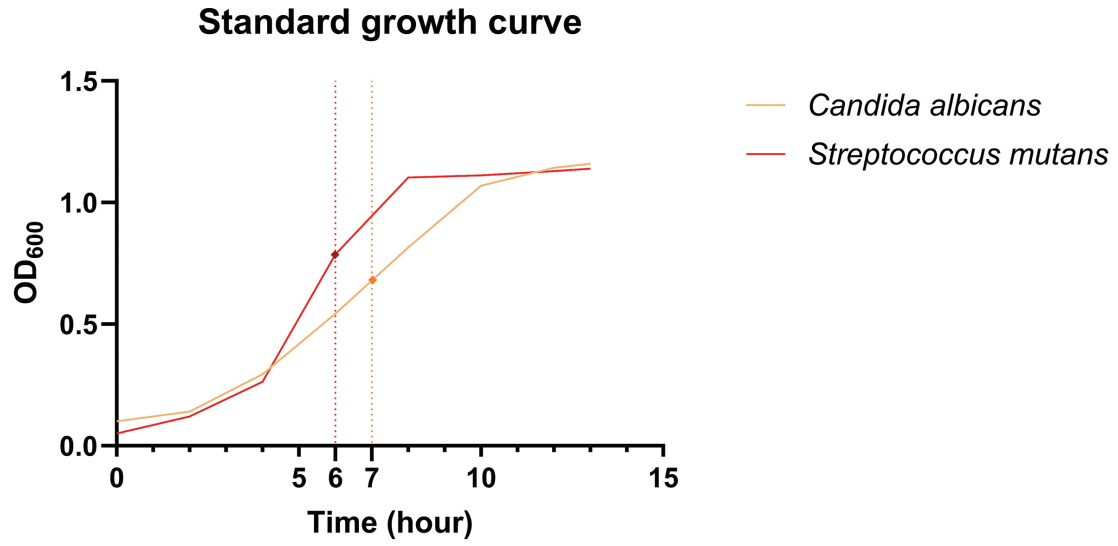

Figure 1: Illustrates the standard growth curves of these two oral pathogens.

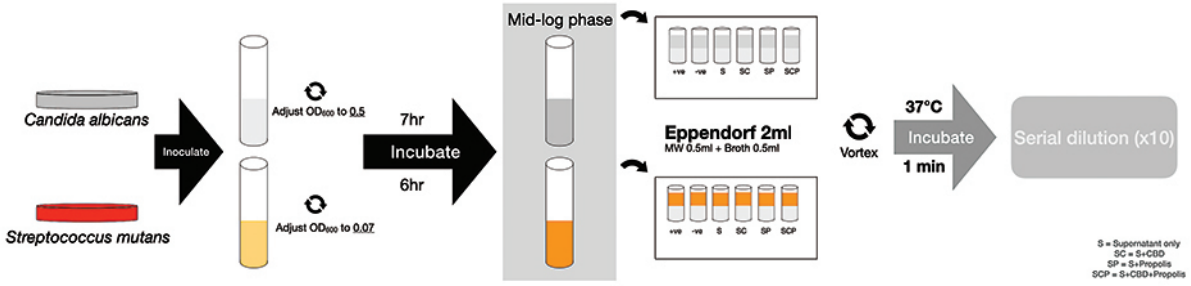

Figure 2: Shows laboratory investigation with direct contact technique.

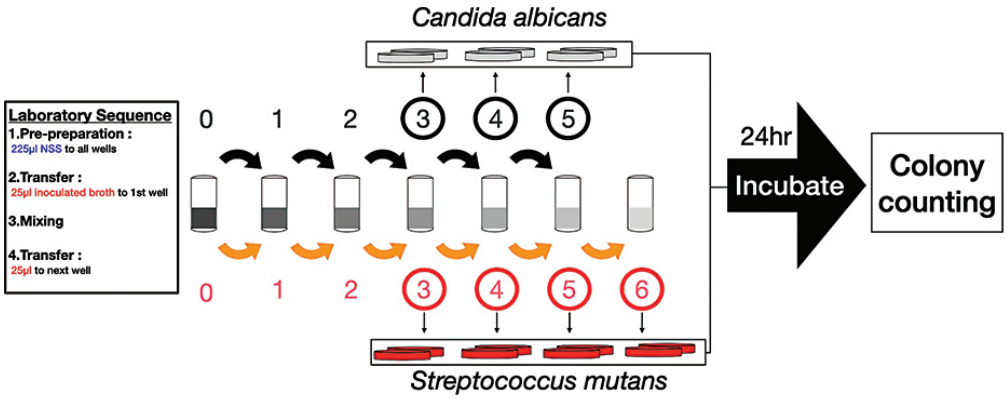

Figure 3: Shows laboratory investigation serial dilution (x10).

Table 2: Shows pH of each mouthwash formular.

Formular	pH
S	4.28
SC	4.33
SP	4.36
SCP	4.31

Table 3: Shows the inhibitory effect of different mouthwash formulas against both pathogens.

Formular	Mean colony (CFU/mL)	
	Candida albicans	Streptococcus mutans
Positive control	0	0
Negative control	1.33×10^7	2.57×10^8
S	1.24×10^7	3.61×10^8
SC	1.40×10^7	3.51×10^8
SP	1.27×10^7	3.14×10^8
SCP	9.30×10^6	1.95×10^8

*Positive control: 0.12% Chlorhexidine mouthwash, Negative control: 0.9% Normal saline solution, S: *Lactobacillus paracasei* supernatant only, SC: *Lactobacillus paracasei* supernatant + cannabidiol, SP: *Lactobacillus paracasei* supernatant + propolis, SCP: *Lactobacillus paracasei* supernatant + cannabidiol + propolis

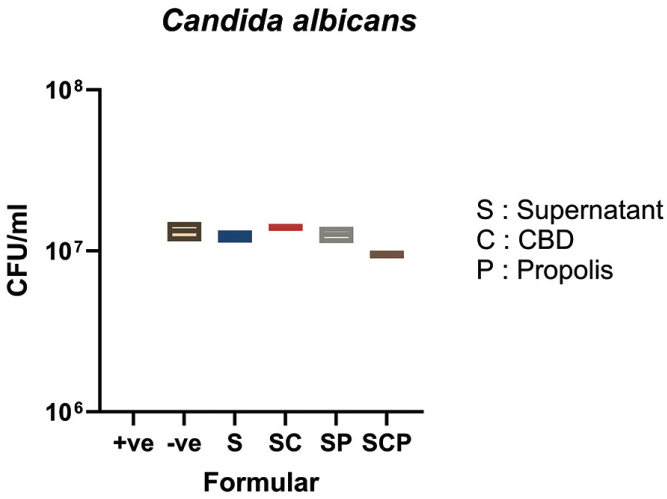

Figure 4: Shows illustration of the test results of different mouthwash formulas on the inhibition of *Candida albicans.*

The results from the direct contact test demonstrated varying inhibitory effects of each mouthwash formulation on the two tested pathogens. These findings are presented in Table 1, which shows the inhibition values of different mouthwash formulations on both pathogenic species.

When the obtained values were subjected to statistical analysis, based on the established hypotheses (H_0: The number of microbes cultured with various mouthwash formulations does not differ from the negative control; H_1: The number of microbes cultured with various mouthwash formulations differs from the negative control).

As for *Candida albicans* (Table 3, Figure 4), despite results of Kruskal-Wallis analysis yield $p<0.05$, no statistically significant differences were found in Dunn's multiple comparison ($p>0.05$). (Table 4 and Table 5)

As for *S. mutans* (Table 3, Figure 5), despite Kruskal-Wallis analysis yield $p<0.05$, statistically significant differences were found only in comparison between positive control to mouthwash containing supernatant with cannabidiol and mouthwash containing supernatant and propolis. (Table 6 and Table 7)

The results indicated that the mouthwash formulations exhibited an inhibitory effect against *S. mutans*; therefore, this pathogen was selected for time-dependent exposure analysis

The final part of the study involved evaluating the mouthwash formulation containing 10% probiotic supernatant, 5% propolis, and 1% CBD against *S. mutans* at various time points. Laboratory testing framework are illustrated in Figures 6 and 7, and the results are shown in Table 8 and Figure 8.

When the obtained values were subjected to statistical analysis using one-way ANOVA, no statistically significant differences were found ($p>0.05$). (Table 9)

Table 4: Shows result of statistical analysis with Kruskal-Wallis test of *Candida albicans.*

Kruskal-Wallis test	
p value	0.0209
Exact or approximate *p* value?	Approximate
p value summary	*
Do the medians vary sig. ($p<0.05$)?	Yes
Number of groups	6
Kruskal-Wallis statistic	13.27

Table 5: Show Dunn's multiple comparison of *Candida albicans.*

Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted *p* value**
+ve vs. -ve	-11.00	No	ns	0.1073
+ve vs. S	-9.000	No	ns	0.4164
+ve vs. SC	-11.00	No	ns	0.1073
+ve vs. SP	-9.000	No	ns	0.4164
+ve vs. SCP	-4.000	No	ns	>0.9999
-ve vs. S	2.000	No	ns	>0.9999
-ve vs. SC	0.000	No	ns	>0.9999
-ve vs. SP	2.000	No	ns	>0.9999
-ve vs. SCP	7.000	No	ns	>0.9999
S vs. SC	-2.000	No	ns	>0.9999
S vs. SP	0.000	No	ns	>0.9999
S vs. SCP	5.000	No	ns	>0.9999
SC vs. SP	2.000	No	ns	>0.9999
SC vs. SCP	7.000	No	ns	>0.9999
SP vs. SCP	5.000	No	ns	>0.9999

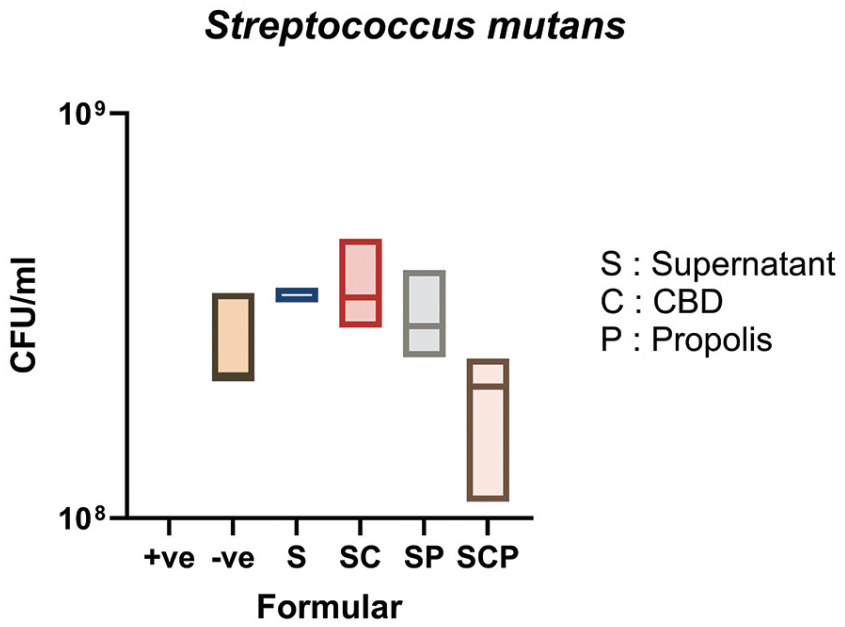

Figure 5: Shows illustration of the test results of different mouthwash formulas on the inhibition of *Streptococcus mutans*.

Table 6: Shows result of statistical analysis with Kruskal-Wallis test of *Streptococcus mutans*.

Kruskal-Wallis test	
p value	0.0030
Exact or approximate *p* value?	Approximate
p value summary	*
Do the medians vary sig. ($p<0.05$)?	Yes
Number of groups	6
Kruskal-Wallis statistic	17.93

Table 7: Shows Dunn's multiple comparisons test of *Streptococcus mutans*.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted *p* value
+ve vs. -ve	-9.125	No	ns	>0.9999
+ve vs. S	-17.63	Yes	**	0.0062
+ve vs. SC	-14.75	Yes	*	0.0466
+ve vs. SP	-13.75	No	ns	0.0876
+ve vs. SCP	-4.750	No	ns	>0.9999
-ve vs. S	-8.500	No	ns	>0.9999
-ve vs. SC	-5.625	No	ns	>0.9999
-ve vs. SP	-4.625	No	ns	>0.9999
-ve vs. SCP	4.375	No	ns	>0.9999
S vs. SC	2.875	No	ns	>0.9999
S vs. SP	3.875	No	ns	>0.9999
S vs. SCP	12.88	No	ns	0.1477
SC vs. SP	1.000	No	ns	>0.9999
SC vs. SCP	10.00	No	ns	0.6748
SP vs. SCP	9.000	No	ns	>0.9999

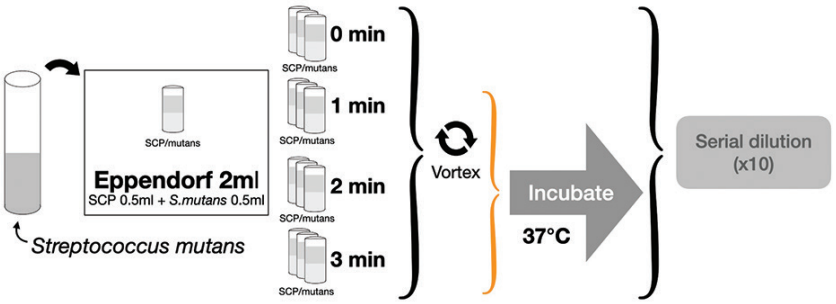

Figure 6: Shows laboratory sequences of various time-point tests.

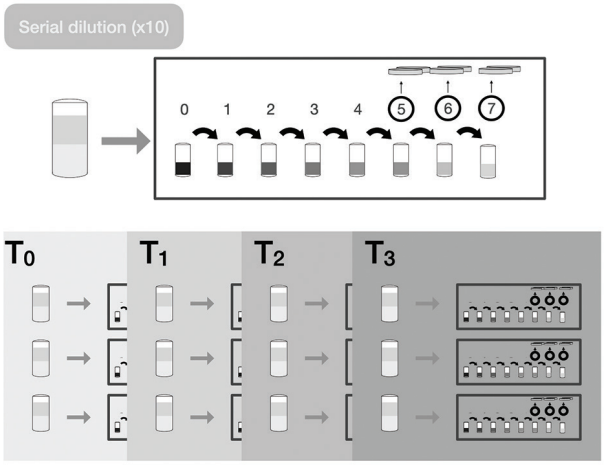

Figure 7: Shows laboratory sequences of serial dilution (x10) after various time-point tests.

Table 8: Shows the test results of different time of contact of mouthwash containing 10% probiotic supernatant, 5% propolis and 1% cannabidiol on the inhibition of *Streptococcus mutans.*

Time (minute)	Mean *Streptococcus mutans* (CFU/ml)
0	3.49×10^8
1	3.70×10^8
2	4.15×10^8
3	3.48×10^8

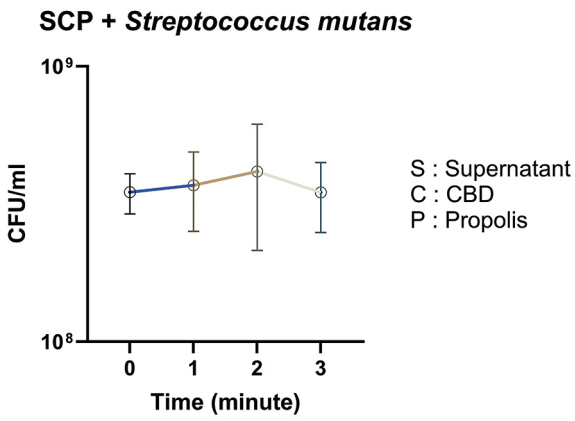

Figure 8: Show a declining trend of bacterial count after exposure to the mouthwash in longer time.

Table 9: Show results of statistical analysis with one-way ANOVA test of *Streptococcus mutans* in various time-point experiments.

ANOVA summary	
F	0.3483
p value	0.7907
p value summary	ns
Significant diff. among means ($p<0.05$)?	No
R squared	0.04965

Discussion

This study was a laboratory-based investigation designed to address a research gap identified by our research group, which aims to extend the findings from the Center of Excellence in Medical Probiotics. Previous research, both *in vitro* and clinical, has demonstrated the anti-inflammatory benefits of *L. paracasei* MSMC39-1 probiotic supernatant.[7,8] Building upon previously established beneficial proportions, this study aimed to explore additional advantages beyond inflammation reduction, specifically focusing on the potential interference or inhibition of oral pathogenic microorganisms. Two common oral pathogens were selected for testing: *S. mutans,* a bacterium associated with dental caries, and *Candida albicans*, a fungal species.

Several interesting points emerged from the experimental results. Firstly, a clear trend and distinctions were observed when analyzing the raw data. For instance, the probiotic supernatant mouthwash at 10%, cannabidiol at 1%, and propolis at 5% formulations showed the greatest reduction in microbial load among the four tested formulations. Furthermore, the positive control group exhibited no growth of either microbial species. However, when the same data set was subjected to statistical analysis, the tests show non-normal distribution of data for both pathogens, so non-parametric statistics were selected. Even Kruskal Wallis test yield significant differences in either pathogen (*Candida albicans p*-value:0.0209, *S. mutans p*-value:0.0030) but when the data were subjected Dunn's to multiple comparison, *Candida albicans* shows no pair of significant difference while *S. mutans* shows two pair of significant difference, which are positive control to mouthwash with only probiotic supernatant and positive control to mouthwash containing supernatant and CBD. This discrepancy may be attributed to statistical reason, an insufficient sample size, precluding robust statistical conclusions. Therefore, increasing the sample size in future experiments could potentially normalize data distribution, allowing for the application of more reliable parametric statistical methods. Another possible reason is short duration of the direct contact test, 1 minute, which may be too short for any mechanism to occur biologically. This duration was chosen based on manufacturer recommendations for mouthwash rinsing, typically ranging from 30 seconds to 1 minute. However, in real-world practice, patients often rinse for less time than recommended.[24] Therefore, even if longer exposures show significant pathogen reduction, these findings may have limited practical relevance if the mouthwash is not retained in the oral cavity long enough. Nonetheless, most commercial mouthwashes are marketed as supplemental cleansing products rather than long-term therapeutic agents, unlike the mouthwash formulated in this study. In clinical use, detailed instructions from dentists could help ensure adequate rinsing time to maximize efficacy.

Another crucial aspect involves the numerous factors influencing pathogen inhibition experiments, such as the inherent characteristics of the microorganisms, the mechanisms of killing/inhibition, the environmental conditions conducive to microbial growth, the duration of exposure to the mouthwash, and the concentration of the different mouthwash formulations. Nevertheless, the experimental design for this study was based on previous research where these formulations were developed and shown to reduce oral inflammation. This reliance on prior formulations might be a limitation, as it does not encompass scenarios where increasing the concentration of certain or all active ingredients in the mouthwash could alter their antimicrobial efficacy, especially at this short duration of time. This observation also applies to the experimental duration, which might have been either sufficient or insufficient for the active ingredients in the mouthwash to exert their full effects on the pathogens. Consequently, a subsequent experiment was designed to test the most

promising formulations over three intervals of time to investigate the trend of microbial reduction with increased exposure time. Although the results of this extended testing do not show district declining trend, the growth of the mouthwash-treated bacteria is diminished compared to the growth of bacteria in mid-logarithm phase which is shown in first part of the studied. These results were subjected to one-way ANOVA. The analysis yield *p*-value of 0.3483, hence no significant differences were found.

An additional relevant factor is the pH of the various mouthwash formulations. All our formulations contain Lactobacillus probiotic supernatant, which retains some acidity due to acid production by Lactobacillus bacteria. Therefore, pH measurements were conducted to clarify any relationship between pH and antimicrobial/antifungal properties. The results shown in Table 2 indicate that the pH values of all formulations were very similar, ranging narrowly from 4.28 to 4.36. This suggests an insignificant role of pH in the observed antimicrobial and antifungal effects. Our findings closely resemble those of Rossoni[25], who evaluated the influence of acids produced by Lactobacillus strains on *S. mutans* and found that all strains exhibited similar acidogenic activity, with no significant pH difference compared to the control, suggesting that pH change may have no effect. Conversely, Lin[26], found that adjusting the pH of Lactobacillus supernatant removed the inhibitory effect of some strains on *S. mutans*, suggesting that growth and biofilm formation are likely inhibited by low pH. Importantly, with the addition of key components such as CBD and propolis, a reduction in colony counts was observed. This indicates that other substances or mechanisms are involved in creating an environment less conducive to microbial growth.[12,13,16,18]

Our mouthwash has a pH below 4.5, which is lower than the critical pH for enamel demineralization; however, this value is comparable to that of several commercially available mouthwashes. In a survey of 47 products, B.W.M. van Swaaij *et al.,*[27] reported that 20 mouthwashes (43%) had pH values below 5.5, including 10 that contained fluoride. Some formulations may be intentionally acidic, as a mildly low pH can facilitate the conversion of hydroxyapatite (HA) to the more acid-resistant fluorapatite (FA) in the presence of fluoride. In another word, our mouthwash might be beneficial to the FA conversion process if used in presence of fluoride which is typically abundantly retained in oral cavity after brushing with fluoride-containing toothpaste.

Regarding the proposed mouthwash which shows best performance, the one with all the ingredients, the result is reasonable and predictable as the formular contains most active ingredients, *L. paracasei* supernatant, CBD and propolis. Although the study cannot reveal what mechanism behind the result or whenever the effect observed is increment of three substances acting independently or synergically, the result shows that they work best together. According to prior studies, *L. paracasei* cell-free supernatant (CFS) contains various bioactive compounds, including bacteriocins, organic acids, and enzymes, which exert antimicrobial and anti-inflammatory effects. The CFS has been sh own to inhibit the growth and biofilm formation of oral pathogens such as *S. mutans* by disrupting cell membranes and interfering with adhesion mechanisms.[25,26] When combined with propolis and cannabidiol, which also target microbial membranes and modulate host inflammation[12,13,18,19], the supernatant may enhance the overall antimicrobial efficacy of the formulation. This multi-targeted approach could provide broader-spectrum pathogen suppression while reducing potential cytotoxicity to beneficial oral microbiota. The complementary actions of these agents suggest a potentially synergistic effect that warrants further exploration in oral health applications

Despite the lack of statistically significant differences, the raw data clearly indicate a trend of reduced microbial load after a single exposure to mouthwash. This information is highly valuable for the development of mouthwash formulations, as it suggests the mouthwash possesses antimicrobial properties. Continued use could potentially reduce the oral microbial load, thereby contributing to the prevention of pathogen-induced diseases. Furthermore, unlike the positive control (0.12% chlorhexidine mouthwash, broad-spectrum antiseptic agent), these novel mouthwash formulations do not eliminate all microorganisms. Complete eradication, if used long-term in real-world scenarios, could disrupt the delicate balance of oral microbiota. Therefore, these new mouthwash formulations may offer a superior long-term option for reducing oral inflammation without disturbing the oral microbial balance.

Conclusions

The four mouthwash formulations demonstrated a trend towards inhibiting both tested pathogenic microorganisms, although this trend was not statistically significant. The formulation containing 10% probiotic supernatant, 5% propolis, and 1% CBD showed the most promising results in inhibiting the cariogenic bacterium *S. mutans*.

Conflicts of Interest

The authors declare no conflicts of interest.

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Shear Bond Strength of Composite Resin Attachments in Clear Aligner Orthodontic Appliances with Different Adhesive Systems

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Abstract

Objectives: Orthodontic treatments with clear aligners are popular. Loss of composite resin attachments during treatment remains a concern. Self-adhesive composite resins were introduced to simplify bonding. This study compared the shear bond strength (SBS) and evaluated the failure modes of self-adhesive and conventional composite resin.

Methods: Eighty-four intact upper first premolars were used, 80 were randomly allocated into five groups with different bonding protocols: Group 1 (etch and rinse + conventional flowable composite resin), Group 2 (self-etching + conventional flowable composite resin), Group 3 (self-adhesive composite resin), Group 4 (etching + self-adhesive composite resin), and Group 5 (self-etching + self-adhesive composite resin). After thermocycling, the SBS was tested using a universal testing machine and analyzed with one-way ANOVA ($p<0.05$). Failure modes were determined under a stereomicroscope. The enamel surface of four unallocated teeth with different preparations were assessed by a scanning electron microscope.

Results: 62.5% of attachments in Group 3 dislodged after thermocycling. The mean SBS (MPa) was significantly higher in Group 1 (17.72±5.37), Group 2 (19.13±5.37), Group 4 (19.03±6.91), and Group 5 (13.21±4.87) than in Group 3 (3.69±1.30); ($p<0.05$). Most failures in Groups 1, 2, 4, and 5 were mixed, while Group 3 exhibited only adhesive failure.

Conclusions: Self-adhesive composite resin alone had the lowest SBS. Pretreatment with 37% phosphoric acid or a self-etching primer significantly improved the SBS.

Keywords: attachment of clear aligner, intact enamel surface, self-adhesive composite resin, shear bond strength

Introduction

Today, orthodontic patients place greater emphasis on aesthetics during treatment, which has led to the widespread use of clear thermoplastic materials for orthodontic aligners.[1] Additional components, such as attachments, are incorporated into these clear aligners to achieve effective tooth movement and address complex malocclusions.[2] These attachments are essential not only for guiding proper tooth movement but also for improving appliance retention.[3]

Composite resin is widely utilized for orthodontic attachments owing to its aesthetic compatibility, ease of manipulation in clinical settings, and ability to establish micromechanical retention with etched enamel surfaces.[4] Moreover, the mechanical properties of composite resins specifically hardness and bond strength are frequently considered integral factors in their selection and performance as orthodontic attachment materials.[4,5] Attachment loss may lead to substantial clinical challenges, including prolonged treatment duration, an increased frequency of rebonding appointments, and diminished efficacy of tooth movement.[3,5-7] In orthodontics, bond strength must be adequate to prevent bond failure during treatment while not being strong enough to damage the tooth surface during debonding.[8] Clear aligners exhibit a retention force as high as approximately 49.3 newtons (N).[9] A shear bond strength (SBS) of 5.9-7.8 MPa is recommended for clinically acceptable direct orthodontic bonding systems to withstand masticatory forces effectively.[10]

The etch and rinse method is considered the most reliable for enamel bonding. However, the rinsing and drying process can introduce complexity and impact technique sensitivity. Recent advances in dental materials aim to simplify bonding procedures, reduce technique sensitivity, and shorten chair-side time.[11] These innovations include two-step etch and rinse, two-step self-etch, and one-step self-etch methods.[11] In addition, self-adhesive composite resins that do not require rinsing have also been introduced. These materials are commonly used for small Class I and II cavity restorations and porcelain repairs. Self-adhesive composite resins are also used for bracket bonding.[12]

Given the restricted data on the bonding of self-adhesive composite resin as an attachment of clear aligner orthodontic appliances, this study aimed to (i) compare the SBS and (ii) evaluate the mode of failure of self-adhesive composite resin and conventional composite resin with different adhesive protocols for fabricating the attachments of clear aligner orthodontic appliances.

Materials and Methods

This study received ethical approval from the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand (No. 37/2022).

Teeth preparation

The inclusion criteria for the teeth were as follows: intact upper first premolars without restorations on the buccal surface, without enamel abnormalities, and recently extracted as part of orthodontic treatment. After extraction, the teeth were rinsed with water to eliminate any residual connective tissue and subsequently stored in a 10% formalin solution, which is recommended for tooth preservation and does not adversely affect shear bond strength.[13,14] The sample size was determined from an earlier study.[15] The total calculated sample size from n4 Studies application was 10 for each group. However, this study involved 84 intact upper first premolars. Then, 80 intact upper first premolars were randomly selected and assigned to five groups based on the adhesive system used (16 per group):

Group 1: Etch and rinse+conventional flowable composite resin

Adper™ Scotchbond Multi-Purpose etchant+ Adper™ Scotchbond Multi-Purpose primer+Adper™ Scotchbond Multi-Purpose adhesive+Filtek™ Z350 XT Flowable Composite

Group 2: Self-etching+conventional flowable composite resin

Transbond™ Plus Self Etching+Adper™ Scotchbond Multi-Purpose adhesive+Filtek™ Z350 XT Flowable Composite

Group 3: Self-adhesive composite resin

Vertise® Flow™ Resin

Group 4: Phosphoric acid etching+self-adhesive composite resin

Adper™ Scotchbond Multi-Purpose etchant+ Vertise® Flow™ Resin

Group 5: Self-etching+self-adhesive composite resin

Transbond™ Plus Self Etching + Vertise® Flow™ Resin

The teeth in each group were embedded in plaster of Paris blocks, with four per block. Next, the models were scanned using an intraoral scanner (iTero Element™ 2 imaging system; Align Technology, Tempe, AZ, USA) to create 3D models, which were exported as Standard Triangle Language (STL) files. Then, a rectangular 2.5×3.0×2.0 mm box was designed using Blender 3.1 software (Blender, Amsterdam, Netherlands) to serve as the shape of the attachment. This attachment was then incorporated into the 3D model and printed as a resin model using a 3D printer (Form 2 3D Printer; Formlabs, Somerville, MA, USA). Then, the resin model was used to fabricate individual templates. Next, the teeth were cleaned using pumice for 10 seconds with a rubber cup, followed by thorough rinsing with water and air drying. Finally, the adhesive systems and composite resins were applied as described in Table 1.

After preparation, all samples were aged using a thermal cycling machine (Model TC 301 with cold and hot water baths, models CWB332R and HWB332R, Medical and Environment Equipment Research Laboratory, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand) for 1,000 cycles between 5°C and 55°C with a dipping time of 20 seconds and a transfer time of 10 seconds.

Shear bond strength test

All samples were sectioned 2 mm apical to the cementoenamel junction using a diamond bur. Then, they were embedded in PVC molds (20 mm in diameter, 15 mm in height) with plaster of Paris, leaving only the buccal surface exposed (Figure 1A). The occlusal and gingival surfaces of the attachment were oriented perpendicular to the horizontal plane of the mold. The SBS test was performed using a universal testing machine (UTM) (Instron 5566; Instron Calibration Laboratory, Canton, MA, USA) at a crosshead speed of 1 mm/min. A load was applied to the occlusal surface of the attachment in the occluso-gingival direction using a 2 mm-wide metal band until bond failure occurred (Figure 1B). The SBS was recorded in Newtons (N), and the SBS was calculated using the formula SBS (MPa) = Force (N) / A (mm^2), where A is the actual enamel surface area located beneath the attachment base, which was calculated for every samples using the Materialise 3-matic Research software (version 13.0; Materialise, Leuven, Belgium). The details and reference landmarks about finding the enamel surface areas were demonstrated in Figure 2A-D.

Evaluation of the mode of failure

All samples were examined under a stereomicroscope (model CK 40 culture microscope and DP 12 digital camera, Olympus, Japan) at 10× and 20× magnification to determine the adhesive remnant index (ARI) after bond failure. The ARI evaluation was repeated after four weeks to assess intra-rater reliability. The criteria used to evaluate the ARI were adapted from Årtun and Bergland.[16] The ARI was classified into the following categories: 1)

Table 1: Materials in this study and their application.

Product	Manufacturer (Lot, Exp. date)	Application
1.Adper™ Scotchbond Multi-Purpose etchant	3M ESPE, St. Paul, MN, USA (Lot: 7574156, 2024-01-26)	Etch for 15 seconds on enamel, rinse for 15 seconds, remove excess water with an air syringe
2.Adper™ Scotchbond Multi-Purpose primer	3M ESPE, St. Paul, MN, USA (Lot: NC73875,2023-07-21)	Apply to enamel and dry gently for 5 seconds
3.Adper™ Scotchbond Multi-Purpose adhesive	3M ESPE, St. Paul, MN, USA (Lot: NE18400, 2024-01-22)	Apply to enamel, light cure for 10 seconds
4.Transbond™ Plus Self Etching	3M ESPE Monrovia, CA, USA) (Lot: 831721, 2023-07-03)	Apply liquid onto enamel while applying some pressure for a minimum of 5 seconds per tooth, gentle air burst for 2 seconds to each tooth to dry primer into a thin film.
5.Vertise® Flow™ Resin	Kerr, Orange, CA, USA (Lot:10136401, 2025-06-09)	Apply a 0.5 mm thin layer to the center of the tooth, then fill the template with Vertise® Flow™ Resin and place it on the corresponding tooth, light cure for 20 seconds
6.Filtek™ Z350XT Flowable Composite	3M ESPE Monrovia, CA, USA) (Lot:10100202, 2025-04-23)	Fill the template with Filtek™ Z350XT and place it on the corresponding tooth, light cure for 20 seconds

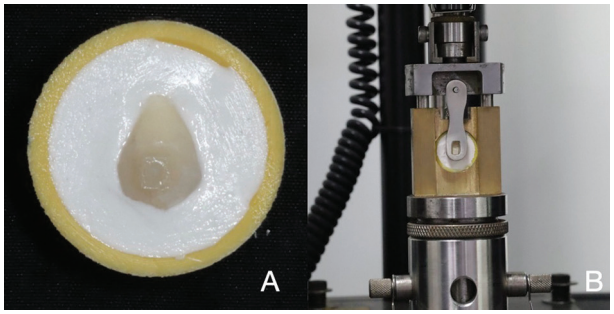

Figure 1: (A) The teeth with attachment was embedded in a mold; (B) The sample was assembled on the universal testing machine with a pull force in occluso-gingival direction and the shear bond strength was recorded.

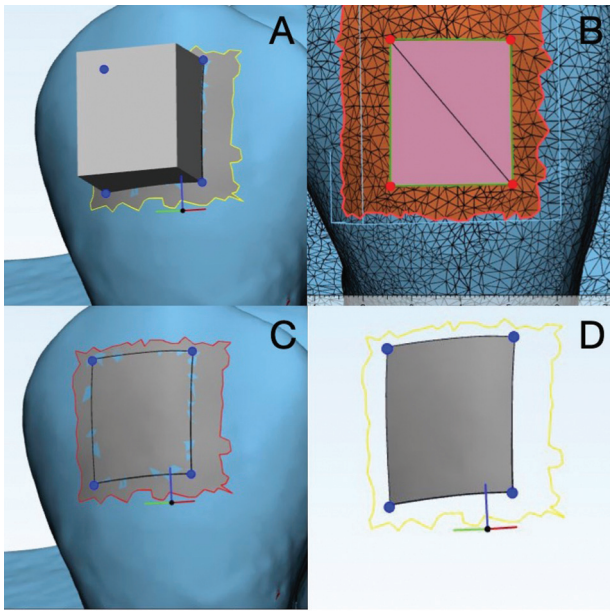

Figure 2: (A) The attachment on intact enamel surface. Four points at the extreme corners of the attachment were located under maximal magnification; (B) Top-view quadrilateral defined by connecting each corner points; (C) The attachment was hidden to reveal the underlying enamel surface bounded by the quadrilateral; (D) Final trimmed surface of bonding under the attachment whose area was measured.

cohesive failure in enamel, 2) interfacial failure between the composite resin and enamel, 3) mixed failure with less than 50% of the bonding area covered by composite resin, 4) mixed failure with at least 50% of the bonding area covered by composite resin, and 5) cohesive failure in the composite resin.

Scanning electron microscope (SEM) analysis

Four teeth were randomly selected to assess the intact enamel surface and enamel surface after preparation with Adper™ Scotchbond Multi-Purpose Etchant, Transbond™ Plus Self Etching, and Vertise® Flow™ Resin. They underwent ultrasonic cleaning, then dehydrated with an ascending ethanol concentration. After that, they were sputter-coated with a thin layer of gold using a sputter coater(model 108; Cressington Scientific Instruments Ltd., Watford, United Kingdom). The surface analysis was performed using SEM (fourth generation VEGA; TESCAN, Brno, Czech Republic) on cross-sectional views in the buccolingual direction at 9000× magnification.

Statistical analysis

The SBS data were presented as mean±standard deviation and analyzed using SPSS Statistics software (version 29.0.2.0[20]; IBM, Armonk, NY, USA). The normality of the data distribution was assessed using the Shapiro-Wilk test. The effect of different attachment bonding protocols on the SBS was evaluated using a one-way analysis of variance (ANOVA) followed by post hoc Dunnett's T3 tests when a significant difference among groups was detected ($p<0.05$). The ARI data were presented as percentages and frequency and analyzed using descriptive statistics.

Results

After thermocycling, most (62.50%) of the attachments in Group 3 dislodged, resulting in only six samples from Group 3 being included in the statistical analysis. The means and standard deviations of the SBS in the five groups are presented in Table 2 and Figure 3. The mean SBS was significantly higher in Groups 1, 2, 4, and 5 than in Group 3 ($p<0.05$). In addition, the mean SBS was significantly higher in Group 2 than in Group 5 ($p<0.05$). However, the mean SBS did not differ significantly between Groups 1, 2, and 4 ($p>0.05$) or Groups 1, 4, and 5 ($p>0.05$).

The ARI evaluation demonstrated good to excellent consistency within each group, with intraclass correlation coefficients (ICC) ranging from >0.75 to >0.90. The remaining composite resins after bond failure are presented as percentages and frequencies in Table 3. Most samples within Groups 1, 2, and 5 exhibited mixed failure with <50% of bonding areas covered by composite resin (score=3) and mixed failure with ≥50% of bonding areas covered by composite resin (score=4). All samples in Group 3 exhibited interfacial failure between the composite resin and enamel (score=2). Most samples in Group 4

exhibited mixed failure with ≥50% of bonding areas covered by composite resin (score=4) and cohesive failure in the composite resin (score=5).

SEM analysis found a demineralization zone using Adper™ Scotchbond Multi-Purpose etchant with a depth of 12.75 µm, Transbond™ Plus Self-Etching Primer with a depth of 13.21 µm, and Vertise® Flow Resin with a depth of 4.04 µm (Figures 4A-D).

Table 2: Mean and standard deviation of shear bond strength in 5 groups.

Group	N	Mean SBS ± SD (MPa)	Maximum (MPa)	Minimum (MPa)	Group difference†
1	16	17.72±5.37	26.35	9.50	A, B
2	16	19.13±5.37	27.24	9.95	A
3	6	3.69±1.30	5.12	1.57	C
4	16	19.03±6.91	32.40	9.30	A, B
5	16	13.21±4.87	21.28	6.16	B

Group 1: Etch and rinse+conventional flowable composite resin, **2:** Self etching+conventional flowable composite resin, **3:** Self-adhesive composite resin, **4:** Etching+Self-adhesive composite resin, **5:** Self etching+Self- adhesive composite resin

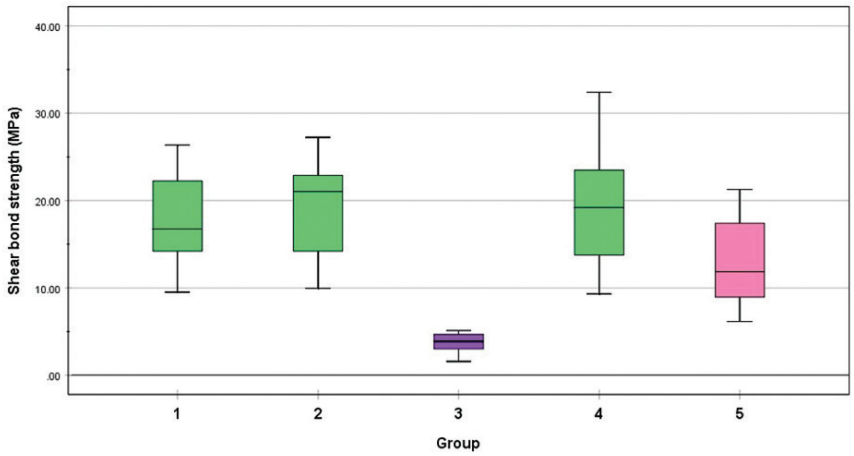

Figure 3: Boxplot of the mean of shear bond strength (SBS). **1:** Etch and rinse+conventional flowable composite resin, **2:** Self etching+conventional flowable composite resin, **3:** Self-adhesive composite resin, **4:** Etching+Self-adhesive composite resin, **5:** Self etching+Self-adhesive composite resin.

Table 3: Percentage and frequency (f) of ARI score in 5 groups.

ARI %(f) / Group	1	2	3	4	5	Total
1	0 % (0)	6.25% (1)	75.00% (12)	12.50% (2)	6.25% (1)	22.86% (16)
2	0 % (0)	6.25% (1)	43.75% (7)	40.63% (6.5)	9.38% (1.5)	22.86% (16)
3	0 % (0)	100% (6)	0% (0)	0% (0)	0% (0)	8.56% (6)
4	0 % (0)	0% (0)	15.63% (2.5)	37.60% (6)	46.88% (7.5)	22.86% (16)
5	0 % (0)	18.75% (3)	53.11% (8.5)	21.88% (3.5)	6.26% (1)	22.86% (16)
Total	0 % (0)	15.71% (11)	42.87% (30)	25.71% (18)	15.71% (11)	100% (70)

Group 1: Etch and rinse+conventional flowable composite resin, **2:** Self etching+conventional flowable composite resin, **3:** Self-adhesive composite resin, **4:** Etching+Self-adhesive composite resin, **5:** Self etching+Self- adhesive composite resin

ARI 1: Cohesive failure in enamel, **2:** Interfacial failure between composite resin and enamel, **3:** Mixed failure with less than 50% of the bonding area covered by composite resin, **4:** Mixed failure with 50% or more of the bonding area covered by composite resin, and **5:** Cohesive failure in composite resin.

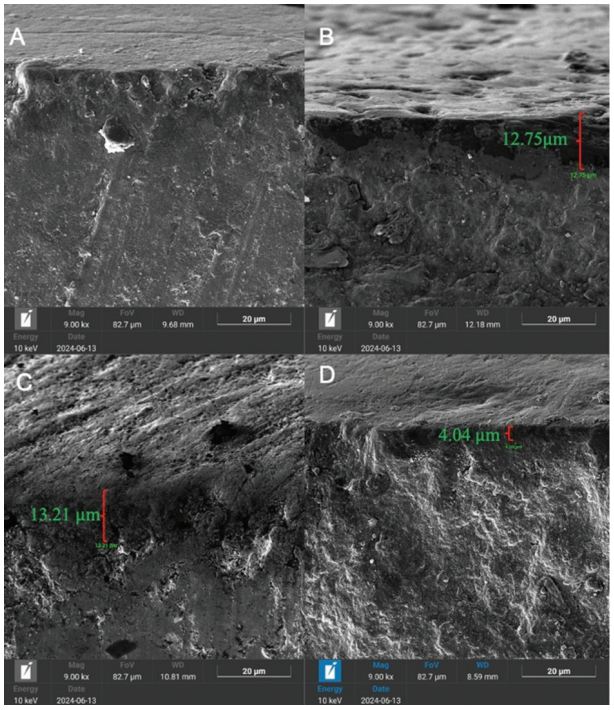

Figure 4: SEM with 9,000x magnification of (A) Intact enamel surface; (B) Demineralization zone using Adper™ Scotchbond Multi-Purpose etchant; (C) Demineralization zone using Transbond™ Plus Self Etching; (D) Demineralization zone using Vertise® Flow Resin.

Discussion

The ideal material for clear aligner attachments should offer resistance to slipping, durability, and ease of application.[17] The etch and rinse system remains the most reliable technique for bonding to enamel. Recent advances in dental materials focus on simplifying bonding procedures to reduce technique sensitivity and chair time.[11] The development of self-adhesive composite resin also offers significant benefits by simplifying the bonding process. Our study aimed to compare the SBS and assess the failure mode of the attachment of clear aligners on intact enamel surfaces with different bonding protocols.

The success of a dental adhesive system depends on its ability to demineralize the tooth surface to enhance its receptiveness and the penetration of monomers into the demineralized zone.[11] A previous study indicated that the etch and rinse and self-etch systems produce comparable SBS on enamel, consistent with our findings that the SBS was similar between Groups 1 and 2.[15]

The bonding mechanism of the Vertise® Flow™ Resin involves two processes: chemical adhesion, where the phosphate group of the glycerophosphate dimethacrylate monomer (GPDM) binds to calcium ions in the tooth structure, and micromechanical interlocking through etching, which creates a network between polymerized monomers and dentin collagen fibers.[18] Our study found that Group 3 had the lowest mean SBS, significantly lower than the other groups. SEM analysis revealed an enamel surface with a shallow demineralized zone in Group 3 (Figure 4D), which can be attributed to the Vertise® Flow™ Resin's low acidity (pH 1.9), which reduces enamel demineralization. In addition, its high viscosity limited monomer penetration into the demineralized zone.[18,19]

Our study observed that 62.5% of the attachments in Group 3 dislodged after thermocycling. These findings are consistent with Goracci *et al*., who reported a significant decrease in the SBS for the Vertise® Flow™ Resin after aging.[15] However, the SBS of the self-adhesive composite resin was increased by pretreating the surface with phosphoric acid etching (Group 4) and self-etching (Group 5). This finding aligns with the results of prior studies.[20,21] Group 3 showed a high failure rate, leaving only six samples for analysis. Post hoc Dunnett's T3 test was performed for this unequal group sizes with valid results and unchanged reliability.[22] Nevertheless, the high early failure rate itself provides clinically relevant information, indicating that self-adhesive resin, when used without additional surface pretreatment, may not provide adequate bonding performance for attachment retention. Surface pretreatment with either phosphoric acid etching or a self-etching primer should be performed prior to its application to ensure adequate bonding effectiveness.

Clear aligners exhibit a retention force as high as approximately 49.3 N.[9] SBS of 5.9-7.8 MPa is recommended for clinically acceptable direct orthodontic bonding systems to withstand masticatory forces effectively.[10] Therefore, our study found that the SBS of Groups 1, 2, 4, and 5 are acceptable for clinical use. Nevertheless, the bond strength in orthodontics should be sufficient to minimize bond failure during treatment but not high enough to damage the substrate surface during debonding.[8] A report indicated that enamel fractures might be associated with a high bond strength, with the risk of enamel damage increasing by nearly 50% when adhesion forces exceed 14.7 MPa.[23] Therefore, clinicians should carefully balance the requirement for reliable attachment stability with the potential risk of irreversible enamel damage by

targeting an optimal range of shear bond strength that is sufficient for clinical retention but not excessively high. Based on the findings of the present study, Group 5 appears to provide the most favorable outcome, offering adequate retention force while minimizing the risk of enamel damage.

It is widely recognized that the failure mode indicates bonding effectiveness, with adhesive or interfacial failure between the composite resin and the enamel signifying low bond strength.[19] Our results indicated a predominance of adhesive failure in Group 3, consistent with the previously reported low bond strengths and corroborating earlier findings.[19] The low acidity and high viscosity of the Vertise® Flow™ Resin might have caused a decrease in the SBS.[18,19] Similarly, the ARI increased with the SBS for self-adhesive composite resins applied after surface pretreatment with phosphoric acid etching (Group 4) and self-etching (Group 5). The increased presence of material remnants in the bonding area leads to surface roughness and the loss of the intact enamel surface after these remnants are removed.[12] Nevertheless, if the SBS is considered acceptable for clinical use, we expect that the attachment will remain adhered to the enamel surface throughout the treatment period. Finally, the attachment will be removed using a dental bur, surface roughness and the loss of the intact enamel surface are unavoidable. Finishing and polishing are necessary after removing the attachment.

The impact of various sterilizing procedures on shear bond strength test has been investigated. The most significant decrease in bond strength was reported for storage in 5.25% sodium hypochlorite (NaOCl) because of enamel deproteinization. NaOCl is not recommended as a storage solution.[14] On the other hand, sterilization and storage in 10% formalin was shown no significant effect on bond strengths.[14] The 10% formalin also demonstrated that sterilization is completely effective.[24] Centers for Disease Control and Prevention (CDC) guidelines suggest immersion in a 10% formalin solution for 2 weeks should be effective for disinfecting.[25] From *in vitro* dental bonding investigations, immersion in 10% formalin might be the best option for storage and sterilizing.[14] In addition, the shear bond strength was not statistically different when storing the teeth in 10% formalin for 24 hours and 2 months.[13] Regarding methodology for surface finding, various studies evaluating the SBS on enamel surfaces conducted successive grinding and polishing to create a uniform flat surface to regulate the size of the bonding area.[26,27] Grinding and polishing to create a uniform flat surface might not represent the SBS on an intact enamel surface. Our study aimed to assess the SBS on intact enamel surfaces. However, the actual enamel surface beneath the attachment base exceeds the area of the attachment base due to the curvature of the tooth surface. Therefore, the Materialise 3-Matic Research software was used to calculate the actual intact enamel surface beneath the attachment base for every sample. (Figure 2A-D).

The limitations of our study were its *in vitro* design and the possibility that its findings may not precisely reflect clinical situations. Moreover, while thermocycling is commonly employed to simulate adhesive aging *in vitro*, it may not fully replicate intraoral conditions such as salivary moisture, enzymatic activity, ionic fluctuations, and occlusal forces. Therefore, the findings should be interpreted with caution, as they may not accurately reflect the long-term clinical performance. A randomized controlled split-mouth clinical trial should be conducted to address these issues.

Conclusions

Attachments made from self-adhesive composite resin had the lowest SBS, which appeared clinically unsuitable. However, surface pretreatment with either 37% phosphoric acid or self-etching primer before applying the self-adhesive composite resin significantly improved the SBS, making it clinically acceptable.

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Conflict of interest

The authors declare no conflicts of interest.

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Comparative Evaluation of Zinc Oxide Eugenol and Its Combination with Manuka Honey as Obturating Materials in Primary Molars: An *In Vitro* Study

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Abstract

Background: An ideal root canal filling material for primary teeth should be non-irritating, antibacterial, and non-inflammatory while ensuring ease of insertion, adhesion, and dimensional stability.

Objectives: This study evaluates the safety and effectiveness of manuka honey (MH) as an obturating material by comparing zinc oxide eugenol (ZOE) alone versus ZOE with MH in extracted posterior deciduous teeth.

Methods: This prospective *in vitro* comparative study assessed antibacterial properties using the agar well diffusion method, measuring the zone of inhibition and minimum inhibitory concentration and evaluating MH's synergistic antibacterial effect with ZOE. Handling properties, including mixing, setting, and obturation times, were recorded, and radiographic quality was evaluated based on taper, density, and length.

Results: Each group included 15 teeth. The mean zone of inhibition was 20.53±1.18 mm for ZOE alone (Group 1) and 22.07±1.09 mm for ZOE with MH (Group 2) (p=0.0021). The MIC was significantly lower in Group 2 (0.248±0.01 mg/mL) than in Group 1 (0.516±0.034 mg/mL, p<0.05), showing greater antibacterial efficacy. The MIC was reduced by 2.08-fold, confirming a strong synergistic effect. However, Group 1 had shorter setting (22.2±28.73 min) and obturation (4.76±0.22 min) times than Group 2 (28.73±1.28 min, 5.47±0.32 min, p<0.00033). Radiographic quality showed no significant difference (p=0.77).

Conclusions: Incorporating MH into ZOE improves antibacterial properties and reduces infection risk without affecting radiographic quality, making it a promising option for primary tooth obturation.

Keywords: antibacterial efficacy, manuka honey, obturation, pulpectomy, zinc oxide eugenol

Introduction

Preserving primary teeth is vital in pediatric dentistry for maintaining arch integrity, mastication, and speech development. Vital pulp therapies like indirect pulp capping, direct pulp capping, or pulpotomy are suitable for reversible pulpitis. However, in irreversible pulpitis or necrosis, non-vital pulp therapy such as pulpectomy is necessary. Pulpectomy involves removing infected pulp tissue and filling the root canals with a suitable obturating material.[1] The ideal obturating material should be antibacterial, biocompatible, resorbable at the same rate as the root, radiopaque, non-irritating, and easy to insert or remove. Zinc oxide eugenol (ZOE), though commonly used for its antimicrobial and sealing properties, has drawbacks like delayed resorption and potential periapical irritation. Despite various available materials, none fulfill all ideal criteria, highlighting the challenge in managing pulpally involved primary teeth effectively until natural exfoliation.[1,2]

Streptococcus mutans (S. mutans) plays a key role in root canal treatment (RCT) failure due to its acid production, biofilm formation, and ability to survive in low-pH environments. It adheres strongly to dentin, resists antimicrobial agents, and persists in secondary infections, contributing to reinfection and periapical disease. Additionally, *S. mutans* degrades dentin and obturating materials, leading to microleakage and secondary caries. Its production of extracellular polysaccharides enhances biofilm protection, making eradication particularly challenging. Studies have shown that *S. mutans* can withstand intracanal medicaments like calcium hydroxide, thereby reducing treatment effectiveness. Given its frequent presence in failed RCT cases, *S. mutans* underscores the need for improved disinfection and antimicrobial strategies to ensure long-term success. Moreover, as a primary facultative anaerobe involved in early biofilm formation and dentinal caries progression, *S. mutans* can thrive under both aerobic and anaerobic conditions, making it a reliable and reproducible model organism for *in vitro* studies assessing antimicrobial efficacy and root canal disinfection.[3,4]

Natural products, such as manuka honey (MH), offer a potential alternative to conventional obturating materials.[5] While promising, their feasibility requires comprehensive biocompatibility, clinical applicability, and efficacy evaluation.[6] Recent studies have explored the effectiveness of natural compounds in paediatric endodontics, particularly their use as irrigants, intracanal medicaments, and root canal filling materials.[7] However, existing research alone may not be sufficient for clinical decision-making. Honey's antibacterial properties stem from hydrogen peroxide production via enzymatic activity, though at lower concentrations than traditional antimicrobial solutions. MH, distinguished by its Unique Manuka Factor (UMF) in New Zealand, demonstrates strong antibacterial activity and excellent biocompatibility, making it a promising candidate for endodontic applications.[8,9]

This study evaluates the safety and effectiveness of MH as an obturating material in primary teeth. While plant-derived products have long been used in therapy, and honey is recognized for its antibacterial and biocompatibility properties, its application in paediatric endodontics remains limited. The study examines Manuka honey's antibacterial effects against *S. mutans* and its potential synergy with ZOE. Additionally, it compares the radiographic quality of obturation, including length, taper, and density, between ZOE alone and ZOE with MH to assess its suitability for root canal treatment in primary teeth.

Materials and Methods

Study settings and sample collection

This *in vitro* study involved thirty freshly extracted primary molars from children aged 4-8 years, collected at a postgraduate dental college in Eastern India, following ethical committee approval. Teeth were extracted for reasons unrelated to the study (e.g., caries or mobility) and stored in saline until use. Inclusion criteria required restorable crowns and at least two-thirds of intact root structure, with exclusions for teeth showing fractures, advanced root resorption, pathological lesions, or previous endodontic treatment. The teeth were randomly assigned into two groups (n=15 each) using a simple randomization method. Although a CONSORT diagram was not applicable due to the *in vitro* design, all procedures related to sample selection, allocation, and handling were documented to ensure transparency and reproducibility.

The sample size was determined using G*Power software, with parameters set at an effect size of 1.35, an alpha level of 0.05, and a statistical power of 80%, based on preliminary data. The calculation indicated a minimum of 11 teeth per group. To enhance reliability and

account for potential variability, 15 teeth were included in each group, yielding a total sample size of 30, which was sufficient for statistical analysis.

Baseline characteristics confirmed that both groups (ZOE and ZOE+MH) were comparable. The mean age of patients was 4.5±1.5 years, with an equal gender distribution (6 males and 9 females in each group). Each group had a similar mix of first and second molars, with most teeth from the maxillary arch. All teeth exhibited adequate root integrity, and caries were the primary reason for extraction. A few teeth had existing restorations. Overall, the groups were well-matched, supporting the validity of comparative analysis (Table 1).

Preparation combination of zinc oxide eugenol & manuka honey

ZOE+MH was prepared by first mixing zinc oxide powder and eugenol in a 1:1 ratio to form a base paste. Then, an equal amount of MH was added relative to the volume of eugenol used (1:1 with eugenol). The mixture was blended thoroughly to achieve a thick, pliable consistency suitable for root canal obturation. The paste was applied incrementally using a folding technique, ensuring homogeneity and optimal canal filling. Fresh preparation was ensured each time to maintain the antimicrobial activity of MH.

In vitro antibacterial assessment & evaluation of synergistic effect

For the antibacterial assessment, the antimicrobial efficacy of MH combined with ZOE was evaluated using the agar well diffusion and microbroth dilution methods. The agar well diffusion method measured the zone of inhibition against *S. mutans* strains cultured on MH agar. At the same time, the minimum inhibitory concentration (MIC) was determined using a microbroth dilution method in a 96-well microtiter plate. Each well contained 100 µL of Mueller-Hinton Agar (MHA) medium (Figure 1). The first column served as a control with Triphenyl Tetrazolium Chloride (TTC) and *S. mutans* (MTCC 890) inoculum, while the second column contained Chlorhexidine, TTC, and *S. mutans* inoculum.[10,11] Serial twofold dilutions of the MH solution were performed starting from 50% (w/v), decreasing stepwise across the wells up to the 12th column, resulting in a dilution range from 50% to 0.024%. The plate was incubated at 37°C for 36-48 hours and analyzed using an ELISA reader to determine MIC values. Samples were divided into two groups: ZOE alone (n=15) and ZOE+MH (n=15). Statistical analysis was conducted using t-tests for significance and Cohen's d for effect size, with $p<0.05$ considered statistically significant.[12]

Root canal procedure

Thirty extracted primary molars meeting the inclusion criteria underwent pulpectomy performed by a single trained postgraduate student under the supervision of an experienced faculty member, ensuring standardized technique, consistency across procedures, and minimal operator-related variability. Carious tissue was removed using a No. 6 round bur to create straight-line access, and the pulp was extirpated with H and K hand files. The canals were irrigated with 2.5% sodium hypochlorite and normal saline to remove debris. A diagnostic radiograph determined the root canal length, and instrumentation was performed up to file size 30. The canals were obturated with two different materials after final irrigation and drying with paper points. In Group I, root canals were filled with ZOE mixed in a 1:2 powder-to-liquid ratio, and added incrementally to achieve a thick consistency. In Group II, ZOE was combined with eugenol and MH in a 1:1 ratio and applied using a folding technique. This study aimed to compare the effectiveness of these

Table 1: Baseline characteristics of extracted primary molars used in the study.

Characteristic	Group 1 (ZOE) (n=15)	Group 2 (ZOE + Manuka Honey) (n=15)	Total (N=30)
Age of patients (mean ± SD)	4.5±1.5	4.5±1.5	4.5±1.5
Gender (Male/Female)	6/9	6/9	12/18
Tooth type (First/Second molar)	8/7	7/8	15/15
Arch location (Maxillary/Mandibular)	9/6	10/5	19/11
Root integrity (≥2/3 intact)	15	15	30
Presence of caries	13	14	27
Existing restorations	2	1	3
Reason for extraction	Caries/Mobility	Caries/Mobility	Caries/Mobility

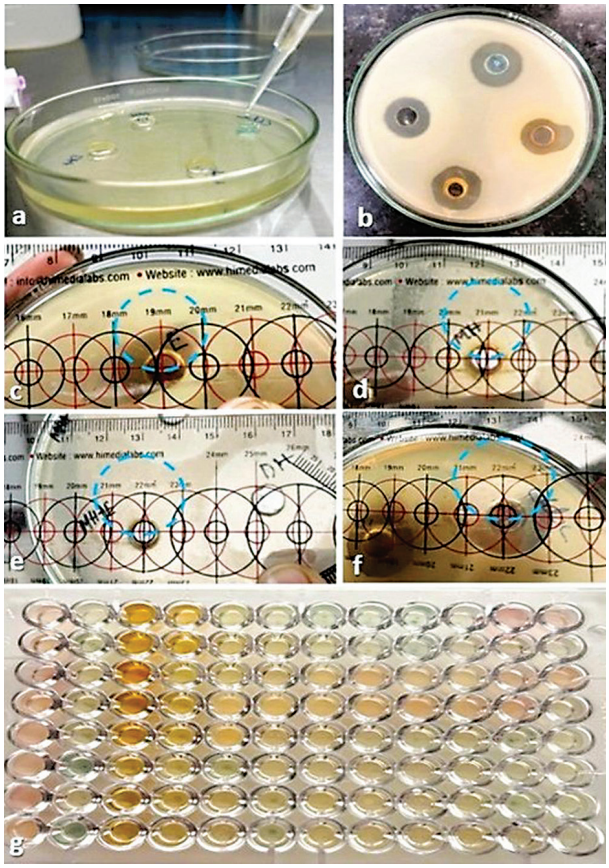

Figure 1: Evaluation of antibacterial properties against *Streptococcus mutans*. (a) application of extracts into wells; (b) zones of inhibition; (c) Eugenol ZOI-19mm; (d) Manuka honey ZOI-21 mm; (e) Manuka Honey with Eugenol ZOI-22mm; (f) Control group (Chlorhexidine) ZOI-22mm; (g) Minimum inhibitory concentration.

materials in primary molars, assessing whether the antimicrobial and therapeutic properties of MH could enhance the sealing ability and clinical performance of ZOE as an obturating agent.

Radiograph analysis

Post-obturation periapical radiographs were taken using the paralleling technique with RVG to evaluate the quality of obturation. The radiographic quality was assessed based on three parameters: length of obturation, density of obturation, and taper of obturation. The length was categorized as adequate (within 0-2 mm of the radiographic apex), underfilled (more than 2 mm from the radiographic apex), or overfilled (beyond the apex). The density of obturation was deemed adequate if no voids were present and inadequate if voids were detected. The taper was considered adequate if it was consistent from orifice to apex and inadequate if inconsistent. To ensure unbiased evaluation, a single evaluator who was blinded to the group allocation analyzed all radiographs. Before the assessment, the evaluator underwent a calibration exercise using ten sample radiographs assessed twice at a two-week interval. Intra-examiner reliability was determined using Cohen's kappa coefficient, yielding a value of 0.87, indicating strong agreement and consistency in interpretation (Figure 2). To quantify obturation quality, a T-score system was used: a score of 4 indicated all three parameters were ideal; a score of 3 indicated two were ideal; a score of 2 indicated one was ideal; and a score of 1 indicated none were ideal (Figure 3).

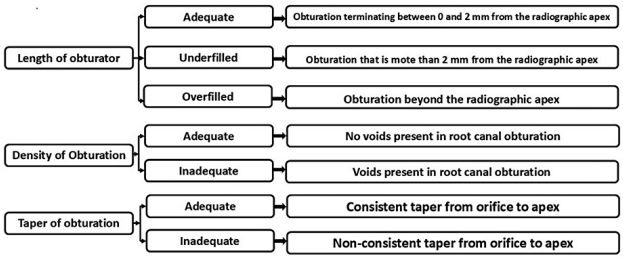

Figure 2: Parameters to assess root-filling radiographic quality.

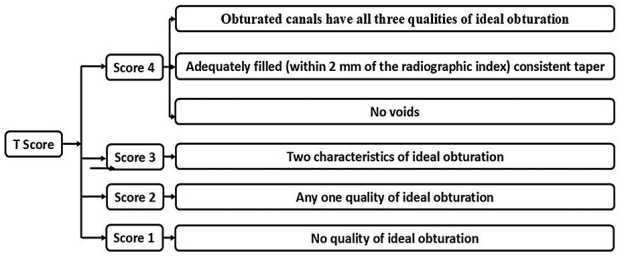

Figure 3: T- Score.

Statistical analysis

Statistical analysis was performed using SPSS version 29. Quantitative data, such as age, were expressed as mean±standard deviation, while qualitative variables, including gender, T-score, and obturation quality, were reported as frequencies and percentages. Statistical analysis included t-tests for significance and Cohen's d for effect size, with $p<0.05$ considered significant. The Chi-square test assessed density and taper quality, while Fisher's Exact test compared obturation length and final T-score. The Shapiro-Wilk test evaluated normality, and an unpaired t-test was used to analyze manipulation time, setting time, obturation time, zone of inhibition, and MIC. A $p<0.05$ was considered statistically significant, and 95% confidence intervals were calculated for all variables.

Results

The combination of ZOE with MH significantly enhanced its antibacterial properties against *S. mutans*. The zone of inhibition increased by 7.48% compared to ZOE alone (from 20.53 mm to 22.07 mm), indicating greater antibacterial efficacy (p=0.0021, Cohen's d=1.35, large effect). Additionally, the MIC was reduced by 51.94%, demonstrating a substantial improvement in antimicrobial potency (p<0.0001, Cohen's d=10.69, very strong effect). The MIC value showed a 2.08× reduction, further supporting the synergistic effect of MH. These findings suggest that incorporating MH into ZOE enhances its antibacterial potential, making it a promising candidate for improved dental applications (Table 2 and Figure 4).

Among 30 extracted teeth, the age ranged from 4 to 9 years, with a mean of 4.5±1.5 years. Males were 12/30 (40%), and females were 18/30 (60%). Fifteen teeth (50%) were treated with ZOE alone (Group 1), while the other 15 (50%) were treated with ZOE combined with MH (Group 2). Mixing, setting, and obturation times were measured for all the teeth. The Shapiro-Wilk test indicated a normal distribution of the data (W (15) = 0.97, p=0.867). Group 1 had a mean mixing time of 1.11±0.14, slightly less than Group 2 (1.17±0.14), but the difference was insignificant. The setting time was significantly shorter in Group 1 (22.2±28.73) compared to Group 2 (28.73±1.28, p<0.0001). The obturation time was also significantly different (p<0.00033), with Group 1 at 4.76±0.22 and Group 2 at 5.47±0.32 (Table 3 and Figure 5).

Periapical radiographs were evaluated after obturation to assess the quality of root canal fillings based on length, density, and taper (Figure 6 and Figure 7). T-scores were calculated accordingly. In Group I (ZOE), 7 out of 15 teeth (46.6%) had adequate obturation length, 5 (33.3%) were underfilled, and 3 (20%) were overfilled. Adequate density was observed in 11 teeth (73.3%), while 4 (26.6%) showed inadequate density. Ten teeth (66.6%) had an adequate taper, and 5 (33.3%) were inad-

Table 2: Antibacterial properties and synergistic effect of zinc oxide eugenol + manuka honey against *Streptococcus mutans*.

Parameter	ZOE (n=15)	ZOE + MH (n=15)	*p*-value	95% Confidence Interval	Synergistic Effect (ZOE + MH)
Zone of Inhibition (mm)	20.53±1.18	22.07±1.09	0.0021	7.6683 to 31.0317	↑ 7.48% (Cohen's d = 1.35, large effect)
SEM (Zone of Inhibition)	0.31	0.28	—	—	—
Minimum Inhibitory Concentration (MIC) (mg/mL)	0.516±0.034	0.248±0.01	< 0.0001	0.35073 to 0.61327	↓ 51.94% (Cohen's d = 10.69, very strong effect)
SEM (MIC)	0.0089	0.0026	—	—	—
MIC Fold Reduction	—	—	—	—	2.08× reduction in MIC

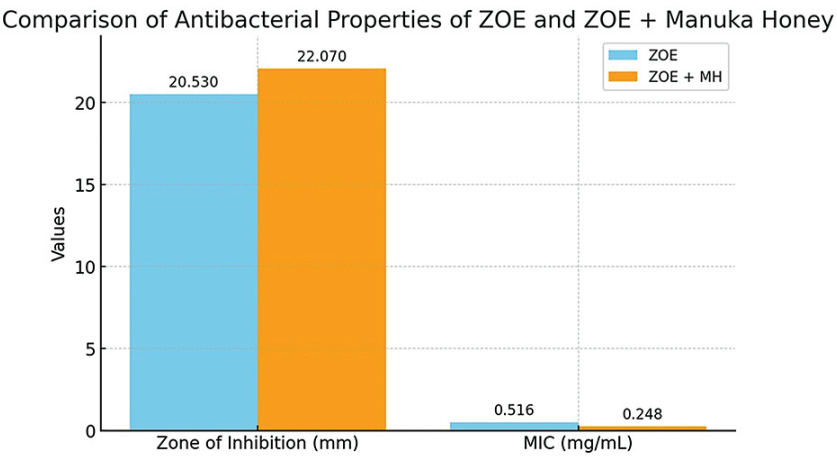

Figure 4: Bar graph comparing the Zone of Inhibition (mm) and Minimum Inhibitory Concentration (MIC, mg/mL) for zinc oxide eugenol and zinc oxide engenol+manaka honey against *Streptococcus mutans*.

Table 3: Mean of manipulation and obturation time for both groups.

Parameters		ZOE (n=15)	ZOE+MH (n=15)	*p*-value	95% Confidence interval
Mixing time	Mean±SD	1.11±0.14	1.17±0.14	0.2504	-0.0486 to 0.1632
	SEM	0.0363	0.0368		
Setting time	Mean±SD	22.2±28.73	28.73±1.28	< 0.0001	-7.98 to -5.08
	SEM	0.63	0.33		
Obturation time	Mean±SD	4.76±0.22	5.47±0.32	0.0033	0.5037 to 0.9203
	SEM	0.0570	0.0842		

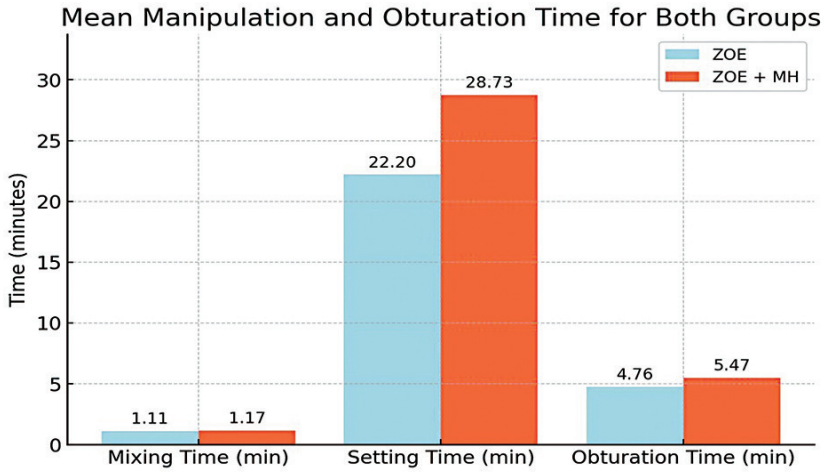

Figure 5: Bar graph comparing the mixing time, setting time, and obturation time for zinc oxide eugenol and zinc oxide eugenol+manuka honey.

Table 4: Radiographic analysis for quality of obturation.

Parameter	Quality	ZOE [n (%)]	ZOE+MH [n (%)]	*p*-value
Density of obturation	Grade 1 (Adequate)	11 (73.3%)	9 (60%)	0.4386
	Grade - 2 (Inadequate)	4 (26.6%)	6 (40%)	
Length of obturation	Grade 1 (Adequate)	7 (46.6%)	5 (33.33%)	0.889
	Grade - 2 (Underfilled)	5 (33.3%)	7 (46.67%)	
	Grade 3 (Overfilled)	3 (20%)	3 (20%)	
Taper of obturation	Grade 1 (Adequate)	10 (66.66%)	12 (80%)	0.4090
	Grade - 2 (Inadequate)	5 (33.33%)	3 (20%)	
T score	Score 1	0	0	0.770008
	Score 2	5 (33.33%)	6 (40%)	
	Score 3	7 (46.6%)	8 (53.33%)	
	Score 4	3 (20%)	1 (6.67%)	

equate. In Group II (ZOE + MH), 5 teeth (33.3%) showed adequate length, 7 (46.6%) were underfilled, and 3 (20%) were overfilled. Density was adequate in 9 teeth (60%) and inadequate in 6 (40%). Twelve teeth (80%) had an adequate taper, and 3 (20%) were inadequate. T-scores were assigned based on how many parameters met the ideal criteria. In Group I, 5 teeth (33.3%) scored 4, 7 (46.6%) scored 3, and 3 (20%) scored 2. In Group II, 1 tooth (6.67%) scored 4, 8 (53.3%) scored 3, and 6 (40%) scored 2. No tooth in either group scored 1. Statistical analysis showed no significant difference between the two groups in obturation quality (p>0.05) (Table 4 and Figure 8).

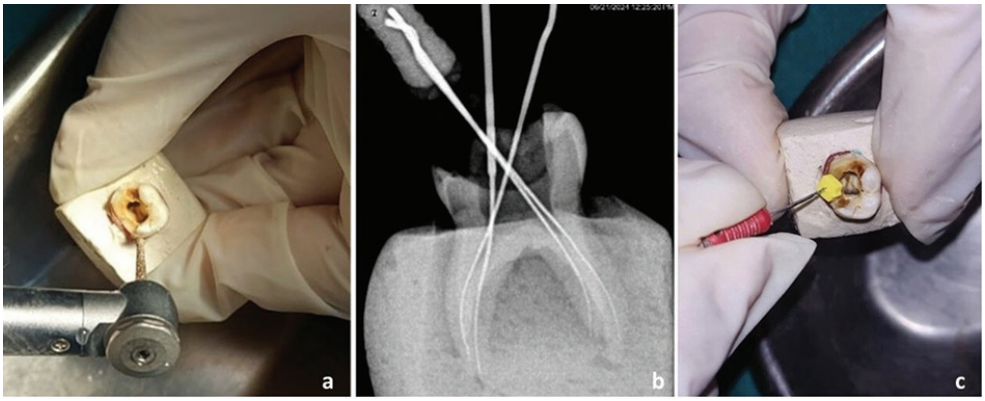

Figure 6: Root canal preparation (a) access opening using high-speed airotor; (b) working length determination using radiographic technique; (c) shaping and cleaning of canals using k-files.

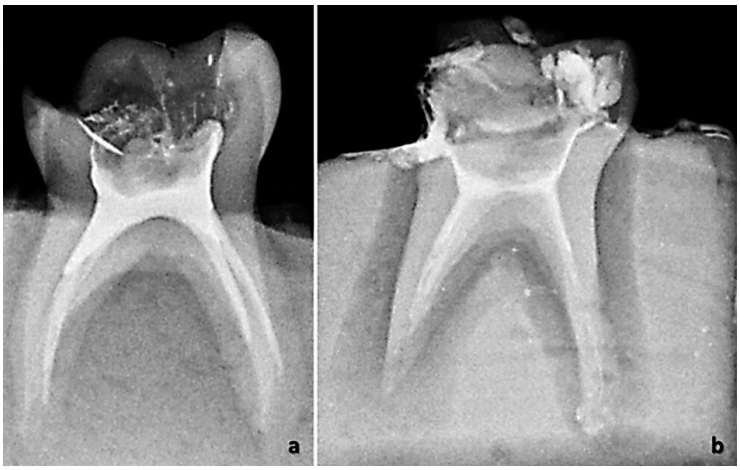

Figure 7: Postoperative Radiographs. (a) Radiograph of Group A; (b) Radiograph of Group B.

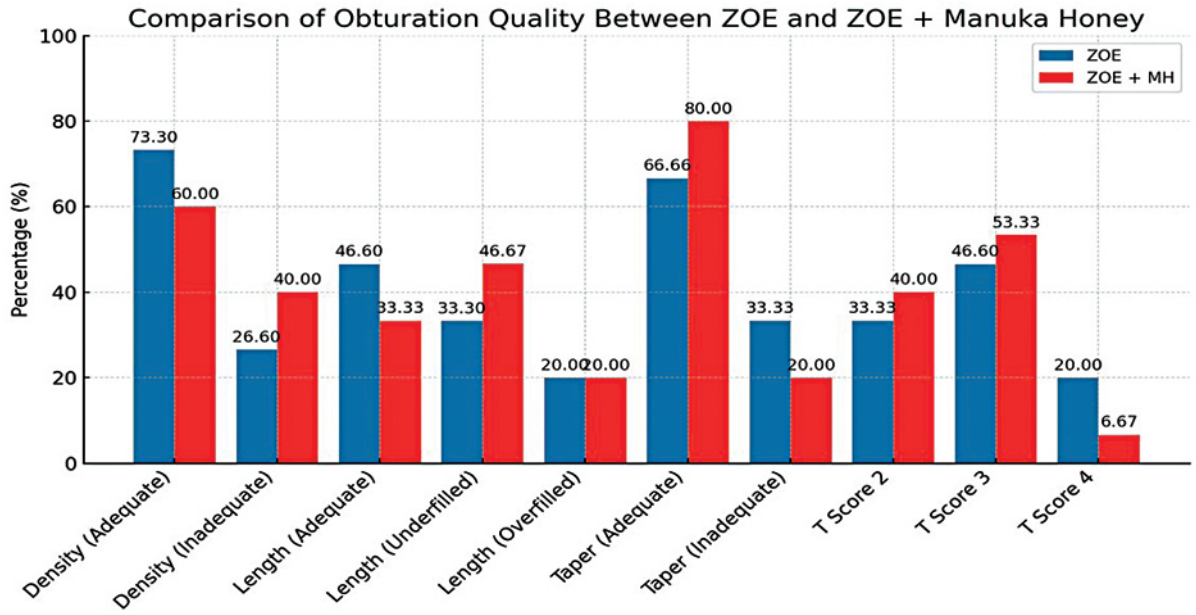

Figure 8: Bar graph comparing the obturation quality parameters (Density, Length, Taper, and T Score) between zinc oxide eugenol and zinc oxide eugenol+manuka honey.

Discussion

This *in vitro* study demonstrated that the incorporation of MH into ZOE) significantly enhanced its antibacterial efficacy. The combination exhibited a larger mean zone of inhibition (22.07±1.09 mm) compared to ZOE alone (20.53±1.18 mm; p=0.0021), and a lower MIC value (0.248±0.01 vs. 0.516±0.034; p<0.0001). These results indicate a statistically significant improvement in antimicrobial activity. However, it is important to acknowledge that *in vitro* antibacterial findings may not directly correlate with clinical effectiveness. Improved inhibition zones or MICs do not necessarily translate to better healing, reduced postoperative infection, or long-term success *in vivo*. Therefore, while promising, these results should be interpreted with caution until further clinical evidence is available.

Manuka honey's antimicrobial effects are attributed to several unique components, including methylglyoxal (MGO), D-glucono-δ-lactone, and hydrogen peroxide, which inhibit bacterial replication, disrupt biofilm formation, and contribute to acidification of the local environment.[13-15] Its low water activity (0.6-0.75) further limits microbial growth.[13,15] Although there is evidence supporting the antibacterial efficacy of MH, studies comparing the duration or sustained effects of MH relative to ZOE remain limited. While MH is known to reduce biofilm formation and promote healing, claims regarding its prolonged antibacterial action over ZOE alone require further validation.[15-19]

Radiographic analysis showed that both ZOE and ZOE-MH groups achieved comparable obturation quality. No significant differences were observed in obturation length, density, or taper between the groups. T-score analysis further supported these findings, with slight, non-significant differences. Although the addition of MH led to marginally increased mixing, setting, and obturation times, these remained within acceptable clinical thresholds, suggesting that usability was not compromised.[16,20]

ZOE remains one of the most commonly used root canal filling materials in primary teeth due to its biocompatibility and antibacterial properties, especially against *E. faecalis, S. mutans*, *E. coli,* and *S. aureus*.[21,22] However, limitations such as delayed resorption, potential irritation of periapical tissues, and interference with permanent tooth eruption have been reported.[14,23] Eugenol, a key component of ZOE, exhibits anti-inflammatory and analgesic effects, though its levels decline significantly within weeks of placement.[24,25] The addition of MH to ZOE may help mitigate these shortcomings by offering complementary antimicrobial and anti-inflammatory benefits.[13-17]

Limitations and future research

Nonetheless, several limitations must be addressed. The sample size in this study was small (n=30), limiting statistical power and generalizability. The radiographic assessment involved a degree of subjectivity, and inter-examiner reliability was not evaluated. Moreover, the use of a single bacterial species *(S. mutans)* does not reflect the polymicrobial nature of pulpal infections. Being an *in vitro* study, it cannot replicate the complexity of clinical conditions, including host immune responses and oral environmental variables.[19] Future studies should include randomized controlled trials with larger cohorts, long-term follow-ups, and assessments against diverse microbial flora. Comparative evaluations with other pediatric obturating materials such as Metapex, Endoflas, and calcium hydroxide would also help determine the broader applicability of ZOE-MH in pediatric endodontics. While MH enhances the antimicrobial potential of ZOE, further clinical validation is essential before it can be recommended as a standard adjunct.

Conclusions

ZOE demonstrated strong antibacterial properties, and the addition of MH further enhanced its antimicrobial efficacy. However, both materials showed comparable results in terms of obturation quality. These findings indicate the potential advantage of incorporating MH into pulpectomy procedures to enhance antibacterial action. Nevertheless, further *in vivo* studies with larger sample sizes are required to validate its clinical applicability.

Funding

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Conflicts of Interests

The authors declare no conflicts of interest.

Ethical Permission

The study was conducted after the approval of the Institutional Ethical Committee vide letter no. IEC-IDS/IDS/SOA/2023/I-31 date 21 December 2023.

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Original Article

Translation and Validation of the Thai Version of the Oral Health-related Caregiver Burden Index (OHBI) Among Caregivers of Patients Living with Neurocognitive Disorders

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Abstract

Objectives: To translate and validate a Thai version of the Oral Health-related Caregiver Burden Index (OHBI) for caregivers in Thailand.

Methods: The Japanese OHBI was translated and culturally adapted into Thai through a structured process involving forward and backward translation, expert committee review, and pre-final version testing. These steps were conducted to ensure content and face validity. The Thai OHBI was then administered to caregivers of outpatients at a general hospital in Thailand, assisting patients with major neurocognitive disorders (MNDs) and Parkinson's disease (PD). A minimum of forty-six participants was required, accounting for dropout rates.

Results: The questionnaire was initially administered to sixty caregivers, with forty-seven caregivers completing the follow-up assessment at week two. Internal consistency was acceptable, (Cronbach's alpha: 0.676 to 0.864). Test-retest reliability, assessed using the Intraclass Correlation Coefficient (ICC), ranged from 0.296 to 0.647. The Mann-Whitney U test demonstrated significant discriminant validity. Over 80% of participants reported difficulties in performing oral hygiene care for PD and MNDs patients, and approximately half of the caregivers perceived a burden in providing oral care.

Conclusions: The Thai OHBI demonstrated acceptable reliability and validity. However, further testing in larger caregiver populations is required to confirm its robustness and applicability.

Keywords: aged, caregiver burden, neurodegenerative diseases, oral care, oral hygiene

Introduction

The 2024 National Survey[1] reported that 6.59% of older adults in Thailand require assistance with daily activities. More specifically, 2.05% of older adults needed a caregiver to assist with grooming and maintain their hygiene.[1] Similarly, some of these older adults also depended on caregivers for daily oral hygiene care.[2]

Providing oral care to dependent patients presents various challenges, such as resistant behavior from care recipients, caregiver's lack of experience or knowledge, and discomfort in managing oral hygiene tasks.[3,4] Chalmers J *et al.,*[5] observed caregivers spent less time on oral care when facing such difficulties. Similarly, Reis *et al.,*[6] identified daily oral care as a contributing factor to caregiver burden.

In Thailand, few studies have discussed caregiver burden arising from performing oral care. A study from the perspective of healthcare stakeholders revealed that a lack of knowledge and skill concerning oral care, together with a high workload was stressful for caregivers.[7] Existing instruments, such as ZBI or CBI, assess general caregiver burden but do not specifically address oral care. Those tools may not comprehensively address certain challenges in delivering oral care to patients, including caregivers' aversion to oral hygiene tasks due to fear of being bitten or feelings of repulsion toward the procedure[8,9], which previously studies indicate that oral care for dependent patients involves unique challenges that may not be present in general caregiving tasks for other parts of the body.[10] Given these distinct characteristics, a specialized instrument is necessary to evaluate caregiver burden related to oral care. Consequently, we adopted the only instrument available to date, called the Oral Health related Caregiver Burden Index (OHBI) by Matsuda Y *et al.,*[11] to be translated into the Thai language and tested with Thai caregivers.

Thus, this study aims to develop the Thai version of the OHBI (T-OHBI) and evaluate its validity and reliability in measuring caregiver burden associated with daily oral care tasks.

Materials and Methods

The OHBI is an assessment tool, which directly measures the effect of oral health care on caregiver burden. Matsuda *et al.* developed OHBI to measure caregiver burden on oral hygiene care. OHBI has 5 subscales: 1) Technique-related burden (TB; questions 1, 2, 3, 6), which arises from the combination of time-dependent burden, emotional burden, and physical burden; 2) Service-related burden (SB; questions 7, 8); 3) Existential burden (EB; questions 4); 4) Risk-related burden (RB; questions 5), which has been added to this tool; and 5) Overall burden (OB). OHBI is a questionnaire with 9 questions asking about the perception of the caregiver's burden when providing oral health care with the answer on a visual analog scale ranging from 0 (never) to 4 (nearly always).[11]

The intraclass correlation coefficient, testing for reliability, yielded 0.789 as the total score (0.743 for TB, 0.779 for SB, 0.758 for EB, 0.449 for RB, and 0.842 for OB). The validity test found that Cronbach's alpha coefficient was 0.691 for SB and 0.847 for TB. The cut-off score for determining high or low caregiver burden is 10. Statistical analysis revealed a significant difference in the total score and across all factors between the high and low burden groups. In the study, developers declared the relationship between oral health-related caregiver burden and general caregiver burden, when testing the relationship between OHBI and Burden Index of caregivers (BIC-11), to be significant ($r=0.620$, $p<0.01$).[11]

The study was conducted in two phases. The first phase involved the translation and adaptation process to develop the Thai version of OHBI. The second phase entailed a pilot testing for reliability of the translated instrument. (Figure 1)

The sample size for face validity was twelve persons with the same characteristics as research participants based on the rule of thumb in a study by Steven A. Julious[12], Sample size calculated based on Croncach's alpha value, followed Bonett's calculation followed Bonett's (2002) method.[13] The sample size calculation was performed using the web-based Sample Size Calculator developed by Wan Nor Arifin.[14] The researchers determined the values used in the calculations as follows: expected Cronbach's alpha = 0.84 (maximum Cronbach's alpha from the original OHBI[11]). precision = 0.15 confident level = 95%, number of items = 2). The minimum target sample required in this study is forty-six. After considering a 10% non-responder rate, the minimum sample size required for this study was fifty-two caregivers. Then, it was rounded up to sixty participants.

Ethical approval was obtained from the Human Research Ethics Committee of Thammasat University

(Science) with protocol number 083/2565. The research was conducted at Thammasat University Hospital, Thailand. Participants were recruited from three outpatient clinics: 1) Dementia Clinic (Department of Psychology), 2) Dementia and Parkinson's Clinic (Department of Neurology), 3) Geriatric medicine clinic (Department of Internal Medicine) and 4) Gerodontology and Special Care Dentistry Clinic. The data was collected from the caregivers who were the main person responsible for the daily oral care of patients living with major neurocognitive disorders (MNDs) and Parkinson's disease (PD). We focused on community dwelling older adults for now as the majority of Thai older adults still live at home.[1] We included both informal caregivers who often were family members and live-in formal or formal caregivers. Participants were excluded if they received any oral care education interventions during the two-week study period or if the patients, they cared for passed away, experienced an acute health crisis, or required emergency treatment. However, we did not run into such conditions in this study.

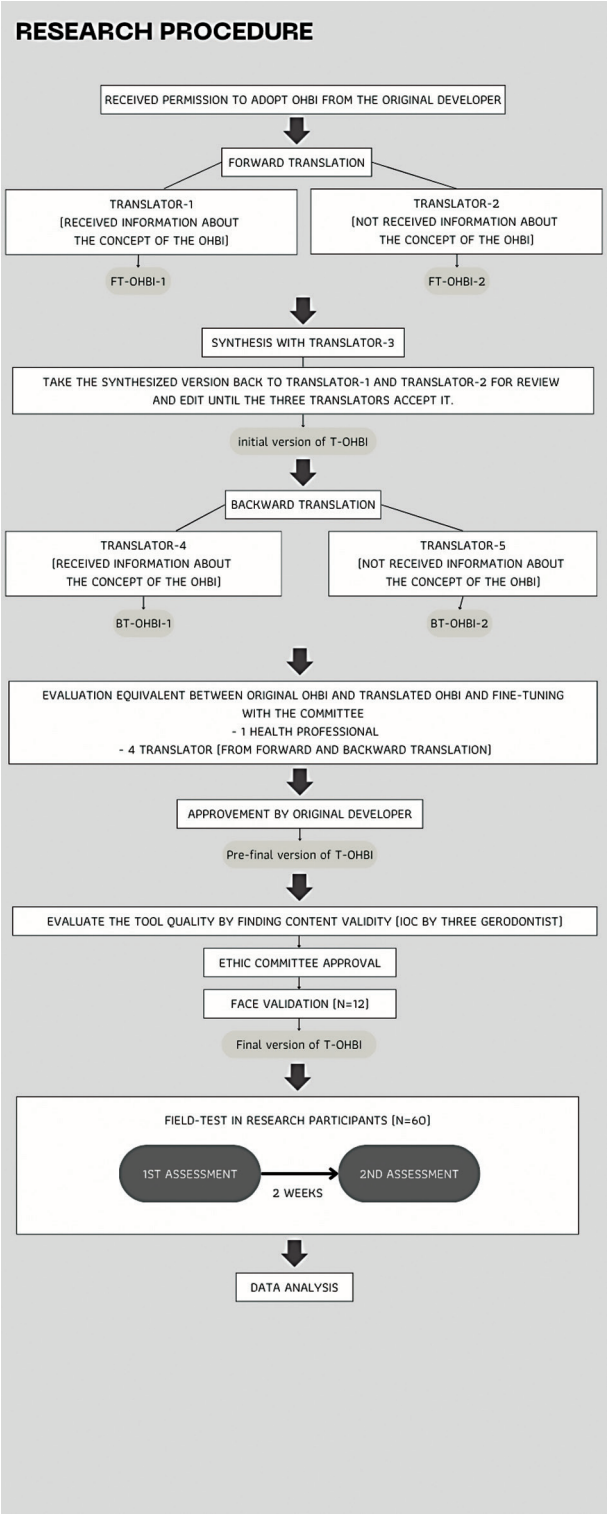

Figure 1: Research protocol.

Phase 1: The translation and adaptation of the OHBI

After obtaining permission from the original author, the translation process followed the "Guidelines for the Process of Cross-Cultural Adaptation of Self-Report Measures" by Dorcas *et al.*,[15] and the "Translation, Adaptation and Validation of Instruments or Scales for use in Cross-cultural Health Care Research: a Clear and User-friendly Guideline" by Sousa & Rojjanasrirat.[16]

1.1 Forward translation

The OHBI was translated from Japanese into Thai by two independent Thai-translators fluent in Japanese (JLPT Level 1 certified). To ensure comprehensive language adaptation:

• One translator had a medical background and was informed of the questionnaire's concept to provide clinical accuracy.

• The second translator, without prior knowledge of the instrument, ensured natural language use.

The two translated versions were then synthesized into an initial Thai version by a third independent translator, who resolved any discrepancies and ambiguities. For example, question number 4: "I experience hardship because caregiving does not give me a sense of satisfaction" was translated as "don't feel proud of cleaning a mouth" and "don't feel the benefits of cleaning a mouth." Each translator interpreted the phrase "sense of satisfaction" differently—one as a feeling of pride, the other as a feeling of usefulness—both of which convey distinct meanings. The third translator pointed out that the translation does not capture the core idea: that the caregiver feels the task is neither important nor worthy of their effort.

It instead emphasizes the nature of the task as lacking emotional or intrinsic value, offering no challenge, and contributing nothing meaningful to their work. Therefore, the sentence should be revised to: “Providing oral care assistance is not regarded as a meaningful task.”

The revised draft was sent back to the original translators for final approval.

1.2 Back translation

To confirm that the words or sentences chosen in the initial version were appropriate, the initial Thai version of OHBI was translated back into Japanese by two independent translators who had never seen the Japanese version of the OHBI.

The translators for this back translation had the same qualifications as in the forward translation process. Two back-translated versions of the OHBI were then obtained.

1.3 Expert committee for cross-cultural equivalence consensus

In this step, a committee comprising one health professional who was a dentist with experience working in Japan and four translators from previous steps evaluated the equivalence between the original OHBI and the translated OHBI. The committee discussed the difference and similarity of the wording, sentence structure, meaning, and relevance of the instructions, questions, and responses among all versions of the translated OHBI and the original OHBI. Any ambiguity or discrepancy due to word choice or cultural aspects was adjusted. The equivalence between the original OHBI and the translated version of OHBI was achieved for “semantic, idiomatic, experiential, and conceptual equivalence” according to the protocol.[15] After a consensus had been reached, the pre-final version of OHBI was created. The back-translated of this pre-final version of OHBI was then sent to the original developer, Dr. Yuhei Matsuda, for his final approval.

1.4 Content validity assessment

The approved translated questionnaire was later evaluated by three experts in geriatric dentistry for its content validity using the index of item-objective congruence (IOC). Any part with an IOC score less than 0.5 was revised according to the experts’ suggestion.

1.5 Preliminary pilot test for face validity

To evaluate if the study’s target population could understand what the instrument wanted to measure, a step was conducted among twelve individual respondents in the target population. Respondents assessed comprehension of instructions, questions, and responses. Their understanding, or lack of it, was measured through questions such as "Is there any part of this questionnaire that you did not understand or found it hard to understand?" and "What do you think this questionnaire is asking you about?". After evaluating the questionnaire, the final version of the T-OHBI questionnaire was obtained. (Appendix 1).

Phase 2: Pilot-testing for the reliability and discriminant validity of the T-OHBI

Finally, the reliability and discriminant validity of the final version of T-OHBI was evaluated by sixty caregivers of patients with MNDs and PD. However, after a gentle reminder, as per the protocol, only forty-seven volunteers completed the questionnaire in week two. Data collection was done through an electronic survey platform (Google Inc., n.d.). After data collection was complete, reliability and validity tests were conducted.

Results

Content validity

The expert panel evaluated the content of the questionnaire. During the first round of evaluation, IOC scores ranged from 0.33 to 1.0. The suggestion for item modification was that this questionnaire needed to clarify whom each question was addressed to. Therefore, specific personal pronoun, “you,” was added to each question. For the second round of evaluation, after the adjustment, the IOC score was 1.0 for all questions; meaning all questions in this questionnaire are considered valid and congruent with the intended purpose.

Face validity

Most participants had no difficulty understanding the instructions in the OHBI. However, one participant each had trouble reading questions 1, 4, 6, and 7, as well as one response choice. For each, there was one volunteer who expressed their opinion that they had problems reading the questionnaire. However, the questionnaire was found to be clear by more than 80% of participants in the pilot test. The majority of participants understood the questionnaire clearly. No further modification was needed.

Internal consistency

Testing for internal consistency using Cronbach's alpha found that the Cronbach's alpha coefficient of the entire questionnaire was 0.864. Cronbach's alpha value of the questionnaire on the topic of TB was 0.676, and SB

was 0.753.

Test-retest reliability

Test-retest reliability, assessed using the ICC, showed values ranging from 0.296 to 0.645, with a total score of 0.647 (Table 1).

Discriminant validity

Discriminant validity was assessed by comparing OHBI scores between caregivers with high and low burden levels, using a cut-off score of 10 points. This criterion was used to categorize participants into high and low burden groups in previous studies.[11] The Mann-Whitney U test was used to compare scores for each factor and the total score. Results indicated a statistically significant difference between groups with high and low caregiver burden. (Figure 2)

Sample characteristics

In this study, most caregivers were female (89.4%), 46.8% of the volunteers were aged sixty years, and older. For the patients under the care of volunteers, 57.4% had symptoms of MNDs, 23.4% had PD, and 19.1 % had symptoms of both diseases.

Most of the volunteers were informal caregivers (93.6%), all of whom were relatives of the patients they cared for. The remaining were informal caregivers 6.4%), of which no one had received training in patient care. Only 23.4% of all the volunteers received training in oral health care for patients (Table 2).

Results from 2nd OHBI questionnaire were presented in figure 3

Of the forty-seven participants who completed the questionnaire:

• 85.1% of participants reported experiencing some type of difficulty in providing oral care.

• Among the 47 participants, 14 (29.8%) reported difficulties in providing oral care did not necessarily perceive it as a burden.

Discussion

This study involved translating and adapting the OHBI questionnaire from the original Japanese into Thai. After passing the inspection, it was found that the T-OHBI questionnaire was equivalent to the OHBI in Japanese and could be used in the context of Thailand. The results from this study showed that Some caregiver of patient living with MNDs and PD in Thailand perceived burden from performed oral care for their care receiver.

Table 1: Test-retest reliability of T-OHBI by intraclass correlation.

	ICC	95% CI
Factor 1: Technique-related burden (Q1, 2, 3, 6)	0.645 (moderate)	0.444-0.785
Factor 2: Service-related burden (Q7, 8)	0.580 (moderate)	0.352-0.743
Factor 3: Existential burden (Q4)	0.296 (poor)	0.023-0.531
Factor 4: Rick-related burden (Q5)	0.574 (moderate)	0.336-0.740
Factor 5: Overall burden (Q9)	0.619 (moderate)	0.408-0.768
Total score	0.647 (moderate)	0.430-0.791

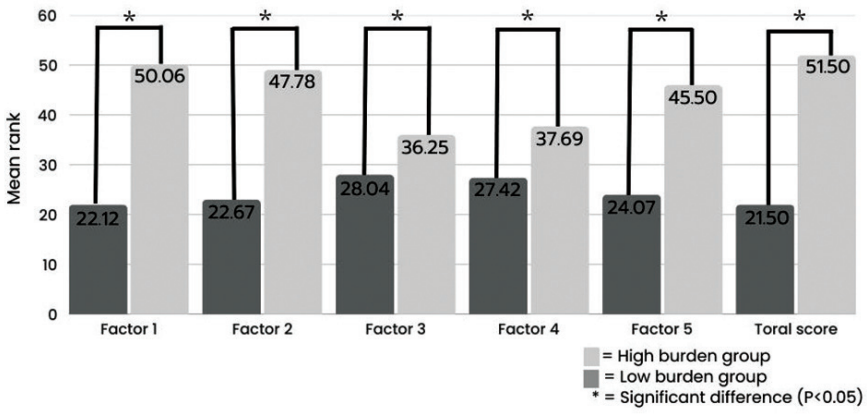

Figure 2: Discriminant validity by Mann-Whitney u test (cut-off score=10).

Table 2: Characteristics of participants (N=47).

Characteristic	Number	%
Sex		
Male	5	10.6
Female	42	89.4
Age		
20-29 years	1	2.1
30-39 years	1	2.1
40-49 years	7	14.9
50-59 years	16	34.0
60 years and above	22	46.8
Type of caregiver		
Informal caregiver	44	93.6
Formal caregiver	3	6.4
Training experience of formal caregiver (n=3)		
Yes	0	0
No	3	100
Care-receiver's disease		
MNDs	27	57.4
PD	11	23.4
Both MNDs and PD	9	19.1
Experience in receiving training in oral care		
Yes	11	23.4
No	36	76.6

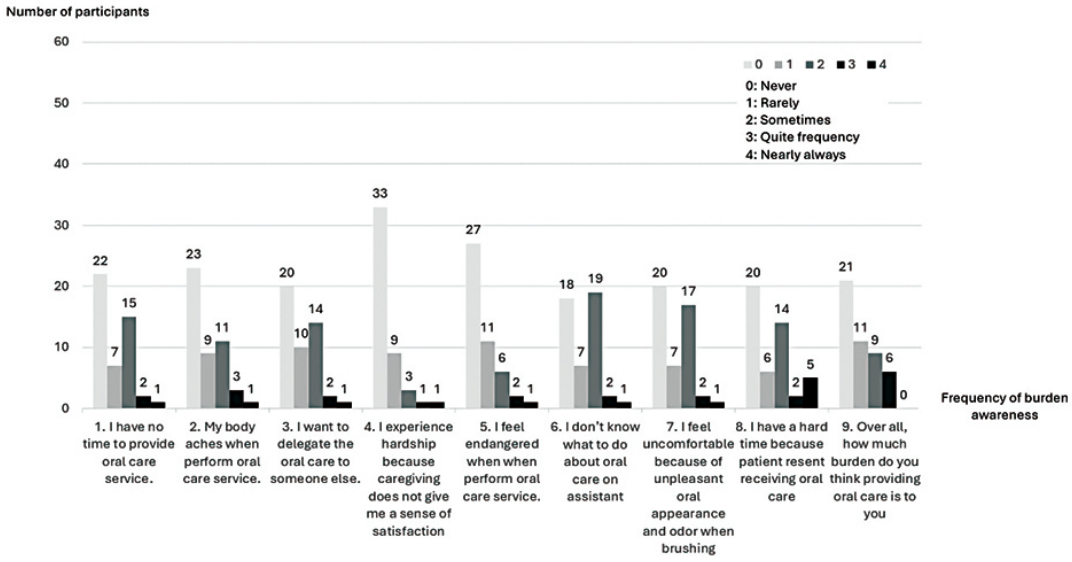

Figure 3: Results of Thai-version of OHIB from research participants (N=47).

A) Psychometric properties of a questionnaire

For psychometric properties of the questionnaire in this research, Cronbach's alpha coefficient of T-OHBI was at a good level. When compared with the test using the Japanese version of the OHBI questionnaire, The TB of the T-OHBI has a lower Cronbach's alpha coefficient. For SB, the T-OHBI has a higher Cronbach's alpha coefficient.[11]

For discriminant validity, it was found that both the original OHBI questionnaire and the Thai version had

the same result. When testing between the high-burden and low-burden groups, it was found that there were differences between the two groups in every topic in the questionnaire and total score as well.[11]

For test-retest reliability, in this study, the reliability of the existential burden of the Thai version of the questionnaire was at the poor level, which was different from the Japanese version of the questionnaire, where the factor with poor reliability was RB. Compared with the original study, this study reported lower ICC level in SB, EB, and OB but higher in RB subscale. The total score shows that the Thai version of OHBI had a lower reliability level.[11]

This study found that test-retest reliability ranged from poor to moderate levels.[17] The low ICC values may have resulted from the measurement tool's instability. The low ICC values may have resulted from the measurement tool's instability. The T-OHBI may not be suitable for evaluating the effectiveness of caregiver intervention programs, as score fluctuations could occur without an identifiable triggering event.[18]

Given that the test-retest reliability coefficient of the questionnaire fell below the acceptable threshold, it might be due to random error or systematic error.

Random error, the various unpredictable factors that interfere with the accurate measurement of a given phenomenon.[19] Respondents' emotional states may have influenced self-assessments, contributing to the low reliability of the questionnaire in this study. Changes in self-evaluation could affect the second assessment, potentially altering retest results even in the absence of an external triggering event. This phenomenon, response shift, might arise from the fact that respondents had a new conceptualization about the events in the questionnaire or a change in priorities. This could occur when there were changes in the respondent's life, such as health changes, or if the retest interval was too long.[20] Systematic error can arise from deficiencies in the measurement tool[21], the observed issue may be due to inadequacies in the questionnaire's construction, indicating potential flaws in item clarity or content.

Take repeated measurements will be necessary to confirm the property of the questionnaire. To control random errors, recollect data with an increase sample size, and utilizing a sampling strategy with greater precision will be helpful.[22] This approach allows for a clearer distinction between sample-related variability and instrument-related flaws, thereby strengthening the psychometric evaluation. If it is a random error, the results will change.[23]

B) Results from a pilot study

This study found that the most common problem caregivers encountered in providing oral care for patients was "knowledge". Slightly more than half (61.7%) of volunteers felt they did not know what to do about oral care. Secondly, we found a problem SB that includes discomfort from oral appearance and odour and had a hard time from patient's behaviour; along with the feeling of wanting to delegate their oral care duty to other people (57.4%). The results showed that most of the problems encountered by volunteers in this research were in the topic of TB and SB. This finding is in the line with the results from the previous study found that most issues in oral cleaning for patients were usually related to a lack of knowledge about oral cleaning for patients, followed by patients' resistance to oral care.[4]

In this study, there were differences in the sample groups used to assess the questionnaire between the Thai and Japanese versions. Regarding the type of caregivers, the Japanese version of the OHIB collected data from formal caregivers, while the Thai version collected data from both formal and informal caregivers. However, the number of informal caregivers in this study was 90% of the total participants. Previous studies have found that the motivation for caring is different between formal and informal caregivers in some aspects. In formal caregivers, financial factors were the main motivation for caring for the elders. Job satisfaction and whether they find caring meaningful or not played a significant role in their continual commitment to patient care.[24-26] While, for informal caregivers, cultural values and social norms were primary motivators. Whether the caregivers has a sense of reciprocity manifested from gratitude or a sense of obligation from the social belief that caring for patients is a familial duty; they accept their role of caregiving.[24,27]

The different types of caregivers between the two studies may have influenced the results. Previous studies identified the barriers to providing oral care for formal and informal caregivers. For informal caregivers, the barriers were lack of knowledge and skill in oral care. By contrast, formal caregivers also claimed a need for more training, but also cited patient resistance, lack of time, and unclear

responsibility in oral care as additional barriers.[4]

Although participants identified issues in questions 1-8 as problematic, they did not necessarily perceive them as burdensome. It may be considered that those research participants consider those problems to be stressors. We proposed that this phenomenon may be consistent with the characteristic of caregiver burden: some factors that caused the perception of that phenomenon. Having a problem with work tasks was not the only thing that caused caregivers to have a caregiver burden. Each caregiver's characteristics include sociodemographic character, background, and physical and psychological state. Environment, social support, and coping method: These factors affect the caregiver's self-evaluation of their status.[28-30] Previous studies showed that caregivers evaluate factors, and all experiences related to patient care, both positive and negative factors, to assess their condition. If negative factors do not affect the caregiver as much as positive factors, the caregiver may not recognize that they have a caregiver burden.[28]

Previous studies have identified barriers to oral care that do not appear in the OHBI questionnaire; for example, oral care equipment: both in terms of oral cleaning equipment and denture cleaning equipment[3,4,10], as well as results from a study on the oral health care status of dependent elderly people in Thailand, it was identified that those involved in oral health care for the elderly expressed the opinion that They do not have appropriate and adequate oral health care equipment for dependent elderly people. Especially among bedridden patients.[7] In addition, it was found that respecting the patient's autonomy was also considered a problem in oral cleaning for the patient.[31] Some caregivers were concerned that cleaning a patient's mouth may cause harm, such as causing bleeding.[4] There is also the issue of the relationship between individuals responsible for caring for patients.[3,10]

Various cultures and support networks across the country may lead to unique challenges faced by caregivers. Therefore, it may be necessary to consider further procedures to improve the questionnaire, such as open-ended questions to find additional problems in oral care to cover other factors that affect caregiver burden in caregivers in those areas.

Future studies may consider collecting additional information on caregivers' experiences related to caregiving, to examine their association with the level or type of caregiver burden, as well as the strategies used to cope with caregiving-related challenges.

In this study, research participants primarily consisted of elderly, female, informal caregivers. The results from this study, therefore, must be interpreted within similar characteristics of the caregivers. However, it was not an intention of the researchers to limit the inclusion but it was the real nature of the profile of caregivers at the general hospital we collected the data. Additionally, certain characteristics of the sample may affect the instrument validation. For example, older adults may experience difficulties in processing and comprehending written content[32], and it was previously reported that they tend to report higher levels of caregiver burden compared to younger age groups.[33] Some studies have shown that different types of caregivers hold differing values regarding patient care, which may lead to variations in how formal and informal caregivers perceive and interpret caregiving-related issues.[24,26,27] Future studies should consider recruiting more diverse samples, potentially through multi-centre/multi-institutional studies in multiple regions of the country. This approach may result in a sample of caregivers with greater age diversity and a higher proportion of formal caregivers. However, given the current trend in Thailand, where most elderly caregivers are female[1], expanding the data collection sites may not significantly alter the gender composition of the sample in the study.

This questionnaire will help dentists and other healthcare providers to gain a clearer understanding of the specific challenges caregivers face in providing oral care to patients. As a result, they will be better equipped to offer targeted and relevant advice or design intervention specifically fit to caregivers' actual burden. The findings from using T-OHBI will reveal if the burden comes from technical issues of oral care, which is easier to intervene or from more complicated issues such as existential crisis of the caregivers or inability to find positive meaning in caregiving or having a tendency of mental health problems. Besides helping to improve oral care for the patient, it may also help detect psychological burden of the caregivers who need further mental health assessment. The researcher suggest to establish a suitable cut-off score for the future application of this tool. This score will serve as a benchmark for directing caregivers to mental health counseling or obtaining further assistance from medical

professionals. It may also be used as a criterion for providing support to caregivers. In addition, from policy or programmatic perspectives, this questionnaire can be used as a survey tool to screen and later suggest policies that would help reduce the burden specifically from oral care related tasks.

Conclusions

The OHBI questionnaire was successfully translated and culturally adapted into Thai, demonstrating acceptable validity for the Thai population However, its reliability requires further evaluation in larger sample sizes. To enhance its effectiveness, the T-OHBI should be further refined, particularly in detecting caregiver burden factors that the current version fails to identify. This instrument is designed to assess caregiver burden related to oral care for patients with neurocognitive disorders in Thailand, facilitating the identification of burden types and informing intervention strategies to ultimately improve caregivers' quality of life.

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The author obtained permission from the developer of the OHBI to use it in this study.

Conflicts of Interest

The authors declare no conflicts of interest.

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Case Report

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Beyond the Blister: A Case Report on Recurrent Herpes Labialis

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Abstract

The most prevalent viral infection in humans, herpes labialis is caused by the herpes simplex virus 1 (HSV-1). This disease is generally seen in childhood and adolescence. It is a contagious viral infection due to direct contact with active lesions and infected body fluids. It can affect the individuals who are immunocompromised like suffering from Human Immunodeficiency Virus (HIV) infection, undergoing chemotherapy and people who had history of oral herpes. The individuals with exposure to ultraviolet rays, fever, hormonal changes under stress can also become the trigger point for the recurrent infection. The present article reports a case of 60 years old female presented with persistent ulcers on her lower lip for 5 days followed by prodromal symptoms characteristically diagnostic of herpes labialis. She was treated by topical antiviral medication. Treatment is necessary with antiviral drugs therapy for reducing the significant pain and discomfort caused during the infection. Early detection and management is of utmost importance to reduce symptoms and avoid further complications. This case report provides valuable clinical insights into the diagnosis and management of recurrent herpes labialis (RHL), a common but often ignored oral viral condition. Also, it emphasizes the efficacy of topical acyclovir and the supportive role of multivitamin therapy in enhancing immune response and tissue healing.

Keywords: acyclovir, antiviral therapy, herpes simplex virus (HSV), immunity, stress

Introduction

Herpes simplex virus type 1 recurrent oral HSV-1 infection causes the common oral mucosal disorders including acute herpetic gingivostomatitis (AHG) and herpes labialis (HL). HL, commonly known as cold sores. This is a condition characterized by the formation of multiple blisters on the vermilion border of the lips or the surrounding skin.[1,2] HSV is highly contagious and usually occurs in childhood. It is highly prevalent viral disease worldwide. Many of the affected patients are asymptomatic. Despite of its majority, prevalence virus remain dormant in the body (latent) and has potential for reactivation. This reactivation can be triggered by ultraviolet rays (sunlight), fever, hormonal changes under stress. Therefore, it is of major concern to prevent the recurrence. Antiviral drugs like acyclovir, famciclovir can be used to manage acute episodes and its recurrences.[3] The present article discusses an occurrence of HL accompanied by AHG in a 60-year-old woman, who was effectively treated with a topical antiviral medication. It is done by evaluating and managing symptoms and preventing future episodes, contributing to a better understanding of the multifaceted treatment strategies for HL. Public awareness and education about the virus and its transmission can also help to reduce the spread of HSV-1 and decrease the stigma associated with the condition.

Case description

A 60-year-old woman presented to the Department of Oral Medicine and Radiology with a chief complaint of persistent ulcers on her lower lip for 5 days. She reported that her symptoms began with a fever and vesicles on lower lip and in oral cavity, which subsequently developed into ulcers. Within 3 days, most of the intraoral vesicles had ruptured. The patient reported a history of occurrence of similar lesions twice in the past, approximately 1 month and 3 months prior, which resolved within a week without treatment. Notably, there was no history of similar lesions on other parts of the body, including the genitals, eyes, or skin. Her medical history revealed a 4-year history of hypertension, for which she was currently receiving medication and her blood pressure is now normal. The patient's family history was non-contributory. All of the vital signs were found to be within the normal range during the general physical examination. Lymph node assessment showed no evidence of regional lymphadenopathy.

Extraoral examination revealed multiple haemorrhagic crusted ulcers on the lower lip and vermilion border with erythematous base, measuring approximately 1 x 0.5 cm in size, with a roughly oval shape. There were 5-6 ulcers in total, characterized by well-defined borders and sloping edges. Ulcers were mildly tender on palpation and no bleeding or pus discharge seen on manipulation (Figure 1).

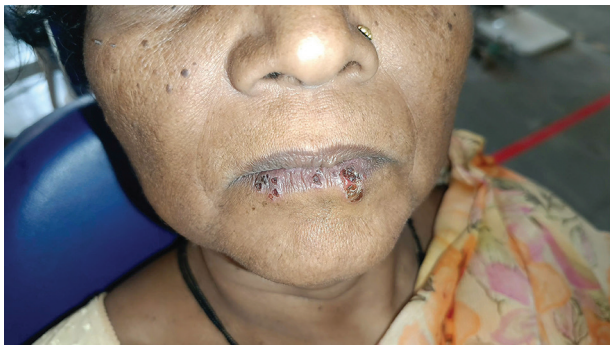

Figure 1: Multiple haemorrhagic crusted vesicles and ulcers on lower lip and vermillion border.

Intra oral examination showed adequate mouth opening and no abnormalities were detected on soft tissue examination. The marginal and attached gingiva in lower arch was observed to be swollen, bright red, and highly tender, with bleeding on probing, consistent with a diagnosis of acute gingivitis (Figure 2). Acute gingivitis is managed through the removal of local irritants that includes scaling and polishing to eliminate plaque and calculus, followed by patient education on oral hygiene practices. Also the antimicrobial mouth rinses, chlorhexidine gluconate 0.12-0.2% was advised for 7days to reduce bacterial load and inflammation.[4]

Based on the patient's history and clinical presentation, a provisional diagnosis of recurrent herpes labialis (RHL) was made, and the patient was subsequently treated with antiviral medication. Topical 5% acyclovir cream was prescribed, to be applied five times a day for 7 days to the affected area. The patient was also prescribed vitamin B complex supplements for 7 days, and the lesion resolved completely within a week. At the follow-up visit seven days later, the lesions had completely resolved (Figure 3). The patient received counselling about her condition, as well as preventive measures regarding recurrence to help avoid HSV infection.

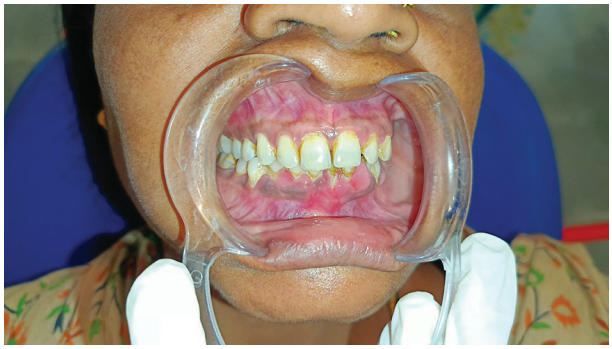

Figure 2: Swollen bright red marginal and attached gingiva in lower arch.

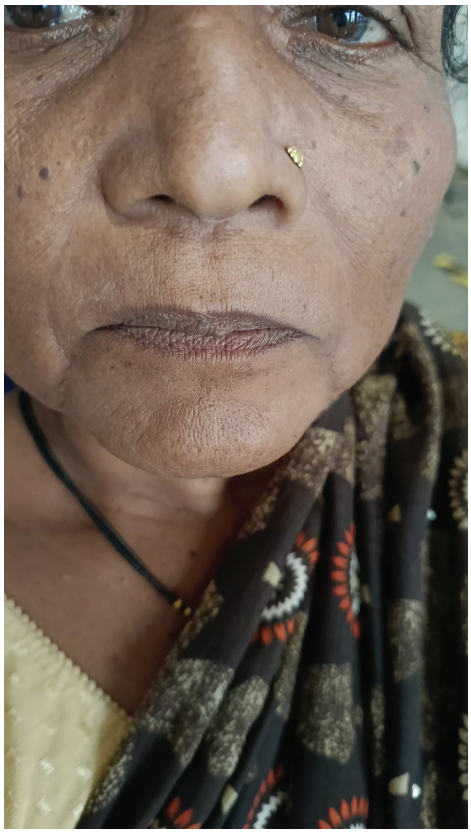

Figure 3: Showing healed lesions after 7 days.

Discussion

The HSV-1 virus will penetrate the mucosal surface and be transmitted through oral secretions, by direct contact with contaminated saliva or respiratory droplets. HSV enters host cells through the interaction of several glycoproteins on the surface of the viral envelope with host cell surface receptors. Glycoprotein B (gB) in the virus will bind to the heparin sulfate (HS) receptor on host cells so that the HSV-1 virus will settle in the dorsal ganglia, especially the trigeminal nerve ganglion and can be reactive if there is a trigger.[5] This form of reactivation of the HSV-1 virus can be triggered if there is a decrease in immunity due to changes in weather, fever, sun exposure, emotional stress, trauma, menstruation, systemic diseases, allergies, and immunosuppression. Recurrent lesions in immunocompromised patients are often more aggressive and take an extended period to heal than normal patients.[6] In this case report, it is suspected that the trigger factor for HSV infection might be reduced immunity with ageing.

The trigger factor becomes a stimulus in the sensory nucleus where the virus is dormant in the latent phase, both in the central and peripheral nerves. When virus reactivation occurs, sufferers usually feel tingling, itching and erythema in the affected area, whereas secondary infection often occurs in the labial area or outside the vermilion border. The next phase is the inflammatory phase which usually occurs on the first day, the virus begins to produce and infect nerve endings. The cells will react to the invasion of the virus which is characterized by erythema. On the second to third day there is a pre-afternoon phase which is characterized by the appearance of papules and vesicles that feel itchy and sensitive to touch.[7]

About 16% and 38% of people are affected by RHL.[1] RHL is usually diagnosed based on the patient's medical history and clinical presentation. One of the interesting or incomparable presentation in this case was bilateral involvement. Nevertheless, it is well known that RHL typically presents as unilateral lesions, often on the vermilion border of the upper or lower lip. The possible explanation for this bilateral involvement could be a wider distribution of viral reactivation across bilateral nerve branches, a compromised immune status due to aging, or a higher local viral load.[8] Although the lesion showed characteristic clinical feature such as confinement to the lower lip and vermillion border, differential diagnosis was considered to rule out other possible ulcerative or vesiculobullous conditions with overlapping clinical features. Similar presentations can occur in conditions such as aphthous stomatitis, erythema multiforme, herpangina, and varicella zoster infection. However, aphthous ulcers typically appear on non-keratinized mucosa (e.g., buccal mucosa, labial mucosa), and lack the prodromal symptoms seen in herpes. Erythema multiforme often involves the entire lip with characteristic target lesions on the skin,

which were absent. Herpangina presents with ulcers in the posterior part of the oral cavity. Similarly, Varicella zoster virus (VZV) infection typically shows a dermatomal, unilateral distribution and may involve skin and mucosa simultaneously. In contrast, this case showed clustered, crusted ulcers localized to the lower lip, preceded by prodromal signs, and a rapid therapeutic response to topical acyclovir, all of which are characteristic of RHL.[8,9]

Laboratory tests such as the tzanck test, immunofluorescence, and serological assays for anti-HSV IgM and IgG can be used to confirm the diagnosis during times of uncertainty. However, viral culture remains the gold standard for confirming the diagnosis as it isolates live virus for strain identification and confirms active infection. It is also useful for performing antiviral sensitivity testing when resistance is suspected.[10]

Most cases of herpes simplex infection resolve on their own. However, antiviral therapy provides substantial clinical benefits for most symptomatic patients and is the cornerstone of treatment. The three antiviral medications commonly used to manage symptomatic HSV infections are acyclovir, valacyclovir hydrochloride, and famciclovir.[1,11] Acyclovir has been demonstrated to be safe for prolonged suppression of RHL and genital herpes infections, despite the availability of other antiviral drugs. Acyclovir systemically at a dosage of 5 mg/kg to 10 mg/kg is the suggested regimen; this should be given every 8 hours for 2 to 7 days, or until clinical improvement is observed. After this oral antiviral therapy should be given to ensure a complete treatment duration of at least 10 days.[11] Acyclovir administered systemically reduces pain and accelerates the healing process and resolution of viral shedding. Lesion location and the infection history (primary or recurring) determine the dosage and duration of the therapy. It is critical to understand that instances of HSV encephalitis and serious HSV infections in people who are immunocompromised require high-dose intravenous acyclovir, which is frequently started therapeutically.

In the present case, therapy was in the form of causative and supportive care using topical antivirals, namely 5% acyclovir cream and multivitamins. The patient was treated with topical 5% acyclovir cream, applied four times a day for seven days. Starting treatment early, ideally within 48 hours of symptoms, helps to speed up healing and reduce discomfort. Because the patient's general condition was good, for the treatment of infection in this case it was enough to use topical drugs that are applied to the lesion area.[12] Acyclovir first breaks down to acyclovir monophosphate by viral thymidine kinase which is found in viruses. Cellular guanylate kinase then transforms this to acyclovir diphosphate, then phosphoglycerate kinase, phosphoenolpyruvate carboxykinase, and pyruvate kinase finally convert it to acyclovir triphosphate. Acyclovir triphosphate is the cause of the inhibitory effect of viral DNA polymerase and viral replication of genomes. acyclovir triphosphate blocks DNA synthesis by serving as a chain terminator. Since acyclovir selectively inhibits normal cellular thymidine kinase and has minimal cytotoxicity, it cannot be efficiently used as a substrate by uninfected cells. Patients with hypersensitivity, particularly those with renal failure, immunocompromised hosts, possible risk of thrombotic thrombocytopenic purpura (TTP) should not use acyclovir.[13]

In this case report, it is suspected that the trigger factor for HSV infection might be reduced immunity with ageing. In this case, the most probable trigger factor for the RHL was age-related immunosenescence, as the patient was 60 years old. With advancing age, the immune system undergoes functional decline, leading to decreased surveillance and reactivation control of latent viruses like HSV-1.[14] Immunocompromised individuals, such as those with Human Immnodeficiency Virus (HIV), are at higher risk of severe HSV infections and may require systemic or intravenous antivirals. Since acyclovir is primarily excreted by the kidneys, monitoring renal function is essential to avoid potential nephrotoxicity.[15]

Host immunity is a key factor in viral infection. Improving the immune system, both natural and adaptive, can be achieved by improving nutrition, improving psychological conditions and administering multivitamins. Multivitamin Becom-zet® which consist of vitamin B complex, vitamin C, E and zinc which acts as a catalyst and regulator of biochemical reactions in the body. Vitamin B is a water-soluble vitamin that serves as a cofactor and coenzyme, playing a significant role in the immune response by supporting the function of CD8 T cells and natural killer (NK) cells. It can increase the patient's immune system through adequate intake of the vitamins. Multi vitamins needed and prevent the occurrence of functional metabolic disorders that cause reduced vitamin

intake also accelerates the change of proline and lysine residues in procollagen to hydroxyproline and hydroxylysine in collagen synthesis.[12,16] This process plays a key role in speeding up the healing process. Patients are also advised to manage stress and adequate rest and maintain nutrition and hydration to prevent recurrence of herpes labialis.

Conclusions

The dentist plays a critical role in the comprehensive management of RHL. From early diagnosis and accurate differentiation of lesions to the initiation of appropriate antiviral therapy and patient education, their involvement ensures timely and effective management. By addressing potential triggers, advising on preventive strategies, and providing supportive care, dentists help to reduce the frequency and severity of recurrences.

Clinical significance

Early recognition and prompt antiviral treatment of recurrent herpes labialis can significantly reduce lesion severity, healing time, and patient discomfort, emphasizing the importance of timely clinical intervention.

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