pH-CYCLING, DYNAMIC CHEMICAL MODEL IN DENTINE CARIES CREATION. A SYSTEMATIC REVIEW AND META-ANALYSIS

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Abstract

Introduction: The pH-cyling (pHc) is a widely used method applied by cariology researches for lesions-like caries creation. This study evaluates the methodological aspects of pHc, proposes a specific protocol to standardize this demineralization method and quantifies the demineralization yielded by this *in vitro* model in dentine. **Materials and methods:** PubMed, Scopus and WOS databases were used to select studies. Meta-analyses were performed for microtensile bond strength test (μTBS), hardness test, microradiography (MRG) and polarized light microscopy (PLM). **Results and discussion:** Sixty-seven references were selected for qualitative assessment and 32 for meta-analysis. Significant differences were found between sound/ untreated dentine and pH-cycled dentine in all analytical techniques. Considerable heterogeneity was showed by meta-analyses. The results for the depth of demineralization by pHc averaged between 194.95 μm and 298.17 μm for MRG and PLM, respectively. **Conclusions:** pHc is an effective and reproducible *in vitro* procedure to simulate dentine caries lesions for cariology and dental materials research.

Keywords: *dental caries, dentine, meta-analysis, pH-cycling, systematic review, tooth demineralization.*

1. INTRODUCTION

Dental caries is defined as a localized acid attack of the dental hard tissue as a result of the metabolism of bacterial plaque biofilm [1]. The caries process consists of rapidly alternating periods of tooth demineralization and remineralization, leading to the initiation of specific lesions at particular anatomical sites on the teeth [2]. *In vitro* models under different experimental conditions simulate high cariogenic situations, aiming at developing artificial lesions comparable to those produced *in vivo* [3].

Ten Cate and Duijsters [4] first identified the mineral imbalance in enamel subjected to pH-cycling (pHc), as an experimental model to create artificial caries lesions *in vitro*. The pHc model involves a series of combined demineralization and remineralization cycles designed to simulate the dynamics of mineral loss and gain involved during caries formation [5]. In addition to the artificial formation of caries processes, the pHc has also been used for many other purposes, *e.g.*, to simulate pH oral fluctuation [6], to test agents for caries formation [7], for prevention and treatment [8], and even for the creation of erosive lesions [9]. Overall, these applications employ different methodologies by modifying some of the experimental parameters (*i.e.*, exposure time, pH, number of cycles and chemical composition of demineralization-remineralization solutions), depending on the particular research objectives.

Considering the large number of reported protocols for the pHc model, the importance of having summarized data, based on a systematic search of the scientific literature should be highlighted for caries research. Current research and analysis provide an accurate and precise assessment for *in vitro* investigations involving carious processes.

Therefore, the objectives of this systematic review were to evaluate the available scientific literature, to characterize and analyze the methodological approach and uses of the pHc model in caries formation. Likewise, this work aimed at quantifying, by meta-analysis, the

demineralization effects produced by the pHc procedure from the mechanical properties of teeth and depth of demineralization in dentine. Additionally, in this review we propose a specific procedure for dental caries research using pHc in order to standardize this *in vitro* demineralization method.

2. MATERIALS AND METHODS

2.1. ELIGIBILITY CRITERIA

The current systematic review was developed according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist methodology [10]. For this search, the included studies satisfied the following criteria: (1) use pHc *in vitro* model for the development of artificial dentine caries lesions. In addition, (2) studies conducted to test the remineralization agents or previous procedures to pHc were included if at least one experimental or control group was not pre-treated. Moreover, (3) studies performed to test the remineralizing products during pHc were also included when there was an independent experimental or control group treated with distilled/ deionized water. Studies performed in enamel, cementum, natural caries, restorations and other dental materials were excluded. Furthermore, studies employing lesion formation by another *in vitro* model (*i.e.*, static or microbiological model) were not considered. Erosion studies, review articles and books were also excluded from the reference search.

2.2. SEARCH STRATEGY

An electronic bibliographic research was performed in the following databases: Medline - National Library of Medicine (PubMed), Web of Science (Thompson Reuters) and Scopus (Elsevier). The search was limited to the English language but not to date. The search was performed using controlled wordings through Medical Subject Headings (MeSH terms) and open terms around "caries", "pH cycling" and "dentine". The search was first carried out in the PubMed database and then fitted to Web of Science and Scopus. From the eligible studies, a reference search was conducted in order to include other related studies.

2.3. SCREENING AND REFERENCES SELECTION

All references were managed with the Ryyan QCRI online software [11]. Abstracts and titles were subjected to a screening process by two reviewers (co-authors: #1 and #2), who followed a flow chart as an assistant tool for references selection (Fig. 1).

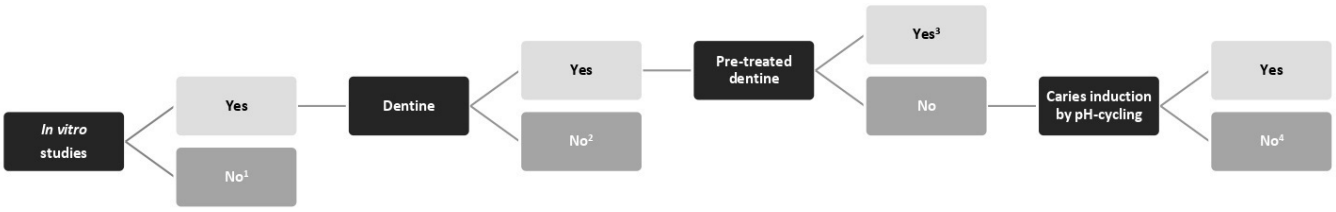

Fig. 1. Flow chart for screening and references selection. Excluding references;
1: *in vivo* studies, erosion studies, reviews, books and off topic studies;
2: studies on enamel, cementum, materials, restorations and natural caries;
3: remineralizing agents or procedures before pH-cycling (pHc);
4: caries formation by microbiological or static model.
Treatment during pHc (including remineralizing agents, artificial saliva, laser therapy, etc.).

2.4. RISK OF BIAS ASSESSMENT

The assessment of risk of bias was performed on the basis of CRIS guidelines (Checklist for Reporting *In-vitro* Studies) [12], fitted to the characteristics of the current systematic review. For this purpose, the following parameters were extracted from the references: sample size calculation, sample selection (*i.e.*, sound/ healthy teeth, caries-free or free crack teeth), explanation of sample preparation, randomization of groups, subgroups and testing machines, presence of a control location/ group [negative (sound) or positive (natural caries) control], description of the solutions and of the pH used during cycling, solutions renovation, and blinding operator of testing machines. In the event that the authors report any of the above parameters, it was set "+" or, inversely, "-" if the data was not reported. Based on these assignments, studies that received between one to three "+" were considered to be high risk of bias (-), four or five were considered as medium risk of bias (½), and those above were considered to be at lowest risk of bias (+).

2.5. DATA EXTRACTION AND STATISTICAL META-ANALYSIS

From the studies included, the following data and information were extracted: dental structure, demineralizing solution composition, immersion time and pH of the demineralizing solution, remineralizing solution composition, immersion time and pH of the remineralizing solution, pHc temperature, agitation/ stirring of solutions, type of induced caries, and pHc evaluation tests and characterization (*i.e.*, analytical techniques).

Meta-analysis was performed separately for the most commonly used pHc evaluation tests. For this purpose, two subgroups were compared: 1) type of dentition: primary *vs.* permanent teeth and 2) type of teeth: human *vs.* bovine teeth. Selected studies performed a negative control group (sound dentine/ untreated dentine) and an experimental group (artificially created caries by pHc). Data extraction was made according to Cochrane Handbook for Systematic Reviews of Interventions [13], indicating mean, standard deviation and sample size obtained from the selected studies, when such data was available. Analysis was carried out with Review Manager software (RevMan version 5.3 software, Cochrane Collaboration; Copenhagen, Denmark) with random effects model, a statistical significance established as a p-value ≤ 0.05 and using as effect measure the mean differences between sound dentine and artificially created caries through pHc. Besides, in the event that no negative control group was performed, an estimation of the effect and standard error of data was done. For quantifying heterogeneity, the I^2 test was interpreted as follows: 0%-40% (not important), 30%-60% (moderate heterogeneity), 50%-90% (substantial heterogeneity), and 75%-100% (considerable heterogeneity) [13].

3. RESULTS

3.1. SEARCH AND STUDY SELECTION

The search strategy using PubMed, WOS, Scopus and other references search included a total of 661 studies. The combinations of terms used in the literature search resulted in a number of 340 duplicates that were excluded. Therefore, 321 records were screened following the eligibility criteria and, as a result, 254 studies were excluded. Sixty-seven studies [14-18,22-31,33-73,75-85] were selected for qualitative analysis and 32 studies for further inclusion in meta-analysis [15-17,22,23,26-28, 35,36,39,43,46,47-51,53-55,58-61,65,67, 68,70-72,85] (Fig. 2).

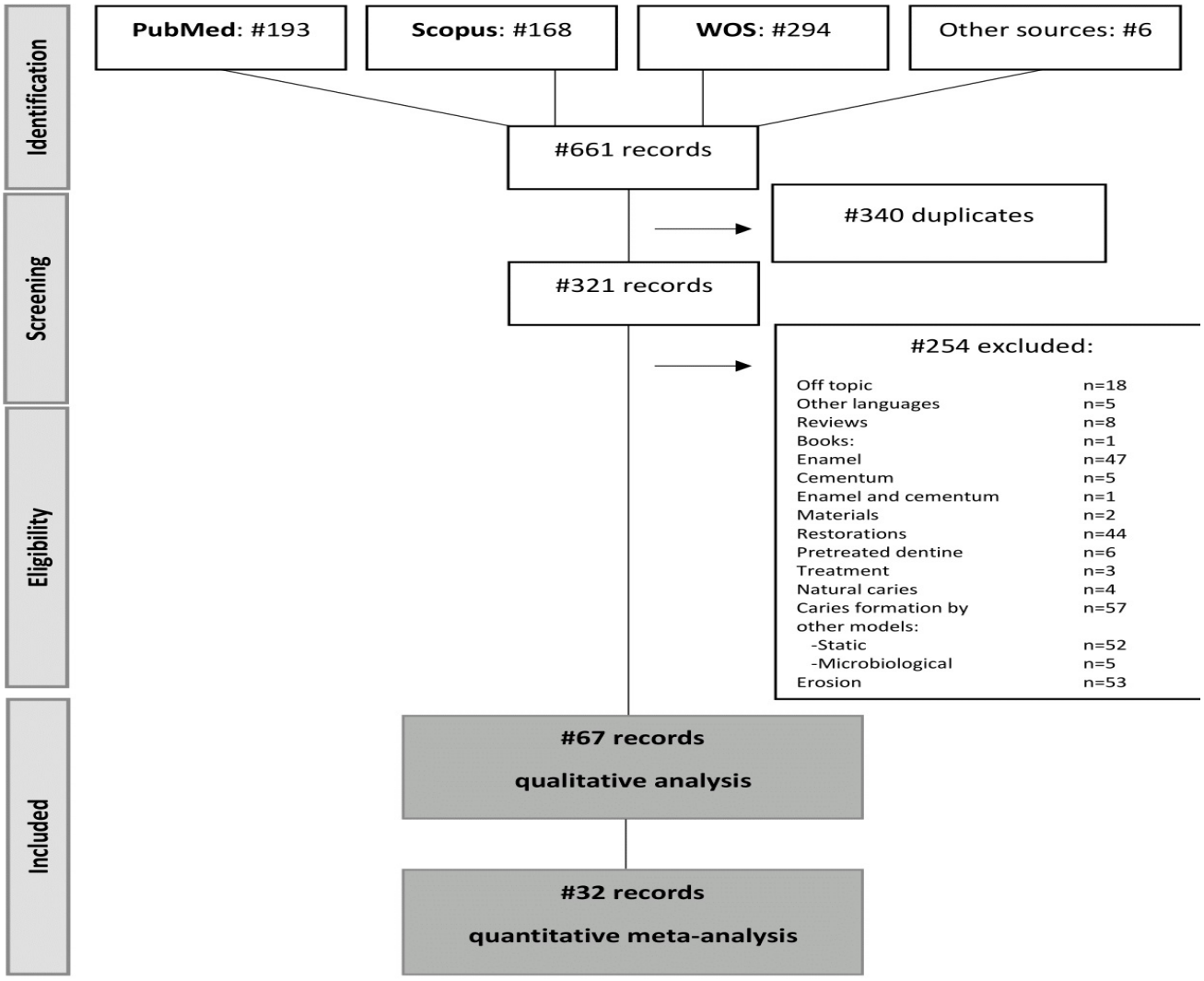

Fig. 2. Flow diagram reference selection based on PRISMA checklist

3.2. METHODOLOGICAL FEATURES

Table 1 summarizes the methodological features from each reference. The dental substrates used for pHc model in dentine were mainly human teeth (50 studies) representing around 75% of the included studies, compared to bovine teeth (16 studies, around 24% remaining). One study compared human dentine with bovine dentine (1%). Considering all studies, crown (45 studies) or root dentine (22 studies) were employed, representing around 67% and 33%, respectively. For human teeth, only three studies used both permanent and primary teeth for comparison, and one study employed bovine and human teeth. Without taking into account the four previously mentioned records, 30 studies used permanent teeth, while 17 studies used primary teeth. For bovine teeth, seven studies used crown and nine, root dentine.

Table 1. References included in the systematic review and methodological features

Reference	Dentine type (structure)	Demineralizing solution	DS: Time/ pH	Remineralizing solution	RS: Time/ pH	Days	T°	Agitation/ Stirring	Type of caries	Evaluation test (properties measurement)
Herkströter et al., 1991 [14]	Human root (premolars)	3 mM $CaCl_2.2H_2O$, 3 mM KH_2PO_4 and 50 mM $C_2H_4O_2$ pH adjusted with KOH	0.5 h/4.5	1.5 mM $CaCl_2.2H_2O$, 0.9 mM KH_2PO_4 and 20 mM HEPES. pH adjusted with KOH	0.5 h-1 h-1.5 h-2 h/7.0	24 h	NR	Yes	NR	MRG (mineral loss)
[1*]**Ettinger et al., 1994** [15]	Human root (anterior teeth)	2.2 mM $CaCl_2.2H_2O$, 2.2 mM KH_2PO_4 and 50 mM $C_2H_4O_2$	6 h/ 4.3	1.5 mM $CaCl_2.2H_20$, 0.9 mM KH_2PO_4 and 150 mM KCl	17 h/7.0	17 days	NR	NR	NR	PLM (lesion depth), MRG (lesion depth, remineralization band), overall demineralization
[1*]**Ettinger et al., 1997** [16]	Human root (anterior teeth)	2.2 mM $CaCl_2.2H_2O$, 2.2 mM KH_2PO_4 and 50 mM $C_2H_4O_2$	6 h/ 4.3	1.5 mM $CaCl_2.2H_20$, 0.9 mM KH_2PO_4 and 150 mM KCl	17 h/7.0	18 days	NR	NR	NR	PLM (lesion depth), MRG (lesion depth, remineralization band), overall demineralization
Shinkai et al., 2001 [17]	Human root (third molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM $C_2H_4O_2$ and 1 ppm of NaF	7 h/4.5	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	17 h/7.0	8 days	37°C	NR	Secondary caries	Cross-Sectional Microhardness (plus mineral profile, integrated area of mineral content, mineral loss measurements)
Featherstone et al., 2003 [18]: White and Featherstone 1987 [19], Featherstone et al., 1990 [20], Featherstone et al., 1998 [21]	Human crown (molars and premolars)	NR	18 h/4.70	NR	6 h/7.0	5 days	NR	NR	NR	PLM (demineralization pattern) Microhardnes (with integrated mineral loss, % inhibition)
[2*]**Hong et al., 2003** [22]	Human root (anterior and premolar teeth)	2.2 mM $CaCl_2.2H_2O$, 2.2 mM KH_2PO_4, and 50 mM $C_2H_4O_2$	6 h/4.3	1.5 mM $CaCl_2.2H_2O$, 0.9 mM KH_2PO_4, and 150 mM KCl	17 h/7.0	21 days	NR	NR	NR	PLM (lesion depth), Contact MRG (remineralization band), net demineralization

Darling et al., 2006 [23]	Human root (canines, premolars and molars)	2.2 mM $CaCl_2.2H_2O$, 2.2 mM KH_2PO_4 and 50 mM $C_2H_4O_2$	6 h/ 5.5 (18 days) 4.7 (16 days)	1.5 mM $CaCl_2.2H_2O$, 0.9 mM KH_2PO_4 and 150 mM KCl	18 h/ 7.0	32 days	NR	NR	NR	PLM (lesion depth)
de Menezes et al., 2007 [24]	Bovine root (incisors)	1.4 mM Ca, 0.9 mM PO_4 and 0.05 mM buffer solution of acetate	4 h/5.0	1.5 mM Ca, 0.9 mM PO_4 and 0.1 mMol Tris buffer	20 h/7.0	2 days	NR	NR	Incipient lesions	Microhardness/ Knoop
Erhardt et al., 2008 [25]	Bovine crown (incisors)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM $C_2H_3NaO_2$ 50 mM, $C_2H_4O_2$ and 1 ppm NaF	3 h/4.5	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 150 mM KCl, 100 mM Tris buffer and 10 ppm NaF	45 h/7.0	16 days	37°C	NR	CAD	µTBS, Microhardness/ Knoop, SEM (interface analysis)
Marquezan et al., 2009 [26]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	16 h/7.0	14 days	Room	No	CAD	Microhardness/ Knoop, SEM (morphological analysis)
Paranhos et al., 2009 [27]	Human crown (third molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM $C_2H_4O_2$ and 1 ppm of NaF	7 h/4.5	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	17 h/7.0	8 days	37°C	NR	CAD	SEM (fractographic analysis)/EDS (% elements determinated area), µTBS
Marquezan et al., 2010 [28]	Human crown (primary second molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	16 h/7.0	14 days	37°C	No	CAD	µTBS, SEM (fractographic analysis)
Esteves-Oliveira et al., 2011 [29]	Bovine root (incisors)	1.4 mM $Ca(NO_3)_2$, 0.91 mM NaH_2PO_4, 50 mM acetate buffer and 0.06 µg NaF/ml	4 h/5.0	1.5 mM $Ca(NO_3)_2$, 0.9 mM NaH_2PO_4, 150 mM KCl, 100 mM Tris buffer and 0.05 µg NaF/ml	20 h/7.0	9 days	37°C	Yes	NR	ICP-OES (Ca, P release)
[3*]**Hiraishi et al., 2011** [30]	Bovine root (incisors)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM $C_2H_4O_2$. pH adjusted with KOH	14 h/5.0	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl and 20 mM HEPES buffer. pH adjusted with KOH	8 h/7.0	8 days	37°C	NR	NR	AAS (Ca ion release), HPLC (degraded collagen/ hydroxyproline), Transverse MRG (mineral loss, lesion depth)

Liu et al., 2011a [31]**:** ten Cate et al., 1995 [32]	Human crown (third molars)	NR	NR	NR	NR	NR	NR	NR	CAD	Micro CT (mineral content profiles, lesion depth, integrated mineral loss), TEM (dimension and hierarchy of apatite collagen matrix)
Liu et al., 2011b [33]**:** ten Cate et al., 1995 [32]	Human crown (third molars)	NR	NR	NR	NR	NR	NR	NR	CAD	Micro CT (lesion depth, integrated mineral loss), Light Microscopy (Goldner's Trichrome), TEM (ultrastructural examination)/EDX (Ca and P distribution)
Marquezan et al., 2011 [34]	Human crown (primary second molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	16 h/7.0	14 days	37°C	No	CAD	µTBS, SEM (nanoleakage)/EDX (chemical elements determination)
[4*]**Pavan et al., 2011** [35]	Human root (third molars)	2.2 mM $CaCl_2.H_2O$, 2.2 mM KH_2PO_4 and 50 mM $C_2H_4O_2$	6 h/4.3	2.25 mM $CaCl_2.H_2O$, 1.35 mM KH_2PO_4, 130 mM KCl and 20 mM HEPES	17.5 h /7.0	18 days	37°C	Yes	NR	Transverse MRG (mineral loss, lesion depth)
Lenzi et al., 2012 [36]	Human crown (primary second molars and third molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	CAD	µTBS
[5*]**Sohn et al., 2012** [37]	Human root (premolars)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM acetate buffer	3 h/4.8	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 20 mM HEPES	20 h/7.0	5 days	37°C	Yes	NR	SEM/EPMA (width demineralization bands, % fluoride), Fluoride Ion Electrode (fluoride concentration)

Alves et al., 2013a [38]	Human crown (primary second molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	16 h/7.0	14 days	Room	No	CAD	µTBS, SEM (nanoleakage)
Alves et al., 2013b [39]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2P0_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	CAD	µTBS, SEM (nanoleakage)
Comar et al., 2013 [40]	Bovine root (incisors)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM lactic buffer	8 h/5.0	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl, 20 mM HEPES buffer and 5 mM NaN_3	16 h/7.0	7 days	37°C	No	NR	Cross-Sectional Hardness/Knoop
de-Melo et al., 2013 [41]	Human crown (third molars)	2 mM $CaCl_2 2H_2O$, 2 mM of KH_2PO_4, 75 mM $C_2H_4O_2$, 0.030 ppm NaF and 0.1 mM Tris buffer	4 h/4.6	1.5 mM of $CaCl_2$, 0.9 mM KH_2PO_4, 0.050 ppm NaF, and 150 mM KCl	20 h/7.4	2 / 4 / 8 days	37°C	NR	NR	Cross-Sectional Hardness (integrated area microhardness loss *vs.* lesion depth, hardness profile), µTBS
Lenzi et al., 2013a [42]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM of $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM of NaH_2PO_4, and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	Carious dentine	µTBS
Lenzi et al., 2013b [43]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	CAD	µTBS, SEM (interface analysis)
Calvo et al., 2014 [44]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2P0_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCI	16 h/7.0	14 days	NR	NR	CAD	µTBS

Erhardt et al., 2014 [45]	Bovine crown (incisors)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM $NaC_2H_3O_2$, 50 mM $C_2H_4O_2$ and 1 ppm NaF	CAD: 3 h CID: 2 h/4.5	Hyper-RS: 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 150 mM KCl, 100 mM Tris buffer and 10 ppm NaF/RS: 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 150 mM KCl and 100 mM Tris buffer	CAD (Hyper-RS): 45 h/7.0 CID (RS): 22 h/7.0	CAD: 16 days CID: 4 days	37°C	NR	CAD and CID	µTBS, SEM (interface analysis)
[6*]**Goettsche et al., 2014** [46]	Human root (posterior teeth)	2.2 mM $CaCl_2.2H_2O$, 2.2 mM KH_2PO_4 and 50 mM $C_2H_4O_2$	2 t X 3 h/4.3	1.5 mM $CaCl_2.2H_2O$, 0.9 mM KH_2PO_4 and 150 mM KCl	1 t X 2.5 h 1 t X ≈15 h / 7.0	12 days	NR	NR	NR	PLM (cavitation, remineralization band, total lesion depths)
Lenzi et al., 2014a [47]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	CAD	µTBS
Lenzi et al., 2014b [48]	Human crown (second deciduous molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2P0_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCI	16 h/7.0	14 days	Room	No	CAD	µTBS, SEM (nanoleakage)
Lenzi et al., 2014c [49]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	16 h/7.0	14 days	Room	No	CAD	µTBS, SEM (nanoleakage)
Rocha et al., 2014 [50]	Human crown (third molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM $C_2H_3NaO_2$, 50 mM $C_2H_4O_2$ and 1 ppm NaF	3 h/4.5	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 150 mM KCl, 100 mM Tris buffer and 10 ppm NaF	45 h/7.0	16 days	NR	NR	CAD	µSBS, Vickers Hardness
Tedesco et al., 2014a [51]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2P0_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCI	16 h/7.0	14 days	NR	NR	CAD	µTBS
Tedesco et al., 2014b [52]	Bovine crown (incisors)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	Carious dentine	µSBS

[7*]**Epasinghe et al., 2015** [53]	Human root (single rooted teeth)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM $C_2H_4O_2$. pH adjusted with KOH	14 h/5.0	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 139 mM KCl and 20 mM HEPES buffer. pH adjusted with KOH	8 h/7.0	8 days	37°C	NR	Root caries	Transverse MRG (lesion depth, mineral loss), CLSM (fluorescent band), Microhardness Test/ Knoop
Lenzi et al., 2015a [54]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	Room	No	CAD	µTBS
Lenzi et al., 2015b [55]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	CAD	µTBS, SEM (nanoleakage)
Lenzi et al., 2015c [56]	Human crown (primary and permanent molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	Room	No	CAD	Microhardness/ Knoop
Melo et al., 2015 [57]	Human crown (third molars)	2.0 mM $CaCl_2.H_2O$, 2.0 mM KH_2PO_4 and 750 mM $C_2H_4O_2$	4 h/4.6	1.5 mM $CaCl_2.H_2O$, 0.9 mM KH_2PO_4, 150 mM KCl, 100 mM Tris buffer and 0.05 ppm NaF	20 h/7.4	2 / 4 / 8 days	37°C	No	CAD	SEM (morphological characteristics after laser irradiation)
[8*]**Silva et al., 2015** [58]	Bovine root (incisors)	2.25 mM $CaCl_2.2H_2O$, 1.35 mM KH_2PO_4, 50 mM acetate and 130 mM KCl	6 t X 30 min/5.0	2.25 mM $CaCl_2.2H_2O$, 1.35 mM KH_2PO_4 130 mM KCl and 20 mM HEPES	6 t X 10 min 1 t X ≈ 19 h /7.0	8 days	NR	Yes	Root caries	Surface and Cross-Sectional Microhardness, PLM (qualitative analysis)
Dias et al., 2016 [59]	Human crown (primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	Room	No	NR	Microhardness/ Knoop, Micro-Raman Spectroscopy (qualitative analysis of mineral composition)
Kucukyilmaz et al., 2016 [60]	Human crown (molar teeth)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	Room	NR	CAD	µTBS, SEM (surface morphology analysis)/ EDS(chemical analysis)

Lenzi et al., 2016 [61]	Human crown (second primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	Room	No	CAD	µTBS (Weibull)
Nicoloso et al., 2016 [62]	Human crown (third molars and primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.5	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	Room	No	CAD	µTBS, SEM (failure mode)
Sung et al., 2016 [63]	Human root (premolars)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM acetate buffer	24 h/4.8	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 20 mM HEPES	24 h/7.0	14 days	37°C	NR	CAD	SEM/EPMA (elemental contents, lesion width, mineral loss of Ca and P and Ca/P ratio)
Curylofo-Zotti et al., 2017 [64]	Bovine crown (incisors)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 150 mM KCl	16 h/7.0	14 days	Room	NR	CAD	Microhardness/ Knoop, SEM (morphological analysis)/EDS (% Ca, P and Ca/P ratio)
[9*]**Epasinghe et al., 2017** [65]	Human root (single rooted teeth)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM $C_2H_4O_2$. pH adjusted with KOH	14 h/5.0	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl and 20 mM HEPES buffer. pH adjusted with KOH	8 h/7.0	8 days	37°C	NR	Root caries	Transverse MRG (lesion depth, mineral loss), CLSM (minerals formation), XRD (crystal characterization), Chloramine T-assay (solubilized collagen peptides/ hydroxyproline release)
Li et al., 2017 [66]	Human crown (third molars)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and tttt5 mM NaN_3. pH adjusted with $C_2H_4O_2$	0.5 h X 50 cycles/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 130 mM KCl and 5 mM NaN_3. pH adjusted with HEPES buffer	2.5 h X 50 cycles/ 7.0	≈6 days	Room	NR	CAD	SEM-BSE/ FEG-EPMA (demineralization depth, remineralization depth, D_{RM}, I_{RM}, elemental composition)

Nicoloso et al., 2017 [67]	Human crown (third molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.5	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCL	16 h/7.0	14 days	NR	NR	CAD	µTBS
de Morais et al., 2018 [68]	Human crown (molar teeth)	NR	NR	NR	NR	NR	NR	NR	NR	µTBS, SEM (fracture patterns)/EDX (Ca and P semi-quantitative analysis)
Dias et al., 2018 [69]	Human crown (primary molars)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	Room	No	Affected dentine layer	Microhardness/ Knoop, SEM (qualitative analysis)/EDS (Ca, P and F analysis), Micro-Raman Spectroscopy (qualitative analysis of mineral composition)
Follak et al., 2018a [70]	Bovine crown (incisors)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$. pH adjusted with 100 mM KOH	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	CAD	µTBS
Follak et al., 2018b [71]	Bovine crown (incisors)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM $C_2H_4O_2$	8 h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 days	NR	NR	CAD	µTBS, SEM (differences between substrates)
[10*]**Velo et al., 2018** [72]	Bovine root (incisors)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM $C_3H_6O_3$ buffer	8 h/5.0	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl, 20 mM HEPES buffer and 5 mM NaN_3	16 h/7.0	7 days	37°C	NR	Root caries	Surface and Cross-Sectional Hardness/Knoop, Transverse MRG (lesion depth, integrated mineral loss, average mineral loss), Fluoride Ion Electrode (fluoride concentration)
[11*]**Al-Hasnawi & Radhi 2019** [73]: [Jeon et al., 2008] [74]	Human crown (maxillary first premolars)	NR	NR	NR	NR	5 days	NR	NR	Caries like lesion	Fluoride Ion Electrode (fluoride quantities)

dos Santos Ferreira et al., 2019 [75]	Bovine root (incisors)	1.4 mM Ca, 0.91 mM PO_4^{3-}, 0.06 ppm F^- and 0.05 M acetate buffer	2 h/5.0	1.5 mM Ca, 0.9 mM PO_4^{3-}, 150 mM KCl, 0.05 ppm F^- and 0.1 M Tris buffer	22 h/7.0	8 days	NR	NR	Initial caries lesions	ATR-FTIR (chemical evaluation), SEM (morphological effects)
Fathy 2019 [76]: Marquezan et al., 2009 [26]	Human crown (maxillary premolars)	NR	8 h/ 3.0	NR	16 h/7.0	NR	NR	NR	CAD	SEM (surface analysis)/EDS (mineral % Ca and P)
Zander et al., 2019 [77]	Bovine root (incisors)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM $C_2H_4O_2$. pH adjusted with KOH	14 h/5.0	1.5 mM $CaCl_2$, 130 mM KH_2PO_4 and 20 mM HEPES. pH adjusted with KOH	10 h/7.0	8 days	37°C	NR	Root caries	Micro CT (lesion depth, mineral loss, surface layer mineral density)
Bassi et al., 2020 [78]	Bovine crown (incisors)	2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 0.05 M acetic acid	8 h/4.5	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl	16 h/7.0	14 cycles	Room	No	CAD	μSBS, Stereomicroscopy (failure mode)
[12*]**Leal et al., 2020** [79]	Bovine root (incisors)	1.4 mM Ca, 0.91 mM P, 0.06 μg F^-/mL and 0.05 M acetate buffer	6 h/ 5.0	1.5 mM Ca, 0.9 mM P, 150 mM KCl, 0.05 μg F^-/mL and 0.1 M Tris buffer	18 h/ 7.0	10 days	NR	NR	NR	Longitudinal hardness (lesion area)
Luk et al., 2020 [80]	Human crown (third molars)	1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, and 50 mM acetate	16 h/4.5	150 mM KCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.9 mM KH_2PO_4 and 1.5 mM $CaCl_2$	8 h/7.0	8 days	25°C	NR	NR	Micro CT (lesion depth), nanohardness, SEM (surface morphology assessment)/EDS (elemental analysis)
Sadoon et al., 2020 [81]: Marquezan et al., 2009 [26]	Human crown (third molars)	NR	8 h/NR	NR	16 h/NR	14 days	Room	No	NR	EDX (surface mineral content), Optical Microscopy (surface analysis), Microhardness/ Vickers
[13*]**Okayuma et al., 2021** [82]	Human root (molars)	3 mM $CaCl_2$, 1.8 mM KH_2PO_4 and 0.2 M lactic acid	6 t X 2 m /4.5	3 mM $CaCl_2$, 1.8 mM KH_2PO_4, 0.02 M HEPES and 130 mM KCl	5 t X 176 m 1 t X 8.9 h/7.0	28 days	NR	NR	NR	PLM (lesion depth), PIXE/PIGE (fluorine, titanium and calcium contents)

Enrich-Essvein et al., 2021a [83]	Human crown (anterior teeth)	2.2 mM $CaCl_2$, 2 mM NaH_2PO_4 and 50 mM acetic acid	8h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 M KCl	16 h/7.0	14 days	Room	No	NR	Three-point bending test (flexural strength) ATR-FTIR (chemical composition), TGA (chemical composition), XRD (mineralogical characteristics)
Enrich-Essvein et al., 2021b [84]	Human crown (upper incisors) and bovine crown (lower incisors)	2.2 mM $CaCl_2$, 2 mM NaH_2PO_4 and 50 mM acetic acid	8h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 M KCl	16 h/7.0	3 / 7 / 14 days	Room	No	NR	Three-point bending test (flexural strength) ATR-FTIR (chemical analysis), TGA (chemical analysis), XRD (microstructural analysis)
Cifuentes-Jimenez et al., 2021 [85]	Human crown (permanent molars)	2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 50 mM acetic acid	8h/4.8	1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 M KCl	16 h/7.0	14 days	Room	No	CAD	ATR-FTIR (chemical composition), XRD (crystalline characteristics), SEM (morphological changes and tubular occlusion)/EDS (elemental analysis), *μTBS*

1: all specimens were treated with one of the test solutions/ materials for 4 minutes;*
2: it took a half-hour twice a day to brush, treat, and move the specimens from one solution to another;*
3: treatment solutions for 2 hours; 4*: treatment solutions for 30 minutes;*
5: one hour is missing to complete 24 hours cycle; 6*: treatment slurries for 6 minutes;*
7: testing solutions or pastes for 2 hours;*
8: treatment (6 t x 10 minutes);*
9: testing solutions or pastes for 2 hours; 10*: testing toothpastes for 5 minutes twice a day;*
11: After pH cycling, specimens were submitted 2 days in remineralizing solutions;*
12: dentine specimens remained for an additional 24 h in the remineralizing solution;*
13: 6 t X 2 m in deionized water.*
Specimens were soaked weekly for 5 min in TiF_4 or NaF solution.
After that, specimens were washed with deionized water for 10 m and then returned to pH cycling.

Abbreviations: CAD, caries-affected dentine; CID, caries-infected dentine; DS, demineralizing solution; RS, remineralizing solution; NR, not reported; t, times; MRG, microradiography; PLM, polarized light microscopy; μTBS, microtensile bond strength; SEM, scanning electron microscopy; EDS/EDX; energy dispersive x-ray spectroscopy; ICP-OES, inductively coupled plasma-optical emission spectrometer; AAS, atomic absorption spectrophotometer; Micro CT, micro computed tomography; TEM, transmission electron microscopy; EPMA, electron probe micro analysis; μSBS, microshear bond strength; CLSM, confocal laser scanning microscopy; XRD, X-ray diffraction; BSE, back scattered electron; FE, field emission-gun; D_{RM}, relative remineralization depth; I_{RM}, relative remineralization intensity; HPLC, high-pressure liquid chromatography; ATR-FTIR, Attenuated total reflection Fourier transformed infrared spectroscopy; PIXE, μ–particle-induced X-ray emission; PIGE, particle-induced gamma emission; TGA, thermogravimetry

During pHc, the composition of demineralizing and remineralizing solutions varied at specific concentrations. For demineralizing solutions, the molar concentrations for calcium compounds ranged from 1.5 mM-3 mM using $CaCl_2$ or 1.4 mM for $Ca(NO_3)_2$, for phosphates salts molar concentrations ranged from 0.9 mM-3 mM for KH_2PO_4 and 0.91 or 2.2 mM for NaH_2PO_4, while the acid buffers employed were acetic acid (50, 75, or 750 mM), acetate buffer (0.05 mM or 50 mM) or 50 mM lactic buffer. Regarding the remineralizing solutions, the molar concentrations for calcium were 1.5 or 2.25 mM for $CaCl_2$ and 1.5 mM for $Ca(NO_3)_2$, for phosphates ranged from 0.9 mM-130 mM employing KH_2PO_4, and 0.9 mM when using NaH_2PO_4. Another compound added to the remineralizing solution was KCl, with molar concentrations ranging from 0.15 mM-150 mM. Other less used compounds were NaF, $C_2H_3NaO_2$, HEPES buffer and Tris buffer added to the remineralizing solution. Only two studies used collagenase in remineralizing solution [30,65].

The immersion times during pHc in demineralizing solutions were mostly 8 h, with an upper and lower limit of 24 h and 0.5 h, respectively. The acidic pH in the demineralizing solution ranges from 3.0 to 5.0. For remineralizing solutions, 16 h was the most widely used time, the upper limit being 45 h and the lower 0.5 h. In the remineralizing solution, the pH was neutral, whereas 7.0 and 7.4 were the most common values. Some studies used a higher number of shorter cycles in the demineralization phase, with the aim of simulating the exposure conditions similar to the usual daily intake [46,58]. Other studies used exclusive time scales to test remineralization products, 2 h being the most common time of exposure.

Although some studies did not report the temperature, the most frequently used values were room temperature and 37°C (body temperature). The application days of the pHc ranged from one to 21 days, 14 days being the most common time to induce caries-affected dentine (CAD). Although some studies only referred to "artificial caries lesions" or even did not report the type of induced caries, most of the included studies reported CAD induction and only one [45] reported caries infected dentine (CID) induction.

The main protocols used for the characterization of the samples subjected to pHc were microtensile bond strength (μTBS) and the hardness tests. In addition, other analytical techniques were also frequently used to characterize the compositional and microstructural properties of the samples: scanning electron microscopy (SEM) coupled with energy dispersive x-ray spectroscopy (EDS/EDX) detectors, microradiography (MRG) and polarized light microscopy (PLM). Other less used analytical techniques employed in these studies were microcomputed tomography (Micro CT), confocal laser scanning microscopy (CLSM), and Raman spectroscopy.

3.3. RISK OF BIAS ASSESSMENT

The study on the risk of bias is reported in Figure 3, indicating the percentage for each item and the average for the total included studies. Thirteen studies presented high risk of bias (score below three "+"), most of them had medium risk (41 studies with score four or five "+"), and 13 studies low risk of bias (score up to six to eight "+"). Among them, the more common

risks of bias were: (1) no sample size calculation and (2) no blinding operator to testing machines.

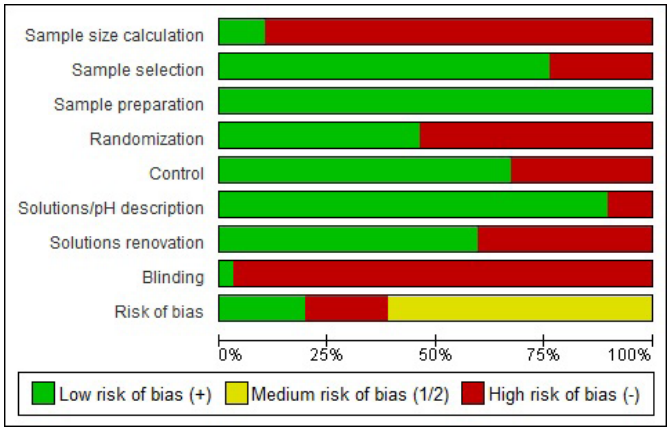

Fig. 3. Summary of quality and risk of bias assessment for each item studied included

Figure 4 shows the detailed report of the risk of bias for the analyzed references. From the studies with low risk of bias, only two described sample size calculation and blinding operator to the testing machine [70,77]. Regarding randomization, several studies received "-", because there was no randomization to experimental subgroups or test machines. In a particular study, due to lack of complete "randomization" in the design of the experimental groups and the evaluation test, the item received "-". In the solutions/ pH description item, only seven studies received "-", including a proceeding paper [18], which did not report solutions composition.

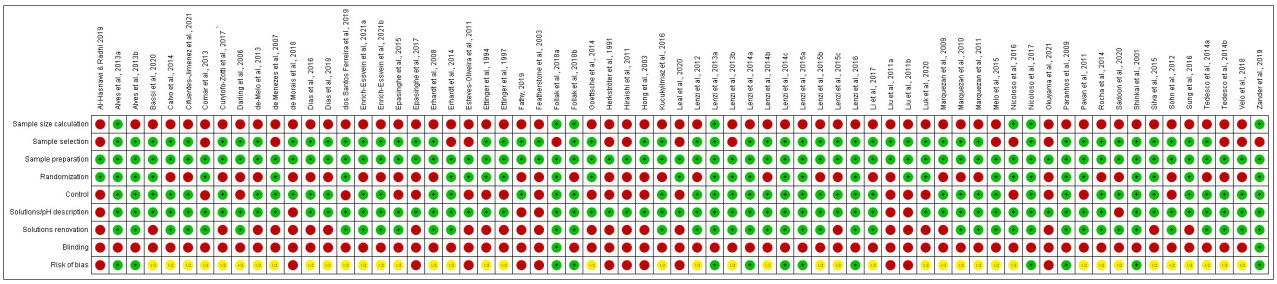

Fig. 4. Risk of bias assessment for the references included in the study

3.4. META-ANALYSES

For meta-analyses, μTBS was the most employed evaluation test, followed by hardness test, MRG and LPM, resulting in 32 studies included for quantitative analyses. For the adhesion test (μTBS), studies that reported immediate bond strength, untreated samples and adhesive systems used according to manufacturer's instructions were considered. Regarding the hardness test, only cross-sectional hardness studies that considered 20 μm depth and immediate pHc were taken into account when measurements were performed at different depths. The effect of pHc demineralization has been limited to 100 μm depth in dentine [25], increasing the heterogeneity of the meta-analysis results up to this limit. Three global analyses were performed, considering the type of dentition (permanent *vs*. primary teeth) and type of teeth (human *vs*. bovine teeth) for the μTBS test, and type of teeth (human *vs*. bovine teeth) for the hardness test. Analyses on the type of teeth for the hardness test were not performed, as only a few studies were conducted on this topic (#3 references: two studies for permanent teeth *vs*. one for primary teeth).

Forest plots of the μTBS and hardness tests meta-analyses are shown in Figures 5-6, respectively. The three analyses showed differences between sound/ untreated dentine and pH cycled dentine ($p < 0.001$), with a heterogeneity $I^2 > 90\%$. With respect to the μTBS test (Fig. 5), there were differences between sound/ untreated dentine and the dentine submitted to pHc in all subgroups. Permanent ($I^2 = 96\%$) and primary ($I^2 = 82\%$) teeth subgroups had *p*-values = 0.008 and < 0.001, respectively (Fig. 4-Left). For human ($I^2 = 93\%$) and bovine

teeth ($I^2 = 0\%$), the *p*-values were < 0.001 for both subgroups (Fig. 4-Right). Regarding the hardness test (Fig. 6), the analyses revealed differences ($p < 0.001$) with $I^2 = 99\%$ for sound/untreated dentine and pHc dentine. Furthermore, significant differences were found for both human and bovine teeth subgroups meta-analysis ($p < 0.001$) with $I^2 = 98\%$. Forest plots for MRG and LPM are shown in Figure 7. The overall effect had *p*-values < 0.001 for both tests ($I^2 = 99\%$). The depth of demineralization was 194.95 µm and 298.17 µm for MRG and LPM, respectively.

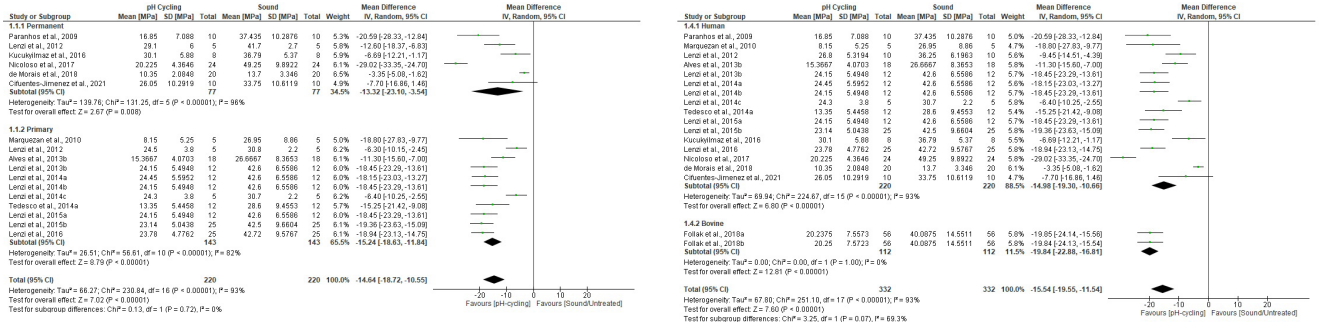

Fig. 5. Forest plots for microtensile bond strength of adhesives systems: permanent *vs*. primary teeth (Left), and for microtensile bond strength of adhesive systems: human *vs*. bovine teeth (Right)

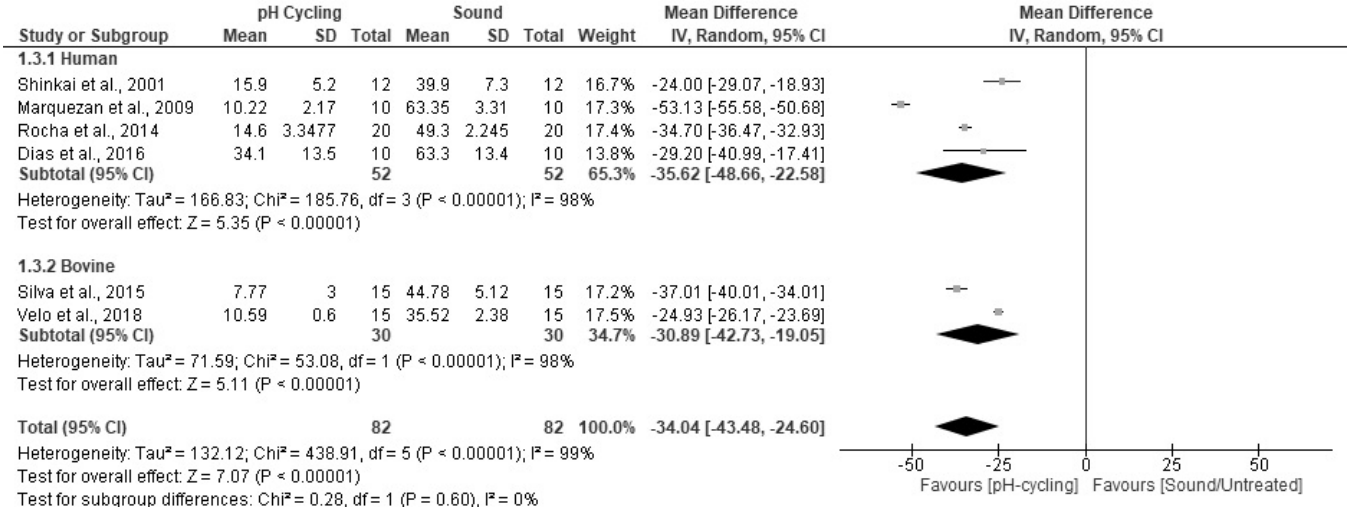

Fig. 6. Forest plot for hardness test: human *vs*. bovine teeth

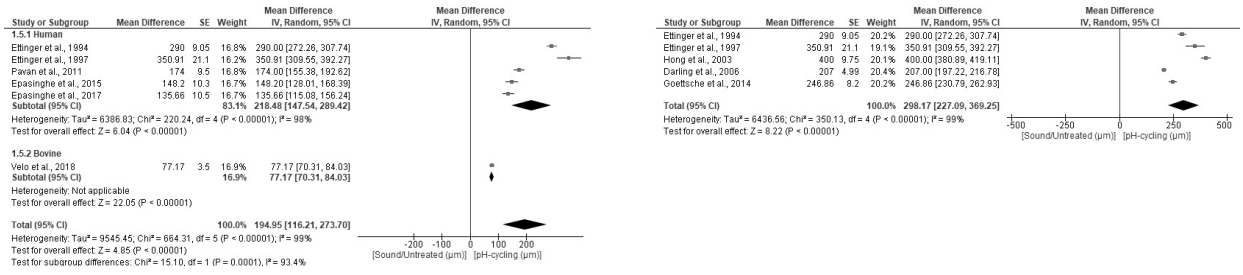

Fig. 7. Forest plots for microradiography – MRG (Left) and for polarized light microscopy - PLM (Right)

4. DISCUSSION

The pHc is considered a simple experimental procedure used as a preliminary step in many investigations, addressing many methodological aspects, depending on the research. The pHc procedure was initially created for the development of artificial caries lesions in the enamel [4], which were later transferred to dentine, simulating the loss or gain of mineral in both substrates. This procedure has the advantage of simulating a dynamic carious process in which demineralization is higher than remineralization, in opposition to the static method using buffers or acid gels, ultimately producing caries lesions. Despite pHc limitations, such as incomplete chemical simulation of oral conditions or lack of bacterial activity [86], it is an *in vitro* model often used in caries research. In this regard, a comprehensive systematic search and meta-analysis of the experimental parameters employed during pHc is of great interest for determining the methodological conditions required for specific studies in dental research.

Worth noting is the large number of pHc studies conducted on primary teeth, despite the difficulty of gathering them. There are several differences between primary and permanent teeth in terms of their chemical composition and microstructural characteristics (*e.g.*, enamel thickness, phosphate and calcium concentration in dentine) [87]. Between these differences, the lower relative content of mineral and organic components in the primary teeth [88,89] makes them more susceptible to demineralization. On the other hand, bovine teeth are presented as an alternative to human teeth due to some compositional and structural similarities [90,91], two systematic reviews performed with meta-analysis supporting their reliable substitution in bond strength studies [92,93]. To date, there is one study comparing the effect of the pHc method between bovine and human dentine [84]. So far, most of these comparative researches employed other methods to induce artificial caries [94,95] or used healthy teeth [96,97]. The limited use of bovine incisors may be due to the fact that the characteristics of human teeth approach more closely to a real clinical situation, allowing the results to be extrapolated to dental practice.

Regarding the compounds and concentrations of the demineralization and remineralization solutions, an extensive part of the research followed the concentration ranges existing in the oral fluid reported by Jenkins [98]. The demineralizing solution is acidic, with critical limit of pH values = 5.5 and 4.5 for the solubility of hydroxyapatite (HAp) and fluorapatite (FAp) [99], being undersaturated with respect to the inorganic phases providing mineral loss, which simulates the acid attack by bacterial metabolism. On the other side, remineralizing solutions aim at acting as the composition of saliva, through the concentration of Ca^{2+}, $(PO_4)^{-3}$, and F^- ions at a neutral pH, promoting the formation of HAp and FAp in the dental substrate [99]. Overall, the demineralizing and remineralizing solutions tend to simulate the loss and gain of minerals, respectively, limiting the effect on the organic components of the dental tissue. Several studies propose to use collagenase in order to promote the degradation of the demineralized collagen matrix, approaching the actual situation in a carious process [7,30,65]. In the pHc, collagenase acts in the remineralizing solution [7] unlike under *in vivo* conditions, where not only bacterial collagenase or dentine metalloproteinases, but also salivary metalloproteinases are active [100,101].

The pHc makes use of different experimental parameters, such as immersion/ exposure time, pH, agitation, temperature and duration (days) of the pH cycle, which decisively influence the resulting demineralization. Exposure times (*i.e.*, demineralization and remineralization duration cycles) are based on the changes occurring in the oral environment during food intake. On one side, demineralization times correspond to the period in which the teeth are exposed to the simulated bacterial acids, while remineralization corresponds to the time intervals between these acid exposures [22,46]. Herkströter *et al*. [14] demonstrated that a 1:1 ratio for the demineralization/ remineralization cycles showed higher mineral loss than other experimental exposure ratios (*e.g.*, 1:2, 1:3, and 1:4). Likewise, de-Melo *et al.* [41] showed that

mineral loss was significantly higher when the number of days/ cycles of pHc increased. Regarding the experimental temperatures, the most common were 37°C and room temperature. However, *in vitro* erosion studies showed an increase in the effects of demineralization erosion with the temperature of acidic solutions [102-104]. This increased mineral alteration is explained by the influence of higher temperatures on the reaction rates in solutions [104]. Moreover, the effect of pH lies in the fact that a decrease in pH condition logarithmically increases the solubility of apatite [105,106]. As previously mentioned, the recommended pH values for pHc associated with the natural process of caries range between 5.0 and 4.5, since a lower pH would result in a dental erosion unrelated to caries process [99]. Regarding the agitation and/or stirring of solutions, Eisenburger and Addy [103] showed the strong relationship between the depth of erosion and the effect of the mechanical stirring of solutions, explained by the enhanced mobility and ion exchange and, consequently, the increasing rate of tooth demineralization.

Regarding analytical techniques, the references mainly employed μTBS and hardness tests to assess the mechanical and physical properties of pHc samples. The μTBS methodology, implemented by Sano *et al*., [107] consists of evaluating the effectiveness in the adhesion of different materials and, consequently, in predicting the longevity of dental restorations [108]. The hardness test consists of measuring the resistance of a material or dental substrate to the penetration of a mechanical indenter. In addition, the hardness data obtained also provides information on the mineral loss in dental substrates [17,41]. Transverse MRG was used to observe and measure the mineral loss in the substrate profile, and to quantify the lesion depth caused during the pHc, the later being also characterized by PLM. SEM observations were also carried out for the morphological analysis of the interfaces or nanoleakage evaluation, as well as for examining the failure mode in bond strenght studies. *In situ* characterization techniques, such as SEM coupled with EDS/EDX detectors or electron probe microanalysis (EPMA), were used to determine the chemical composition of the mineral component. Furthermore, other spectroanalytical methods, such as atomic absorption spectrophotometer (ASS) and inductively coupled plasma optical emission spectrometer (ICP-OES), were employed for elemental analysis.

In order to quantify the demineralization yielded by pHc, a meta-analysis was performed on a total of 31 studies, including the four main analytical techniques for sample characterization (*i.e.*, μTBS, hardness, MRG and PLM). All analyses showed considerable heterogeneity (I^2 > 90%) in all subgroups, except bovine teeth subgroup (I^2 = 0%) for μTBS, as only two references with similar data (*i.e.*, mean, standard deviation and sample size) met the inclusion criteria. Since heterogeneity is inevitable by methodological diversity [109], a common factor for this heterogeneity was the variety of pHc protocols and, even if less considered, the biological variability of samples. The increased heterogeneity for μTBS results may be due to the type of adhesive systems considered (etch-and-rinse, self-etch and multimode adhesives). Comparing the studies on human *vs*. bovine teeth, the human subgroup included permanent and primary teeth so that the morphological and structural differences between them favor the observed heterogeneity. Similarly, the heterogeneity for the hardness test is also increased by considering the permanent and primary teeth together, as well as by the analytical differences in the results between the microhardness and hardness tests in the included studies. In PLM meta-analysis, all studies used human teeth while, in MRG, five studies used human and only one study used bovine teeth. The heterogeneity observed for these results can be explained by some of the reasons mentioned above (*i.e.*, variety of pHc protocols, biological variability of samples and differences between human and bovine teeth). Despite the considerable heterogeneity, the forest plots showed that the direction of effects is clearly favoring pHc (*i.e.*, producing a demineralization of substrates), where the μTBS and hardness values decreased with respect to the sound/ untreated dentine. Likewise, the comparative analysis of the results describing

the depth of the lesion using MRG and LPM techniques gave ranges between 200-300 µm, considering the heterogeneity of the included studies.

Through the years, many pHc models have been developed and described in literature. In accordance with the research objectives, we distinguish the pHc protocols as aiming: 1) to induce a previous caries lesion, and 2) to test the effects of the targeted remineralizing agents. The first approach for evaluating a method to induce CAD was proposed by Erhardt *et al*., [25] although this procedure was not validated with natural caries. Later on, Marquezan *et al*. [26] aimed at evaluating artificial caries by using three different caries induction methods (*i.e*., static, pHc and microbiological), based on hardness analysis and morphological characterization techniques. From this procedure, several methodological variations were introduced, for example; removing NaF of the solutions, increasing the immersion time and pH of the demineralizing solution, reducing the immersion time in the remineralizing solution, duration of the procedure and room temperature and agitation conditions. Nowadays, the principle of minimally invasive dentistry has prompted research on the induction of artificial caries that reproduces the characteristics of residual caries remaining underneath the restorations after selective removal [110]. In the current research, only one of the studies included in the analysis [45] was aimed at inducing CID through pHc, although it was not evaluated with positive controls (natural caries). Summarizing, on the basis of the methodological aspects discussed in this review, the recommended parameters to develop the pHc procedure are: 1) maintaining the composition and concentration for the de- and remineralizing solutions in the ranges described, 2) immersion in demineralizing solution for three periods of 2 h each, 3) immersion in remineralizing solution for three periods: two per 1.5 h (between demineralization periods) and one for 15 h (overnight), 4) maintaining the pH between 4.5 and 5.5 in the demineralizing solution, 5) keeping the pH at 7.0 in the remineralizing solution, 6) methodological interest of incorporating collagenase in the remineralizing solution, 7) agitation and 37°C temperature, 8) the days of execution must be validated with natural residual caries.

5. CONCLUSIONS

The current study reports that the pHc model, widely accepted by cariology researchers, is a feasible and reproducible procedure to simulate different types of caries lesions in dentine. Although most of the included studies have a medium risk of bias and heterogeneity of results, the performed quantitative meta-analysis shows a well-defined effect that indicates pHc as an effective procedure to produce *in vitro* demineralization.

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