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In vitro demineralization techniques used to form white spot as an initial caries lesion: a systematic review

UPDATE

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Técnicas de desmineralização *in vitro* utilizadas para formação de mancha branca como lesão inicial de cárie: uma revisão sistemática

Abstract

Objective: The objective of this systematic review was to describe and compare the most commonly used *in vitro* demineralization techniques for the formation of white spot lesions as an initial caries lesion.

Methods: This study was conducted in accordance with the updated PRISMA 2020 guidelines for the publication of systematic reviews. A systematic search of the scientific literature was performed to identify original research that met the eligibility criteria, using the PICO model for systematic reviews. The search terms were: caries, white spot lesion, *in vitro*, and demineralization. *In vitro* studies with clear and replicable methodologies were included, while systematic reviews, *in vivo* studies, and those lacking sufficient methodological detail were excluded. The search was carried out in LILACS, PubMed, and Web of Science, for articles published in 2024.

Results: A total of 29 full-text articles that met all eligibility criteria were included in the systematic review. The demineralization technique using acid solutions was applied in 65.52% of the studies (20 of 29 articles), with acetic acid being the most commonly used solution (44.83%). The pH cycling technique was employed in 31.03% of the studies (9 of 29 articles), with acetic acid predominating in 27.59% of cases. The use of acid solutions allows for controlled demineralization conditions; the pH of the acids ranged from 4.0 to 5.5, with 4.5 being the most frequent. Exposure times varied from 36 hours to 22 days, most commonly around 4 days. The use of pH cycles simulates the dynamic conditions of demineralization and remineralization found in the oral environment. Solutions with a pH of 4.4 were used for demineralization and a pH of 7.0 for remineralization. The cycles had variable exposure times—6 hours for demineralization and 18 hours for remineralization—repeated for 5 to 10 days.

Conclusion: Demineralization with acid solutions is the most widely used technique due to its simplicity and experimental control. Demineralization using pH cycling offers greater similarity to clinical conditions but requires a more complex protocol. No *in vitro* technique presents an accepted standard formula for the formation of white spot lesions as an initial caries lesion, highlighting the need to establish uniform guidelines in future studies.

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Keywords: enamel caries; white spot lesions; *in vitro*; demineralization; acetic acid (MeSH).

Resumen

Objetivo: El objetivo de esta revisión sistémica fue describir y comparar las técnicas *in vitro* de desmineralización más usada para la formación de mancha blanca como lesión de caries inicial.
Métodos: El presente estudio está enmarcado en la guía actualizada para la publicación de revisiones sistemáticas PRISMA 2020. Se realizó una búsqueda sistemática de la literatura científica para identificar investigaciones originales que cumplieran con los criterios de elegibilidad, usando el modelo PICO para revisiones sistémicas. Las palabras de búsqueda fueron: caries, lesión de mancha blanca, *in vitro* y desmineralización. Se incluyeron estudios *in vitro* con metodología clara y replicable, se excluyeron revisiones sistemáticas, estudio in vivo y aquellas sin suficiente detalle metodológico. Se realizó la búsqueda en LILACS, Pubmed y Web of Science, de artículos publicados durante el año 2024.
Resultados: Fueron 29 los artículos completos incluidos para la revisión sistémica que cumplieron con todos los criterios de elegibilidad. La técnica de desmineralización con soluciones ácidas fue utilizada en el 65,52% de los estudios (20 de los 29 artículos), siendo el ácido acético la solución ácida más común con el 44,83%. La técnica de ciclos de pH fue empleada en el 31,03% de los estudios (9 de 29 artículos), con un predominio del ácido acético en el 27,59% de los casos. El uso de las soluciones ácidas permite una condición controlada de desmineralización, el pH varió entre 4,0 y 5,5 de acidez, con una frecuencia mayor de pH de 4,5; el tiempo de exposición osciló desde 36 horas hasta 22 días, siendo más común el rango de 4 días. El uso de los ciclos de pH simulan condiciones dinámicas de desmineralización y remineralización, similares al medio oral, se alternaron soluciones con pH de 4,4 en la desmineralización y pH de 7,0 para la remineralización; los ciclos tuvieron tiempos de exposición variable, 6 horas para la desmineralización y 18 para la remineralización, repetidos durante 5 a 10 días.
Conclusión: la técnica de la desmineralización con soluciones ácidas es la más utilizada debido a su simplicidad y control experimental. La desmineralización mediante los ciclos de pH ofrece mayor similitud con las condiciones clínicas, pero requieren un protocolo más complejo. Ninguna técnica *in vitro* presenta una formula estándar aceptada para la formación de mancha blanca como lesión de caries inicial, resaltando la necesidad de establecer lineamientos uniformes en futuros estudios.

Palabras clave: caries en esmalte; lesiones de mancha blanca; *in vitro*; desmineralización, ácido acético (DeCS).

Resumo

Objetivo: O objetivo desta revisão sistemática foi descrever e comparar as técnicas de desmineralização *in vitro* mais comumente utilizadas para a formação de mancha branca como lesão inicial de cárie.
Métodos: O presente estudo está enquadrado no guia atualizado para publicação de revisões sistemáticas PRISMA 2020. Foi realizada uma busca sistemática na literatura científica para identificar pesquisas originais que atendessem aos critérios de elegibilidade, utilizando o modelo PICO para revisões sistemáticas. As palavras de busca foram: cárie, lesão de mancha branca, *in vitro* e desmineralização. Foram incluídos estudos *in vitro* com metodologia clara e replicável, foram excluídas revisões sistemáticas, estudos in vivo e aqueles sem detalhamento metodológico suficiente. A busca foi realizada nas bases LILACS, Pubmed e Web of Science, por artigos publicados durante o ano de 2024.
Resultados: Foram incluídos 29 artigos completos para a revisão sistemática que atenderam a todos os critérios de elegibilidade. A técnica de desmineralização com soluções ácidas foi utilizada em 65,52% dos estudos (20 dos 29 artigos), sendo o ácido acético a solução ácida mais comum com 44,83%. A técnica de ciclagem de pH foi utilizada em 31,03% dos estudos (9 de 29 artigos), com predomínio do ácido acético em 27,59% dos casos. A utilização de soluções ácidas permite uma condição controlada de desmineralização, o pH variou entre 4,0 e 5,5 dos ácidos, com maior frequência de pH de 4,5; O tempo de exposição variou de 36 horas a 22 dias, sendo mais comum o intervalo de 4 dias. A utilização de ciclos de pH simula condições dinâmicas de desmineralização e remineralização, semelhantes ao meio oral, soluções com pH 4,4 foram alternadas na desmineralização e pH 7,0 na remineralização; Os ciclos tiveram tempos de exposição variáveis, 6 horas para desmineralização e 18 para remineralização, repetidos por 5 a 10 dias.
Conclusão: A desmineralização com soluções ácidas é a técnica mais amplamente utilizada devido à sua simplicidade e controle experimental. A desmineralização por ciclagem de pH oferece maior similaridade com as condições clínicas, mas requer um protocolo mais complexo. Nenhuma técnica *in vitro* apresenta uma fórmula padrão aceita para a formação de manchas brancas como lesão inicial de cárie, destacando a necessidade de estabelecer diretrizes uniformes em estudos futuros.

Palavras-chave: cárie do esmalte; lesões de manchas brancas; *in vitro*; desmineralização, ácido acético (DeCS).

Introduction

White spot lesions, considered the initial carious lesion in tooth enamel, are defined as the initial manifestation of dental caries. When air is applied and the tooth surface is dried, opaque spots become visible, which are unequivocal indicators of an active lesion. The enamel loses its brightness, and the surface becomes porous and rough, giving it a chalk-white appearance. This appearance results from differences in the refractive index between healthy and demineralized hydroxyapatite.[1]

On smooth tooth surfaces, these lesions appear as a cone with a wide base toward the surface and an apex directed toward the amelodentinal junction. At the level of pits and fissures, the process begins on the lateral walls, forming an inverted cone with its base toward the amelodentinal junction and its apex toward the surface.[2] Because surface enamel contains more organic material, it is more resistant and about 0.1–0.2 mm thick. Thus, in white spot lesions as initial carious lesions, the superficial layer remains relatively intact, with incipient demineralization and minimal porosity. Beneath this surface layer lies the body of the lesion, characterized by a significant mineral loss and greater pore volume.[3]

Biochemically, microorganisms in dental plaque produce acids that penetrate the enamel by diffusion through its porous matrix. These acids compete with the proteins and lipids in the organic matrix, dissolving the hydroxyapatite crystals. Lactic acid is the most aggressive, but other acids such as formic, acetic, and propionic are also produced. They can penetrate through diffusion channels in the enamel's organic matrix. Crystal damage occurs through selective dissolution of hydroxyapatite, producing slight etching on the enamel surface. This process typically reaches the critical pH of 5.5 for hydroxyapatite.[4]

To demineralize the enamel surface and induce white spot lesions *in vitro* as an initial carious lesion, various techniques are used to simulate conditions in the oral cavity. These techniques are primarily employed in research to study the early stages of caries formation and to evaluate the effectiveness of different remineralizing treatments. The most common approach involves the use of acid solutions, such as lactic or acetic acid, to lower the pH and replicate the acidic environment that promotes enamel demineralization. Concentrations and exposure times vary by protocol, but solutions with pH values between 4.0 and 5.5 are generally used.[2,5–7] Another frequently used method is pH cycling, which reproduces the pH fluctuations that occur in the mouth during food and beverage consumption. Samples are alternately immersed in acidic and neutral or slightly alkaline solutions. This demineralization–remineralization cycle simulates the dynamic conditions of the oral cavity.[8–10]

Each of these techniques can be adapted and modified according to the specific objectives of the study, allowing different aspects of demineralization and the effectiveness of preventive or therapeutic treatments to be investigated. The *in vitro* formation of white spot lesions as initial carious lesions is essential for simulating early caries conditions and for assessing the efficacy of remineralizing treatments. Despite advances in research, there is no standardized technique for this simulation, which makes comparison between studies difficult and limits the application of findings in clinical protocols. Therefore, this systematic review aims to analyze the methodologies used, identify the most commonly applied techniques, and emphasize the need for standardization to ensure reproducible and clinically relevant results. The objective of this systematic review was to describe and compare the methodologies and techniques of acid-solution demineralization and acid pH cycling for the *in vitro* formation of white spot lesions as initial carious lesions in studies published in 2024.

Methodology

The systematic literature search followed the updated PRISMA 2020 (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines for the publication of systematic reviews and meta-analyses. These guidelines enabled the identification, selection, evaluation, and synthesis of the studies included in this review.[11] To develop the research question: *What is the most commonly used demineralization technique for the formation of white spot lesions in vitro?*, the PICO model (Population, Intervention, Comparison, Outcome) for systematic reviews was used.

SEARCH STRATEGY

To address the research question, searches were conducted in the LILACS database (in Spanish) and in PubMed, Web of Science, and Scopus (in English) for articles published between January and August 2024. The short search period was chosen to ensure completeness and allow a rigorous analysis aligned with PRISMA guidelines. This approach also aimed to focus on recent studies that are more representative and relevant for discussing the current methodologies for generating white spot lesions as initial carious lesions. The search terms used were "caries," "*in vitro*," and "demineralization" (**Table 1**).

Tabla 1 Estrategias e Indicadores de búsqueda

Database	Keywords:
LILACS	Caries, white spot lesion, *in vitro*, and demineralization
PubMed	Caries, white spot lesion, demineralization, and *in vitro*
Web of Science	Caries, white spot lesion, demineralization, and *in vitro*

ELIGIBILITY CRITERIA

All publications were evaluated by the main reviewer according to the inclusion criteria: studies with title, abstract, and full text; *in vitro* studies; original articles on the use of acid solutions for the *in vitro* formation of white spot lesions as initial carious lesions; original articles on the use of pH cycling for the same purpose; and, primarily, open-access publications to ensure transparency, reproducibility, and accessibility of data. This facilitates their use by other researchers and professionals without economic or access restrictions, promoting a more inclusive and collaborative science. Exclusion criteria included original articles using fermentable carbohydrate solutions; studies involving prolonged acid exposure; studies on acid erosion or biofilm demineralization; articles without full text available; letters to the editor; and book chapters. Systematic review articles were included only for cross-referencing purposes. Eligibility criteria were defined using the PICO model for systematic reviews (**Table 2**).[12,13]

Table 2 PICO criteria.

P	Population/Patient	White spot lesions as initial carious lesions in dental enamel created *in vitro*
I	Intervention	Demineralization techniques: use of acid solutions and pH cycles
C	Comparison	Comparison between acid solution and pH cycling techniques
O	Outcome	Identification of solution composition, pH levels used, and time required to produce white spots

FILTERING AND SCREENING

Search results were exported to Zotero software, and duplicates were removed. Two calibrated reviewers (CDH and LLO) independently evaluated the articles to identify the most relevant ones. If the search terms appeared in the title and abstract, the article was selected for full-text review. Two reviewers independently examined the full texts of the selected studies and determined final eligibility.

ELIGIBILITY ASSESSMENT AND VERIFICATION

The articles retrieved from all databases that met the eligibility criteria were processed for data extraction. Disagreements among the reviewers were resolved through discussion; if disagreement persisted, a third reviewer was consulted to resolve the conflict until consensus was reached.

DATA EXTRACTION

Two reviewers (CDH and LLO) extracted data from the full-text articles that met the eligibility criteria using Microsoft Excel and previously designed data extraction forms. The extracted data included the methodology employed, the composition of the solutions used in both the demineralization and pH cycling techniques, the pH values induced in each technique, and the type of acid used for demineralization.

RISK OF BIAS ASSESSMENT

The GRADE system (Grading of Recommendations Assessment, Development, and Evaluation)[14] was used to determine the certainty of the evidence related to enamel demineralization using the two techniques, the use of different acids, and the critical pH values. The GRADE process for systematic reviews followed these steps: the research question was formulated, outcomes were selected, evidence was identified, summaries were prepared, and the quality of the evidence was evaluated using an evidence table.

The data used for the final analysis included: research objectives, sample characteristics, the acidic substance used in the demineralization or pH cycling technique, the critical pH employed for white spot formation, the temperature used, and the exposure time of the samples during demineralization or pH cycling.

Results

Following the search, identification, filtering, and selection process outlined by the PRISMA guidelines (**Figure 1**), a total of 170 publications were identified across the three databases. Of these, 27 were duplicates, 31 were deemed ineligible, and 37 had restricted access. This left 50 publications, which were screened according to the eligibility criteria applied to titles and abstracts. During the selection process, 33 full-text articles were reviewed; of these, four were excluded because their methodological components were conducted in vivo, and seven were excluded because they could not be retrieved from the databases. Ultimately, 29 full-text articles were included for evaluation and verification in this systematic review. Consensus among reviewers was achieved for the final inclusion.

The initial distribution of the results was organized according to the two evaluated techniques. The demineralization technique using acid solutions was employed in 65.52% of the studies (20 of 29 articles), with acetic acid being the most common component (44.83%), followed by lactic acid (20.69%) and formic acid (3.45%). The pH cycling technique was applied in 31.03% of the studies (9 of 29 articles), with acetic acid predominating in 27.59% of cases (**Table 3**).

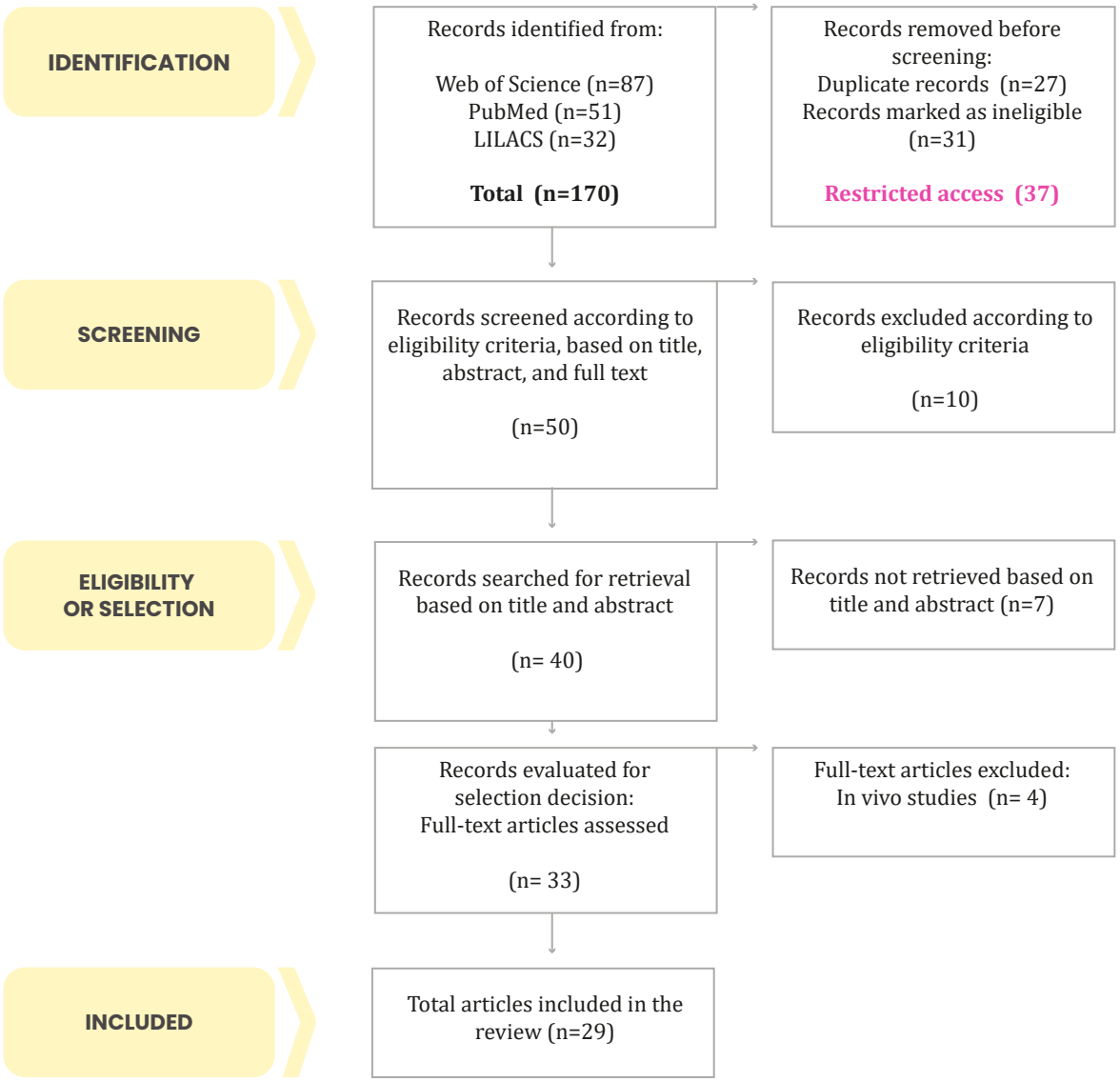

Figure 1 PRISMA flow diagram summarizing the process of searching, identifying, and selecting publications from databases and registries for the systematic review.

Table 3
Technique and acid used for the formation of white spot lesions as an initial carious lesion *in vitro*

Technique	Acid used	N	%
Demineralization with acid solutions	Acetic	13	44,83%
	Lactic	6	20,69%
	Formic	1	3,45%
	Total	**20**	**65,52%**
pH cycles with acid	Acetic	8	27,59%
	Lactic	1	3.45%
	Formic	0	0,00%
	Total	**9**	**31,03%**
Total		**29**	**100%**

The key experimental parameters for acetic acid demineralizing solutions ranged from 0.05 M to 50 mM, depending on each study's design and objectives. In most cases, the demineralizing solution was supplemented with salts such as calcium chloride and monopotassium phosphate to simulate the ionic environment of enamel. The pH values ranged between 4.4 and 5.5, with 4.5 being the most frequent. This range is considered optimal for inducing enamel demineralization without completely disintegrating its structure. All studies were conducted at a temperature of 37 °C to replicate physiological conditions. Demineralization times varied widely, from 16 hours to 22 days, with daily renewal of the solution. On average, four days were sufficient to produce visible and homogeneous lesions (**Table 4**).

The studies that used acetic acid with this technique pursued various objectives, including the evaluation of remineralizing treatments—for example, the effectiveness of agents such as nanohydroxyapatite, casein phosphopeptide, and fluoride in the remineralization of demineralized enamel—or the analysis of dental materials, assessing the effects of infiltrating resins on microhardness, color, and surface roughness of treated enamel. Some studies used acetic acid to develop experimental models that closely replicated early caries conditions.

An analysis of similarities among the studies showed convergence in maintaining a pH close to 4.5 and a constant temperature of 37 °C, reinforcing the validity of these parameters as standards for this technique. All studies included calcium and phosphate in their solutions—essential elements for replicating the oral environment and generating controlled lesions. Regarding differences, exposure times varied considerably, from hours to weeks, reflecting the absence of a standardized protocol. Although acetic acid was the most frequently used, variations in its concentration may affect reproducibility across studies.

Table 4
Technique Using an Acid Solution or Demineralization with Acetic Acid (CH_3COOH) for the Formation of White Spots as Initial Carious Lesions *In Vitro*

N	Objective of the research	Sample	Demineralization components	pH	Temp	Time
1	Evaluate changes in white spot lesions treated with resin infiltration. [6]	56	• 2.2 mM calcium chloride • 2.2 mM monopotassium phosphate • 0.05 M acetic acid	4,4	37°C	4 days
2	Compare enamel composition at the level of amorphous calcium phosphate with fluoride and biomimetic nanohydroxyapatite for enamel remineralization.[15]	96	• 2.2 mM/L calcium chloride • 2.2 mM/L monosodium phosphate 50 mM/L acetic acid	4,5	Not mentioned	4 days, renewed daily

3	Evaluate *in vitro* the effect of protocols and anticaries agents containing casein phosphopeptide–amorphous calcium phosphate, sodium trimetaphosphate, and fluoride on caries lesion remineralization.[16]	60	• 1.3 mM calcium chloride • 0.78 mM monopotassium phosphate • 50 mM acetic acid	5,0	37	16 hours
4	Determine the effectiveness of infiltrating resin on roughness and hardness.[2]	80	• 2.2 mM calcium nitrate • 2.2 mM monopotassium phosphate • 50 mM acetic acid	4,5	37 °C	72 hours, renewed every 24 hours
5	Evaluate biomimetic enamel remineralization using a biomimetic analog to interact with and absorb bioavailable calcium and phosphate ions and induce crystal nucleation in demineralized enamel.[17]	45	• 50 mM acetic acid • 2.2 mM calcium nitrate • 2.2 mM monopotassium phosphate • 5.0 mM sodium azide • 0.5 ppm sodium fluoride	4,5	37°C	3 days
6	Evaluate the effect of bleaching after resin infiltration on surface roughness.[5]	25	• 0.3 g/L calcium chloride • 0.4 g/L potassium dihydrogen phosphate • 2 mL/L methylenediphosphonic acid • 3 mL/L acetic acid • 5 mL/L potassium hydroxide	4,95	37°C	5 days
7	Assess the suitability of high-resolution time-of-flight secondary ion mass spectrometry for imaging cross-sectional changes in enamel microstructure and chemical composition during treatment and remineralization cycles.[18]	50	• 2 mM monosodium phosphate monohydrate • 2 mM calcium chloride dihydrate 75 mM glacial acetic acid • Carbopol 907	4,3	37°C	36 hours
8	Evaluate the release of fluoride, calcium, and phosphate ions from Predicta® Bioactive Bulk-fill composite compared with EQUIA Forte® and Filtek™ Z350.[19]	39	• 2.0 mM calcium nitrate dihydrate 2.0 mM monopotassium phosphate • 75 mM acetic acid	4,4	37	48 hours
9	Compare the remineralizing potential of Moringa oleifera extract, eggshell, and sodium fluoride varnish on the microhardness of artificially demineralized enamel.[20]	44	• 50 mM acetic acid • 2.2 mM calcium nitrate dihydrate 2.2 mM monopotassium phosphate • 0.1 ppm sodium fluoride	4,8	37°C	96 hours

10	Evaluate the potential of different fluoride varnishes to inhibit the progression of incipient caries lesions after cariogenic challenge.[21]	75	• 0.05 M acetic acid • 1.28 mM calcium nitrate • 0.74 mM monopotassium phosphate • 0.03 mM sodium fluoride	5,0	37°C	48 hours
11	Evaluate and compare the remineralization efficacy of flaxseed paste and chicken eggshell paste with standard fluoride toothpaste in primary teeth.[22]	40	• 0.4723 g calcium nitrate • 0.2722 g potassium dihydrogen phosphate • 4.5083 g acetic acid	4 and 4,5	37°C	96 hours
12	Evaluate the effect of artificial enamel lesion infiltration with calcium coacervates and their individual components, including polyacrylic acid.[23]	90	• 50 mM acetic acid • 3 mM calcium chloride dihydrate • 3 mM monopotassium phosphate 6 µL methylhydroxy diphosphonate	4,95	37°C	22 days
13	Evaluate the synergistic effect of eggshell-derived nanohydroxyapatite and carboxymethylchitosan on remineralization.[24]	64	• 2 mM calcium chloride dihydrate 0.0476 mM sodium fluoride • 2.2 mM potassium dihydrogen phosphate • 50 mM acetic acid • 10 mM potassium hydroxide	5,5	No mentioned	72 hours

The pH cycling technique with acetic acid to generate white spots *in vitro*, alternating between demineralization and remineralization phases, aims to reproduce the dynamic conditions of the oral cavity, allowing for a more realistic evaluation of remineralizing treatments. Of the nine studies that employed pH cycling, eight used acetic acid, representing 27.59% of the total studies reviewed (**Table 5**). In all studies, the demineralization phase used an acetic acid solution combined with calcium and phosphate salts (calcium chloride and monopotassium phosphate) to simulate the ionic environment of dental enamel, typically at a concentration of 0.05 M. The remineralization phase included neutral or slightly alkaline solutions (pH 7.0) composed of potassium chloride, calcium, and sodium phosphate—key elements for promoting enamel remineralization. The demineralizing pH (4.4) was appropriate to induce mineral loss in hydroxyapatite, while the remineralizing pH (7.0) simulated a neutral oral environment.

Each cycle consisted of two phases: a 6 to 8 h demineralization phase representing acid exposure episodes in the oral cavity, followed by a 16 to 20 h remineralization phase simulating the action of saliva and the intervals between meals. The total duration of experiments ranged from 5 to 10 days, maintaining a constant temperature of 37 °C to reproduce physiological conditions.

Studies using pH cycling with acetic acid pursued diverse objectives, including the evaluation of dentifrices and varnishes to analyze the effects of remineralizing agents such as functionalized tricalcium phosphate toothpastes and gallic acid varnishes. In addition to studies on color and surface roughness, some works focused on how pH cycling affects the esthetic and physical properties of treated enamel. All studies used a consistent pH range for demineralization (4.4) and remineralization (7.0). The remineralizing solutions contained similar components, such as calcium, potassium, and phosphate, ensuring a degree of experimental uniformity. A standard temperature of 37°C was maintained in all cases. Some studies applied pH cycles for 5 days, whereas others extended the period up to 10 days, depending on the research objectives. Although acetic acid was used consistently, variations in the concentrations of mineral salts in the remineralizing solutions could have influenced the results.

Table 5
pH cycling technique with acetic acid (CH_3COOH) for the formation of white spot lesions as initial carious lesions *in vitro*

N	Objective of the research	Sample	Demineralization components	Remineralization components	pH	Temp	Time
1	Evaluate the remineralization potential of sodium calcium phosphosilicate and functionalized tricalcium phosphate dentifrices. [8]	32	• 2.2 mM calcium chloride • 2.2 mM monopotassium phosphate • 0.05 M acetic acid	• 1.5 mM calcium chloride • 0.9 mM sodium phosphate • 0.15 M potassium chloride	4,4 / 7,0	37 °C	10 days 2 hours demineralization 20 hours remineralization
2	Compare the efficacy of chitosan nanoparticle pretreatment with four different remineralizing agents on artificial white spot lesions. [25]	100	• 2.2 nM potassium phosphate • 2.2 mM calcium chloride • 0.05 M acetic acid	• 0.9 mM sodium phosphate • 1.5 mM calcium chloride • 0.15 M potassium chloride	4,4 / 7,0	Not mentioned	96 hours
3	Evaluate the effect of resin infiltration technique and remineralization of enamel caries induced with fluoride solution on color masking of white spot lesions and surface roughness.[26]	90	• 2.2 mM calcium chloride • 2.2 mM monosodium phosphate • 0.05 M acetic acid	• 1.5 mM calcium chloride • 0.9 mM monosodium phosphate • 0.15 M potassium chloride	4,4 / 7,0	Not mentioned	7 days
4	Evaluate the remineralizing efficacy of freeze-dried coconut extract and coconut milk from freshly grated coconut.[27]	35	• Calcium chloride • Acetic acid • Monobasic potassium phosphate	• Hydrogenated dipotassium orthophosphate Potassium chloride • Calcium chloride	4,4 / 7,0	Not mentioned	72 hours demineralization and 14 days remineralization.
5	Evaluate the efficacy of a remineralizing gallic acid varnish for artificial enamel caries lesions.[28]	55	• 2.2 mmol/L calcium chloride • 2.2 mmol/L sodium phosphate • 50 mmol/L acetic acid • 0.2 mmol/L sodium benzoate	• 1 mmol/L calcium chloride • 3 mmol/L sodium phosphate • 100 mmol/L sodium chloride • 0.1 ppm sodium fluoride • 0.2% sodium benzoate	4,5 / 6,5	Not mentioned	6 days 6 hours demineralization 18 hours remineralization
6	Evaluate the influence of different silver fluoride pretreatment protocols on the adhesive interface of composite resin restorations.[9]	64	• 2.2 mM calcium chloride • 2.2 mM sodium hypophosphite NaH_2PO_2 • 50 mM acetic acid	• 1.5 mM calcium chloride • 0.9 mM sodium phosphate • 0.15 M potassium chloride	4,8 / 7,0	Not mentioned	14 days 8 hours demineralization 16 hours remineralization

7	Establish the development of biomimetic remineralization using organic peptide molecules resembling hydroxyapatite (HA) mineralization in tooth enamel.[29]	50	• 2 mM calcium chloride dihydrate • 2 mM monopotassium phosphate • 50 mM sodium acetate, and • 0.879 mL acetic acid	• 1.2 mM calcium chloride dihydrate • 0.72 mM dipotassium phosphate • 16 mM potassium chloride • 0.2 mM magnesium chloride hexahydrate 50 mM HEPES • 4.5 mM ammonium chloride	4,6 / 7,2	37 °C	7 days 3 hours demineralization 20 hours remineralization
8	Evaluate *in vitro* the effects of the combination of TF and MW, supplemented with sodium trimetaphosphate (TMP) or not, on enamel demineralization.[10]	50	• 2.0 mmol/L calcium phosphate • 0.075 mol/L acetic acid • 0.04 µg/mL fluoride	• 1.5 mmol/L calcium chloride • 0.9 mmol/L dipotassium phosphate • 0.15 mol/L potassium chloride • 0.02 mol/L sodium cacodylate • 0.05 µg/mL fluoride (F)	4,7 / 7,0	37°C	5 days 6 hours demineralization 18 hours remineralization

Studies used lactic acid as a demineralizing agent to generate white spot lesions on tooth enamel *in vitro*. Although less common than acetic acid, this technique stands out for its ability to simulate acidic environments present in the oral cavity, such as those produced by cariogenic bacteria. Of the studies included in the systematic review, 6 used lactic acid as a demineralizing solution, representing 20.69% of the studies that applied demineralization with acid solutions and 20.69% of all studies reviewed (**Table 6**). This percentage positions it as the second most commonly used acid in this technique, after acetic acid, due to its biological nature and its capacity to induce demineralization in a controlled manner.

Lactic acid was used at concentrations between 0.05 M and 0.1 M, often combined with other substances such as calcium chloride, monopotassium phosphate, or thickening agents like hydroxyethylcellulose. The pH of the lactic acid solutions ranged from 4.5 to 5.0, levels considered optimal for inducing demineralization without causing irreversible damage to the enamel structure. This range is close to the critical pH for hydroxyapatite, ensuring the effectiveness of the demineralization process. The demineralization periods varied considerably, from 33 hours to 28 days, depending on the aims of each study. The most common exposure time was 10 days, with periodic renewal of the demineralizing solution. In all studies, the temperature was maintained at 37°C, ensuring conditions similar to those of the human body.

All studies used a pH between 4.5 and 5.0, ensuring that the enamel reached optimal levels of demineralization. The experiments consistently maintained a temperature of 37°C, a common standard in *in vitro* studies. The inclusion of calcium chloride and phosphate in the demineralizing solutions reproduced the mineralized environment of enamel. Some studies used short exposure periods (33 hours), whereas others extended the process up to 28 days, showing variability in protocols according to their objectives. Although the use of lactic acid was consistent, the variation in concentration reflects a lack of standardization in its application.

Lactic acid is naturally produced by cariogenic bacteria, making it a demineralizing agent that more closely represents actual clinical conditions. Lactic acid solutions allow the formation of uniform lesions, facilitating the assessment of treatments. Their ability to maintain a stable pH supports experimental control and repeatability of results. However, exposure times and acid concentrations vary widely across studies, making direct comparison of results difficult. Although biologically relevant, lactic acid is less frequently used, limiting the volume of data available for analysis.

Table 6
Technique using acid solution or lactic acid demineralization for the formation of white spot lesions as initial carious lesions *in vitro*

N	Objective of the research	Sample	Demineralization components	pH	Temp	Time
1	Evaluate the microhardness and surface roughness of human enamel treated with resin infiltration. [7]	80	• 5% methylcellulose • 0.1 M lactic acid	4,6	37°C	10 days
2	Determine the effectiveness of nanohydroxyapatite-enriched universal adhesive resin on color and hardness.[30]	80	• 6% hydroxyethylcellulose gel • 0.05 M lactic acid	4,95 / 5,0	37 °C	10 days with 3 renewals
3	Evaluate the impact of resin infiltration treatment on enamel color stability and surface roughness. [31]	47	• 35 g/250 ml hydroxyethylcellulose gel • 0.1 M lactic acid	4,5	Ambient	9 days
4	Evaluate the combined effect of Biomin F toothpaste and diode laser on remineralization of white spot lesions. [32]	30	• 2.2 mM calcium chloride • 2.2 mM monobasic sodium phosphate • 0.05 M lactic acid • 0.2 ppm fluoride	4,5	37°C	96 hours
5	Evaluate the performance of a new fluoride toothpaste containing microfibrillated cellulose and entrapped silica. [33]	118	• 0.1 M lactic acid • 0.2% Carbopol C907 • 50% hydroxyapatite	5,0	Not mentioned	33 hours
6	Develop a new *in vitro* model for the formation of non-cavitated carious lesions that reflect caries lesion activity. [34]	44	• 0.1 M lactic acid • 1% Carbopol • 50% hydroxyapatite	4,8	37°C	28 days, replaced every 4 days

An article based on pH cycling with lactic acid was also presented, with the aim of simulating the formation of initial caries lesions in a way that more closely reflects real conditions in the oral cavity (**Table 7**).

Table 7
Lactic acid pH-cycling technique for the formation of white spot lesions as initial carious lesions *in vitro*

N	Objective of the research	Sample	Demineralization components	Remineralization components	pH	Temp	Time
1	Evaluate the penetration depth of Icon resin infiltrant using laser scanning microscopy.[35]	22	• 12 mM calcium chloride • 10 mM monopotassium phosphate • 100 mM sodium chloride • 50 mM lactic acid	• 1.5 mM calcium chloride • 5 mM monopotassium phosphate • 100 mM sodium chloride	4,5 / 6,5	37 °C	14 days 6 h demineralization 18 h remineralization

In addition, one article described a more aggressive and direct technique using 5% formic acid. The process consisted of continuous and sustained demineralization (**Table 8**).

Table 8
Technique using acid solution or formic acid demineralization for the formation of white spot lesions as initial carious lesions *in vitro*.

N	Objective of the research	Sample	Demineralization components	pH	Temp	Time
1	Evaluate the diagnosis of proximal carious lesions through different image acquisition and visualization parameters (36)	52	• 5% formic acid	Not mentioned	37 °C	10 hours 20 hours 30 hours

Discussion

This systematic review evaluates and compares acid demineralization techniques and pH cycling using different acids. The selection of demineralization techniques with acid solutions and pH cycles as the only methodologies for the formation of white spots *in vitro* responds to scientific, methodological, and practical reasons that position them as the most relevant approaches to achieving the objectives of this review.

Both techniques are widely used because they allow the controlled reproduction of conditions that induce enamel demineralization, a key process in the formation of white spot lesions as an initial carious lesion. The acid solution technique simulates the damage caused by a constant acidic environment, while pH cycling more dynamically represents changes in the oral environment, including episodes of demineralization and remineralization. Likewise, these techniques are the most frequently employed and documented in the scientific literature. Previous studies have shown that both acid solutions and pH cycling are highly effective in generating reproducible and uniform lesions, allowing different remineralizing treatments to be evaluated consistently. Of the 20 studies that used the acid demineralization technique, 13 used acetic acid, representing 44.83% of the reviewed articles. This result identifies acetic acid as the most widely used compound in this technique due to its ability to generate a critical pH for hydroxyapatite, its ease of handling, chemical stability, and experimental reproducibility.[6,15,16,17]

Methodologies used for *in vitro* white spot formation were described and compared, focusing on widely recognized and practical techniques. The exclusion of other, less common techniques is justified because they are not specifically oriented toward the formation of white spots or present greater complexity and variability. Demineralization with acid solutions allows evaluation of the enamel's response to prolonged acidic environments, such as those associated with a diet rich in sugars. pH cycling replicates physiological fluctuations in the oral environment, making it useful for investigating the effectiveness of preventive and remineralizing therapies under conditions close to reality.

Both techniques are methodologically clear, reproducible, and more accessible than other methods, which facilitates their implementation and comparison of results across different studies. In addition, the simplicity of their experimental design allows greater control of variables, reducing the risk of bias and improving the internal validity of studies. These techniques are frequently used in research but still lack a uniform protocol. Including both in this review highlights the similarities and differences between them and underscores the need for standardization to optimize their use in future studies.

Regarding the use of the acid solution technique or demineralization with acetic acid, the reasons for generating white spot lesions as an initial carious lesion *in vitro* varied, the main one being to assess the effectiveness of infiltrating resin in treating white spot lesions.[2,5,6,23] The pH of the demineralizing solution ranged from 4.4 to a maximum of 5.5, with 4.5 being the most frequent value. Almost all demineralization processes were carried out at 37 °C. In terms of duration, some procedures exposed samples for 36 hours, most used

four days of decalcification, and one study employed 22 days of demineralization (**Table 4**).

It can be argued that the use of acetic acid has the advantage of allowing the formation of reproducible and uniform white spot lesions as initial carious lesions; ideal for evaluating treatments. The protocol is simple and requires fewer resources compared to more dynamic techniques such as pH cycling. A limitation observed with the use of acetic acid is its lack of dynamism; it does not reproduce the pH fluctuations in the oral cavity, which limits the direct clinical applicability of results [17,19,21].

The use of acetic acid to form white spots *in vitro* has been widely validated as an effective experimental model. However, the results highlight the need to standardize protocols—that is, to unify pH, time, and composition parameters—to improve comparability between studies. This analysis shows that studies using acetic acid have made significant advances in understanding dental demineralization but also emphasize the need for greater methodological consistency to maximize the usefulness of this technique in dental research [6,16,19,21].

The pH cycling technique with acetic acid was used in most cases to observe surface remineralization using toothpastes with different active ingredients, evaluating surface color, hardness, and roughness after the formation of white spot lesions (8,25). The process involved subjecting samples to a demineralization cycle lasting 2 to 8 hours, most commonly at a pH of 4.4, followed by exposure to a remineralizing solution for 16 to 20 hours at pH 7.0. Almost all methodologies were performed at 37 °C for 5 or 10 days (**Table 5**).

The advantages of pH cycling are presumed to provide clinical realism, simulating the dynamic conditions of the oral cavity and offering a more representative model of enamel behavior in response to pH fluctuations. This method also allows analysis of both demineralization and remineralization, which is essential for evaluating the effectiveness of preventive and therapeutic treatments. A limitation of pH cycling is its experimental complexity, which requires a more detailed design and handling, increasing the risk of variability between experiments. Furthermore, variability in the duration and composition of cycles makes direct comparison between studies difficult. pH cycling is ideal for evaluating remineralizing therapies under conditions that mimic pH fluctuations in the mouth, such as those caused by food and beverages. This makes it a valuable tool for developing products such as toothpastes, varnishes, and preventive treatments. The technique provides robust and clinically relevant data, but its implementation requires standardization to improve reproducibility and facilitate its use in future studies.[8,25,26,28]

The importance of defining uniform parameters for cycle duration, solution composition, and evaluation criteria is emphasized, as this would allow comparison of results across studies and optimize their applicability to treatment development. This analysis shows that pH cycles with acetic acid offer an advanced and representative—though more complex—methodology for studying the dynamics of enamel demineralization and remineralization.[8,10,26,28]

The demineralization technique using lactic acid employed different concentrations with pH levels ranging from 4.5 to 5.0, most commonly at 37 °C for periods between 33 hours and 28 days to create *in vitro* white spot lesions as initial carious lesions (Table 6). The most frequent research objective was to evaluate resin infiltration treatment for white spot lesions.[7,30,31]

Lactic acid offers a relevant experimental model for analyzing the development of initial carious lesions and the effectiveness of remineralizing treatments. This approach could be particularly useful for studying biomimetic materials designed to interact with real cariogenic conditions. Although less commonly used than acetic acid, lactic acid provides a complementary approach that enhances understanding of demineralization processes and their interaction with therapeutic treatments. However, its implementation requires further standardization to facilitate reproducibility and comparison between studies.[7,30,31]

The importance of defining uniform parameters for concentration, pH, and exposure time is emphasized to optimize its use in dental research and ensure the validity of results. Demineralization with lactic acid is a biologically relevant and effective method for forming *in vitro* white spot lesions, although less used than acetic acid. Despite its potential, high methodological variability and lack of standardization limit its comparability and general applicability. This analysis highlights the importance of including this technique in future studies, particularly for evaluating treatments under realistic cariogenic conditions.[7,30,32,34]

The pH cycling technique with lactic acid was reported in only one article, whose objective was to evaluate the infiltration capacity of a resin for the treatment of white spot lesions. The demineralizing pH was 4.5, applied for 6 hours, and the remineralizing pH was 6.5, applied for 18 hours. These cycles were repeated for 14 days (**Table 7**).[35] Only one article was found that used the demineralization technique with formic acid, aimed at evaluating carious lesions through image visualization. The pH of this acid was 3.75, and it was applied for varying exposure times (**Table 8**).[36]

The most important limitation of this systematic review was the methodological variability in creat-

ing white spot lesions as initial *in vitro* carious lesions, which made it difficult to standardize the methodology according to each type of technique.

White spot formation as an initial carious lesion *in vitro* is a crucial tool for studying the early stages of dental caries and evaluating the effectiveness of remineralizing treatments. The results of this review highlight the need to move toward unified protocols that ensure comparability between studies while providing results relevant to clinical practice. Standardization would allow more precise identification of which treatments are most effective and under which specific conditions they should be applied. Based on this review, it is suggested that international guidelines for *in vitro* simulation of white spot lesions be developed. These guidelines could include uniform composition of demineralizing and remineralizing solutions, predefined pH levels for each technique (for example, critical pH 4.5 for demineralization and pH 7.0 for remineralization in pH cycles), standard exposure times (for example, 6-hour demineralization and 18-hour remineralization cycles for dynamic techniques), controlled temperatures (37 °C as the standard to simulate oral conditions), and clear criteria for evaluating the success of the simulation, such as changes in microhardness, surface roughness, or enamel color.

Conducting further research that directly compares these techniques under controlled conditions, evaluating their reproducibility and clinical applicability is essential. In addition, it is recommended to explore the integration of new technologies, such as mass spectrometry analysis and advanced microscopy, to improve the characterization of white spot lesions as initial carious lesions and to assess the effects of treatments.

Overall, this systematic review highlights the lack of standardization among current methodologies and the urgent need for unified protocols for *in vitro* white spot formation. Such standardization would not only optimize academic research but also enhance the development and validation of preventive and therapeutic treatments for dental caries.

Conclusions

The demineralization technique using acidic solutions, particularly acetic acid, was identified as the most widely employed method for the formation of white spot lesions *in vitro*. However, there is a notable lack of uniformity in solution composition, pH levels, and exposure times, reflecting the absence of a standardized protocol. This methodological variability may compromise the reproducibility of results and their extrapolation to clinical contexts.

The pH cycling technique stands out for its ability to simulate dynamic conditions of demineralization and remineralization, representing a model that more closely reflects fluctuations in the real oral environment. This approach is especially useful for evaluating the effectiveness of remineralizing agents and preventive therapies. However, its implementation is more complex and also lacks standardization in terms of immersion times, pH, and composition of the solutions.

No standard formula was identified for demineralizing or remineralizing solutions, and exposure times varied widely, from hours to weeks. These inconsistencies make it difficult to establish reference protocols that can be applied uniformly in future studies.

Finally, the analysis identified that both acid-solution demineralization techniques and pH cycling present significant limitations in terms of standardization, making comparison between studies difficult.

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Data availability

All data supporting the findings of this study are included within the article

Conflict of Interest

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