Int. J. Morphol.,
41(3):926-936, 2023.

Histological Techniques for the Study of the Dentogingival Junction: A Scoping Review Using the Anatomical Quality Assurance Checklist (AQUA)

Técnicas Histológicas para el Estudio de la Unión Dentogingival: Una Revisión de Alcance Utilizando el Anatomical Quality Assurance Checklist (AQUA)

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CORREA-ARAVENA, J.; VÁSQUEZ, B.; OTZEN, T.; MANTEROLA, C. & OTTONE, N. E. Histological techniques for the study of the dentogingival junction: A scoping review using the Anatomical Quality Assurance checklist (AQUA). *Int. J. Morphol., 41(3)*:926-936, 2023.

SUMMARY: The dentogingival junction (DGJ) is an adaptation of the oral mucosa composed of epithelial and connective tissues intimately related with the mineralised tissues of the tooth. The histological evidence available is mainly based on studies in animals, separate evaluations of hard and soft tissues, and studies using conventional histological techniques that eliminate the enamel from preparations. The aim of this study was to carry out a review of the existing evidence on histological techniques available for study of the tooth and periodontium in conjunction in humans. A scoping review was carried out of the available literature referring to study of the tooth and the periodontium in conjunction in humans, in the Web of Science (WoS), EMBASE, Scopus and SciELO databases, using the terms "Histological Techniques"[Mesh]) and "Epithelial Attachment"[Mesh]. One hundred and fifty-nine articles were found, of which 54 were selected for full-text reading. Ten were finally included in the qualitative synthesis, and we applied the Anatomical Quality Assurance (AQUA) checklist for analysis the methodological quality of the selected articles. The results showed that the only articles with a low risk of bias in all five domains according to the AQUA criteria corresponded to Silva *et al.* (2011) and Agustín-Panadero *et al.* (2020). Finally, we conclude that the quality of the histological sections to observe tissues that simultaneously contain the tooth and the periodontium, is conditioned by the selected technique and by the care required in certain specific tasks during the histological processing of the samples.

KEY WORDS: Histological techniques; Epithelial insertion; Histological analysis; Junctional epithelium; Dentogingival junction; AQUA checklist.

INTRODUCTION

The concept “dentogingival junction” (DGJ) is used in dentistry to refer to how a soft tissue, the gum, adheres to a hard tissue, the enamel in an intact periodontium, or to the cementum in the case of a diminished periodontium (Gargiulo *et al*., 1961; Braescu *et al*., 2020). In the 1920s and 1950s, authors like Weski (1922) and Waerhaug (1952) wondered about the nature of the junction between the gum and the tooth, and this debate led in 1959 to the establishment of the concept of DGJ by Sicher (1959).

The DGJ is an adaptation of the oral mucosa which consists of epithelial and connective tissues; it is the first line of defence in the innate immune system against the permanent microbial challenge posed by the presence of the

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Funded (partially) by Project DI22-2023. Dirección de Investigación de la Universidad de La Frontera, Chile.

Received: 2023-02-13 Accepted: 2023-03-27

oral biofilm (Nanci & Bosshartdt, 2006). Other concepts used to refer to this anatomical zone are: epithelial adhesion; junctional epithelium (Bernick *et al*., 1951; Soskolne & Bimstein, 1977); and supracrestal gingival tissue (SGT) which has recently replaced the traditional term "biological width" at the suggestion of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions co-presented by the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) (Jepsen *et al*., 2018).

Histologically, dental tissue is made up of enamel, a highly mineralized extracellular matrix, made up of 95 % inorganic matter, 1 % organic matter, and the rest by water. The calcified substance of the enamel is contained in hydroxyapatite crystals that are organized to form the enamel prisms. Being a highly mineralized structure, enamel disappears after the decalcification process. Dentin, calcified tissue composed of 70 % inorganic material, 12 % water, and 18 % organic matrix, made up almost entirely of collagen (93 %). Its degree of calcification is not uniform, the less calcified areas are the peripheral dentin, the amelodentinal limit, the newly formed dentin next to the pulp. After the decalcification process, organic matter still remains in the dentin, so it is visible microscopically (Lindhe, 2011).

Dental pulp, a specialized loose connective tissue, is composed of cells, fibers, amorphous ground substance, nerves, blood vessels, and lymphatics. It has 75 % water and 25 % organic substance in the young tissue. Finally, the cementum that covers the root surface and maintains the tooth in its socket thanks to its relationship with the periodontal ligament and alveolar bone. Periodontal tissue is divided into protective periodontium (gingiva) and insertion periodontium (periodontal ligament, cementum and alveolar bone). The epithelium covering the free gingival margin is differentiated as: a) Epithelium of the oral cavity, facing the oral cavity, is keratinized stratified squamous type, divided into cell layers (basal, spinous, granular, and horny), including dendritic cells, inflammatory cells, keratinocytes, melanocytes and tactile epithelial cells; b) Sulcular epithelium, facing the tooth without being in contact with the enamel surface and c) Junctional epithelium, responsible for the contact between the gingiva and the tooth through a basal lamina similar to the one used to relate to the underlying connective tissue. It is pyramidal in shape with its vertex pointing to the dental apex and its base towards the gingival sulcus, where the usual desquamation of these tissues occurs (Lindhe, 2011).

The actual epithelial adherence to the tooth is effected by the hemidesmosomes and the internal basal lamina, which adhere to the tooth surface (enamel, cement) and even to the surface of titanium implants. Adhesion with the gingival connective tissue is achieved by means of the external basal lamina. The richness of desmosomes is lower than in the oral cavity and sulcular epithelium, which makes it more permeable to the passage of molecules and other transient cells. Therefore, the adhesion of the junctional epithelium to the tooth can be disrupted relatively easily. When it occurs, the cohesion between the epithelial cells and the other tissue layers of the dentogingival unit is weakened, and an inflammatory change will be promoted, setting the stage for periodontal destruction. The predominant tissue component in the gingiva is connective tissue (lamina propria or chorion). Its main components are collagenous fibers (about 60 % of connective tissue volume), fibroblasts (about 5 %), vessels and nerves (about 35 %), embedded in amorphous ground substance (matrix). Additionally, different types of cells such as fibroblasts, mast cells, macrophages and inflammatory cells (Lindhe, 2011).

The current accumulated knowledge of this small but vital territory for periodontal health rests mainly on histological studies in animals (Waerhaug, 1952) and in human cadaver material (Gargiulo *et al*., 1961; Vacek *et al*., 1994), in attempts to detect the relation between the hard and soft tissues by macroscopic and microscopic study (Schroeder, 2003).

In this context, it is well known that the study of dental tissues demands even more histological technology and customised procedures than studies of bone; the established methods, such as routine techniques and the Cutting-Grinding Technique (CGT) still suffer limitations due to the hard, fragile, heterogeneous nature of the teeth and related tissues. There is therefore a constant demand for the development of better techniques for quick, safe, repeatable, easily executed research into the conjunction of hard and soft tissues, and to detect specific structures or molecules at cell or even sub-cellular level (Willbold & Witte, 2010).

The object of this study was to carry out a systematic review of the existing evidence on histological techniques available for study of the tooth and periodontium in conjunction in humans.

MATERIAL AND METHOD

The design of the article corresponded to a systematic scoping review. In relation to the variable under study, the principal variable that we sought to detect in the primary

studies was simultaneous histological evaluation of tooth and periodontium in the same human sample, by either optical or electron microscope, considering as a fundamental requirement the conservation of the tooth enamel on completion of preparation of the samples.

No search filters were applied for research design, year of publication or language, mainly because the majority of the histological evidence available is based on *in vitro* and/or animal studies.

The following inclusion criteria were established: studies in humans and *in vitro* by optical or electron microscope, without restriction of language or research design, which included analysis of the tooth tissue and its surrounding periodontium in conjunction. The following were excluded: studies in tooth implants, immunological studies, image-based studies, systematic reviews, letters to the editor and comments by specialists. Likewise, to contextualize and carry out a detailed discussion, articles that used bone processing techniques, with decalcification and without decalcification, but in samples other than those included in the scoping review and the AQUEA analysis (dentogingival junction) were also included.

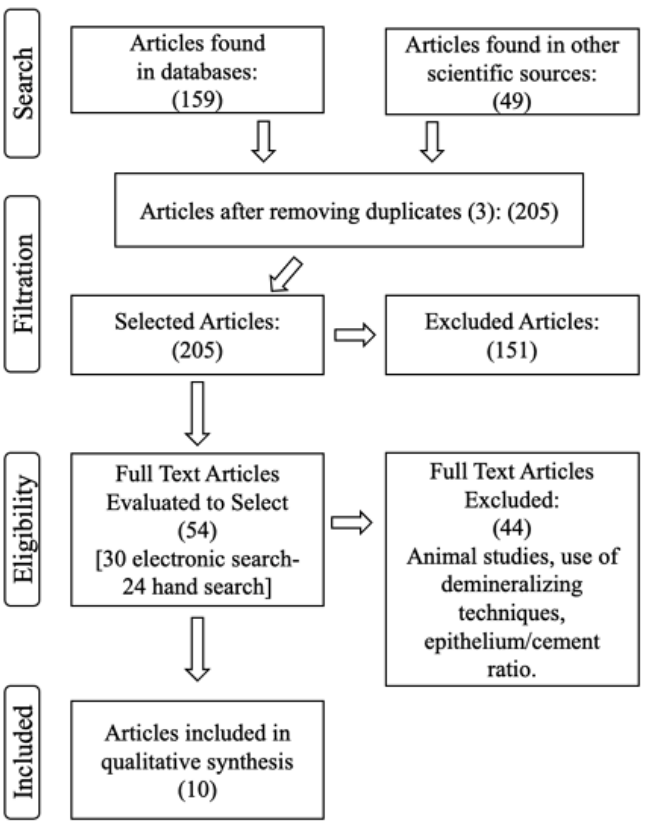

Fig. 1. Article search flowchart.

Various concepts related with the junction between tooth and periodontium were used in a preliminary search in PubMed, e.g.: "histological technique", "histological analysis", "dentogingival junction", and “junctional epithelium”, with inconclusive results. It was therefore decided to use the terms "Histological Techniques"[Mesh]) and "Epithelial Attachment"[Mesh] in order to standardise the search in the four databases selected. The same criteria were applied in the manual search, always considering the presence of the principal study variable.

The search was carried out in Web of Science (WoS), EMBASE, Scopus and SciELO. All the articles collected were identified and filtered systematically following the flow chart of the PRISMA-ScR declaration, together with application of theinclusion and exclusion criteria detailed above (Fig. 1). The initial electronic search produced a total of 159 articles, 30 of which were selected for full-text reading. Furthermore, 49 articles identified manually from other sources of information were considered, 24 of which were selected for full-text reading. The details of the above are shown in Figure 1.

Related to the quality assessment and the risk of bias analysis, two reviewers evaluated methodological quality using the Anatomical Quality Assurance (AQUA) checklist for anatomical studies (Tomaszewski *et al*., 2017), and a second reviewer confirmed their accuracy. Differences between the reviewers were resolved by discussion.

RESULTS

The information sources reviewed produced 159 articles, of which 3 were duplicates. To these were added 49 articles found in other sources of information. Of this total, 151 were discarded for one or more of the following reasons: studies in tooth implants, immunological studies, image-based studies, studies of the medical area and studies without an abstract due to the age of the publication. Fifty-four studies were read in full text, of which 43 were discarded, leaving 10 primary articles for qualitative review and quality assessment (Table I).

Table I. Distribution of primary articles identified by databases.

Databases	Papers found	Papers selected
WoS	19	5
Embase	91	18
Scopus	49	7
SciELO	0	0
Total	159	10

Quality assessment

Application of the AQUA criteria revealed that 60 % of the studies included in this meta-analysis had a low risk of bias in domain one (objective(s) and characteristics of the subject) (Gargiulo *et al*., 1961; Soskolne & Bimstein, 1977; Günhan *et al*., 1996; Majzoub *et al*., 2001; Silva *et al*., 2011; Agustín-Panadero *et al*., 2020), though four articles had a high risk (Bernick *et al*., 1951; Donath & Breuner, 1982; Vacek *et al*., 1994; Calleja Gómez, 2015). In relation to domain two (study design), 60 % (6 articles) presented a high risk (Bernick *et al*., 1951; Gargiulo *et al*., 1961; Soskolne & Bimstein, 1977; Donath & Breuner, 1982; Vacek *et al*., 1994; Günhan *et al*., 1996) and 40 % (4 articles) a low risk (Majzoub *et al*., 2001; Silva *et al*., 2011; Calleja Gómez, 2015; Agustín-Panadero *et al*., 2020). In contrast, in domain three (methodology characterization) 5 articles presented high risk (Bernick *et al*., 1951; Gargiulo *et al*., 1961; Soskolne & Bimstein, 1977; Günhan *et al*., 1996; Majzoub *et al*., 2001) and the other 5 articles low risk (Donath & Breuner, 1982; Vacek *et al*., 1994; Silva *et al*., 2011; Calleja Gómez, 2015; Agustín-Panadero *et al*., 2020). Related to domain four (descriptive anatomy), majority of articles (9, 90 %) showed a low risk of bias (Gargiulo *et al*., 1961; Soskolne & Bimstein, 1977; Donath & Breuner, 1982; Vacek *et al*., 1994; Günhan *et al*., 1996; Majzoub *et al*., 2001; Silva *et al*., 2011; Calleja Gómez, 2015; Agustín-Panadero *et al*., 2020), and only 1 article with high risk of bias (Bernick *et al*., 1951). In domain five (reporting results), also most articles (8, 80 %) presented a low risk of bias (Gargiulo *et al*., 1961; Soskolne & Bimstein, 1977; Vacek *et al*., 1994; Günhan *et al*., 1996; Majzoub *et al*., 2001; Silva *et al*., 2011; Calleja Gómez, 2015; Agustín-Panadero *et al*., 2020), while the remaining 2 articles (20 %) had a high risk of bias (Bernick *et al*., 1951; Donath & Breuner, 1982). There was one article that presented high risk in all domains, and it corresponded to Bernick *et al*. (1951). Finally, the only articles with a low risk of bias in all five domains according to the AQUA criteria corresponded to Silva *et al*. (2011) and Agustín-Panadero *et al*. (2020). Details of the application and assessment of the AQUA tool are given in Tables II and III.

Table II. Details of the application and assessment of the AQUA tool.

References	Study design	Domain 1				Domain 2					Domain 3					Domain 4					Domain 5				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Bernick *et al*., 1951	Cadaveric study	Y	N	N	Y	Y	Y	Y	Y	Y	N	N	N	N	Y	Y	Y	N	N	Y	N	NA	Y	N	NA	N
Gargiulo *et al*., 1961	Cadaveric study	Y	Y	N	Y	Y	Y	Y	Y	Y	N	N	N	N	Y	Y	Y	Y	N	Y	N	Y	Y	Y	NA	N
Soskolne & Bimstein, 1977	Case report	Y	Y	N	Y	Y	Y	Y	Y	Y	N	N	N	N	Y	N	Y	Y	N	Y	N	Y	Y	Y	NA	N
Donath & Breuner, 1982	Cadaveric study	Y	N	N	N	Y	Y	Y	Y	Y	Y	N	Y	N	Y	N	Y	Y	Y	Y	N	N	Y	Y	NA	N
Vacek *et al*., 1994	Cadaveric study	Y	N	N	Y	Y	Y	Y	Y	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	NA	N
Günhan *et al*., 1996	Cadaveric study	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	Y	N	Y	Y	Y	Y	N	NA	Y	Y	NA	N
Majzoub *et al*., 2001	Case report	Y	Y	Y	N	Y	Y	Y	Y	N	N	N	N	N	Y	Y	Y	Y	Y	Y	N	NA	Y	Y	NA	N
Silva *et al*., 2011	Cadaveric study	Y	Y	N	N	Y	Y	Y	Y	N	Y	N	Y	Y	Y	N	Y	Y	Y	Y	N	NA	Y	Y	NA	N
Calleja, 2015	Cadaveric study	Y	N	N	N	Y	Y	Y	Y	N	Y	N	Y	N	Y	N	Y	Y	Y	Y	N	Y	Y	Y	NA	N
Agustín-Panadero *et al*., 2020	Case report	Y	Y	Y	N	Y	Y	Y	Y	N	Y	N	Y	Y	Y	N	Y	Y	Y	Y	N	NA	Y	Y	NA	N

Domains & Questions: Domain 1: Objective(s) and subject characteristics 1. Was (Were) the objective(s) of the study clearly defined? 2. Was (Were) the chosen subject sample(s) and size appropriate for the objective(s) of the study? 3. Are the baseline and demographic characteristics of the subjects (age, sex, ethnicity, healthy or diseased, etc.) appropriate and clearly defined? 4. Could the method of subject selection have in any way introduced bias into the study? Domain 2: Study design 5. Does the study design appropriately address the research question(s)? 6. Were the materials used in the study appropriate for the given objective(s) of the study? 7. Were the methods used in the study appropriate for the given objective(s) of the study? 8. Was the study design, including methods/techniques applied in the study, widely accepted or standard in the literature? If "no", are the novel features of the study design clearly described? 9. Could the study design have in any way introduced bias into the study? Domain 3: Methodology Characterization 10. Are the methods/techniques applied in the study described in enough detail for them to be reproduced? 11. Was the specialty and the experience of the individual(s) performing each part of the study (such as cadaveric dissection or image assessment) clearly stated? 12. Are all the materials and methods used in the study clearly described, including details of manufacturers, suppliers etc.? 13. Were appropriate measures taken to reduce inter- and intra-observer variability? 14. Do the images presented in the study indicate an accurate reflection of the methods/techniques (imaging, cadaveric, intraoperative, etc.) applied in the study? 15. Could the characterization of methods have in any way introduced bias into the study? Domain 4: Descriptive anatomy 16. Were the anatomical definition(s) (normal anatomy, variations, classifications, etc.) clearly and accurately described? 17. Were the outcomes and parameters assessed in the study (variation, length, diameter, etc.) appropriate and clearly defined? 18. Were the figures (images, illustrations, diagrams, etc.) presented in the study clear and understandable? 19. Were any ambiguous anatomical observations (i.e., those likely to be classified as "others") clearly described/depicted? 20. Could the description of anatomy have in any way introduced bias into the study? Domain 5: Reporting of results 21. Was the statistical analysis appropriate? 22. Are the reported results as presented in the study clear and comprehensible, and are the reported values consistent throughout the manuscript? 23. Do the reported numbers or results always correspond to the number of subjects in the study? If not, do the authors clearly explain the reason(s) for subject exclusion? 24. Are all potential confounders reported in the study, and subsequently measured and evaluated, if appropriate? 25. Could the reporting of results have in any way introduced bias into the study? (Tomaszewski *et al*., 2017).

Table III. Anatomical Quality Assurance checklist.

References	Study design	Domain 1	Domain 2	Domain 3	Domain 4	Domain 5
Bernick *et al*., 1951	Cadaveric study	High	High	High	High	High
Gargiulo *et al*., 1961	Cadaveric study	Low	High	High	Low	Low
Soskolne & Bimstein, 1977	Case report	Low	High	High	Low	Low
Donath & Breuner, 1982	Cadaveric study	High	High	Low	Low	High
Vacek *et al*., 1994	Cadaveric study	High	High	Low	Low	Low
Günhan *et al*., 1996	Cadaveric study	Low	High	High	Low	Low
Majzoub *et al*., 2001	Case report	Low	Low	High	Low	Low
Silva *et al*., 2011	Cadaveric study	Low	Low	Low	Low	Low
Calleja, 2015	Cadaveric study	High	Low	Low	Low	Low
Agustín-Panadero *et al*., 2020	Case report	Low	Low	Low	Low	Low

Anatomical Quality Assurance Checklist: Domain 1: Objective(s) and subject characteristics; Domain 2: Study design; Domain 3: Methodology Characterization; Domain 4: Descriptive anatomy; Domain 5: Reporting of results. According to Table II, assessment of each domain ends with a risk of bias question which is marked in bold in the grey box. (Each domain has a set of signaling questions to assist in evaluations and judgements about risk of bias pertaining to the domain). The signaling questions are answered as "Yes", "No", or "Unclear". For these signaling questions, "Yes", "No", and "Unclear" indicate low, high, and unclear risk of bias, respectively. On the other hand, the risk-of-bias question is judged as "Low", "High", or "Unclear". If all signaling questions for a domain are answered "Yes", then risk of bias can be judged "Low". If any signaling question is answered "No", this indicates the potential for bias. Review authors should then reach a consensus regarding this. The "Unclear" option should be used only when the reported data are insufficient to allow for a clear judgment (Tomaszewski *et al*., 2017).

Description of the studies included

Routine histological technique with demineralised samples. Bernick *et al*. (1951) showed the relation of the epithelial proliferation of the junctional epithelium with the principal adjacent fibres of the periodontal membrane in tooth resorption in human teeth and monkey mandibles. The samples were fixed in acetic acid formol with alcohol fixer (5 ml of formalin, 5 ml of acetic acid and 90 ml of alcohol 80 %) for more than a week; they were decalcified using nitric acid 10 % in formalin. Paraffin, paraffin-celloidin and celloidin sections of the blocks were prepared after decalcification. Sections 10 to 150 µm thick were cut and stained with haematoxylin and tryosine, Mallory's connective tissue stain, Verhoeff's elastic tissue stain and Pearson's silver gelatin impregnation.

Gargiulo *et al*. (1961) investigated and established dimensions and relations of the dentogingival junction in humans. Autopsies of human mandibles were fixed in buffered formalin 10 % and included in celloidin blocks. Sections 15 to 20 µm thick were cut and stained with haematoxylin and eosin.

Soskolne & Bimstein (1977) carried out routine histological techniques with decalcification in order to view temporal teeth in humans and epithelial insertion in the buccal and lingual surfaces, among other aspects. The teeth were fixed in buffered formalin and demineralised in formic formalin 5 %; they were then dehydrated and included in paraffin wax. Once the blocks had been obtained, serial sections 7 µm thick were taken and stained with haematoxylin and eosin for viewing by optical microscope. To maintain the adherence between the gum and the tooth, a bevelled internal incision was practiced below the free gum level before extraction.

Majzoub *et al*. (2001), assessed the histological results of two connective tissue grafts combined with a flap of partial thickness repositioned to coronal to cover the root of Miller Class I recessions in the upper right and left canines and first premolars of a 24-year-old woman. These teeth were subsequently extracted following orthodontic indications. The extractions were accompanied by mesial and distal incisions up to 6 mm below the gingival margin, including the gum (and graft) and alveolar bone. Then the samples were placed in physiological serum, fixed in neutral buffered formalin 10 %, decalcified in formic acid 5 % for 4 weeks, processed for inclusion in paraffin and sections 5 to 6 µm thick were cut along the longest axis. The sections were stained with haematoxylin and eosin for viewing and histomorphological analysis. Although the samples were demineralised, it was observed that most of the root surface exposed in both specimens was covered by a band of junctional epithelium located completely above the exposed dentine.

Silva *et al*. (2011) presented a protocol for a routine histological technique with decalcification for easy reproduction in any research or teaching laboratory. Human teeth and fragments of rat maxilla were fixed in neutral buffered formalin 10 % for 48 hours and decalcified with EDTA 10 % aqueous solution for 50 to 60 days. They were then dehydrated, clarified in xylene and embedded in paraffin blocks. Sections 6 mm thick were cut by microtome and stained with haematoxylin and eosin, and Gomori trichrome.

The main methodological characteristics are summarised in Table IV.

Cutting-Grinding Technique (CGT). Donath & Breuner (1982) described a method for studying mineralised teeth and bone with adhering soft tissues which they called the

Table IV. Routine histological technique for the study of the dentogingival junction in human samples.

Authors	Aim of study	Fixative	Decalcification	Inclusion	Sawing	Section thickness	Staining
Bernick *et al.,* 1951	Show the relationship of the epithelial proliferation of the junctional epithelium with the main adjacent fibers of the periodontal membrane in dental resorption.	5 ml formalin, 5 ml acetic acid, 90 ml 80% alcohol for more than a week	10% nitric acid in formalin	Paraffin, paraffin-celloidin, and celloidin	Not mentioned	10-150 µm	Hematoxylin and triosin, Mallory's connective tissue stain, Verhoeff's elastic tissue stain, and Pearson's silver impregnation method.
Gargiulo *et al.,* 1961	Evaluate and establish dimensions and relationships of the dentogingival junction in humans.	10% buffered formalin	Not mentioned	Celloidin	Disk microtome	15-20 µm	Hematoxylin and eosin
Soskolne & Bimstein, 1977	To establish the chronology of the histological changes and to evaluate the participation of the junctional epithelium, the inflammatory process and the multinucleated odontoclasts in the resorption of deciduous teeth.	Buffered formalin	5% formic formalin	Paraffin	Not mentioned	7 µm	Hematoxylin and eosin
Majzoub *et al.,* 2001	Detail the histological nature of the insertion of two grafts to the root surface.	10% neutral buffered formalin.	Formic acid 5%	Parafin	Microtome	5-6 µm	Hematoxylin and eosin
Silva *et al.,* 2011	To present a standardized routine histology protocol for evaluation of human teeth and demineralized rat maxilla fragments.	10% neutral buffered formalin.	EDTA 10%	Parafin	Microtome	6 µm	Hematoxylin and eosin, Gomori trichrome

Cutting-Grinding Technique (CGT). This method has become a reference for histological study of hard and soft tissues in conjunction. The authors used samples of human and animal mandibles (from autopsy or biopsy) of 5 x 10 cm, which is a good size for the cutting machine and for fixing; ideally, they should not be more than 1 cm thick. The technique consists in saturating and fixing the samples in methyl methacrylate; cutting and grinding is carried out by machine, instead of by hand as was done previously. Cutting is carried out using a wood saw in which the standard blade has been replaced by a diamond-tipped blade and a cooling system has been installed. Grinding is done with a machine fitted with a grinding disc 30 cm in diameter, and a water-based cooling system. The samples obtained, with a thickness of 5 to 10 µm, can be stained with any of the stains normally used for tissues embedded in hard plastic.

Vacek *et al*. (1994) examined the natural dimensions of the DGJ in 10 mandibles from adult human cadavers using the recently described CGT in blocks of mineralised tissue; they obtained clear pictures and precise measurements of the junction of the connective tissue, the epithelial junction, the loss of the junction and the depth of the sulcus. From the cadaver material they obtained blocks of two to three teeth using a cutting and grinding machine (Exakt I), following the manufacturer's instructions. This machine can be used to cut serial sections 10 to 30 µm thick of mineralised tissue every 0.75 mm. The resulting thin, non-decalcified sections contained structures for observation and reference, such as the enamel and tooth restorations. The sections were stained with Masson's trichrome or haematoxylin and eosin, and histomorphometric examination was carried out with an optical microscope at magnification of 40x.

The technique proposed by Donath & Breuner (1982) was confirmed by Günhan *et al*. (1996). Although animal samples were used, the method produced clear pictures of the intimate relation between the junctional epithelium and the tooth enamel under optical microscope. Four premolars and segments of surrounding tissue were treated. The samples were fixed in neutral buffered formalin 10 % and dehydrated in ascending series of alcohol at 70, 90, 96 and 100 % for one day at each concentration. These were then infiltrated in resin diluted in ethyl alcohol at concentrations of 30, 50, 70 and 100 %, for one day at each concentration. The samples were not exposed to daylight at any time during the process. Finally, they were embedded in resin moulds at 100 % and left in the sun for 5 to 6 days until the desired hardness was reached. Cutting and grinding was then carried out to obtain sections 25 ± 5 µm thick, which were stained with toluidine blue and covered with a coverslip.

Silva *et al*. (2011) presented a detailed protocol of CGT for the histological evaluation of isolated non-demineralised teeth, and routine histology for analysis of demineralised samples to allow reproduction by any research or teaching laboratory. For CGT, the teeth were embedded in crystal polyester resin (3061) prepared with styrene 10 % and one drop of hardener (MEKP) per 5 mL of solution for 24 hours; then each resin block was sectioned along the longest axis of the embedded tooth with an IsoMet cutting machine fitted with a diamond-tipped cutting disc 0.5 mm

thick. Each section was approximately 10 µm thick. For the grinding phase the authors used a power-driven sander-grinder with wetted sandpaper (600, 1000, and 1200 grains/mm2, giving sections 30 µm thick. For routine histological processing of demineralised samples, care was given to specific tasks during processing, such as formation of access conduits for rapid penetration of the fixing solution, complete removal of the demineraliser, and additional time for dehydration, clarification and setting in paraffin. The authors presented satisfactory results with buffered formalin 10 % as fixing solution and EDTA 10 %, pH 7.3, as demineralising solution. The 6-µm histological sections obtained from samples embedded in paraffin and stained with HE were adequate for the majority of the morphological and morphometric evaluations carried out.

Calleja Gómez (2015) made a detailed analysis of the most common techniques used to characterise the DGJ and alveolar bone, and determined the most important characteristics of the complex for tooth implants. After analysing the bibliography, the author evaluated the morphometry of the DGJ in samples from human cadavers, using radiographic images of individual teeth and histological pictures of mineralised samples obtained through optical microscopy. In the latter case, the samples were washed to remove the excess formol, dehydrated in alcohol at 70 % and 100 % and clarified in xylene. They were processed at ambient temperature and with permanent stirring. They were than impregnated with methyl methacrylate for 7 days, methyl methacrylate with 1 % benzoyl peroxide for 4 days and methyl methacrylate with 5 % polymethyl methacrylatefor 3 days. The whole process was carried out at ambient temperature, without stirring and in darkness. Finally, the block with the embedded samples was placed on a previously prepared methacrylate base and both were submerged in methacrylate and left to polymerise for seven days at ambient temperature and without protection from light. In the cutting phase, diamond-tipped cutting discs were used to obtain central sections of the samples; then the sections were reduced with a grinding machine and wet-sanded with disposable silicon carbide abrasive discs of various particle sizes (Struers®). Finally, to obtain a crystalline appearance, a second sanding phase was added using pastes of diamond in an oil suspension. The samples were stained successively with eosin 1 % in water, and toluidine blue 1 % at pH 3.5 and pH 8. The samples were viewed through a Leica digital optical microscope.

Agustín-Panadero *et al*. (2020) made a histological description of the responses of the soft tissues in an anterior human tooth extracted with the surrounding periodontal tissues intact after restoration by prosthesis using the Biologically Oriented Preparation Technique (BOPT). The sample was fixed in buffered formalin 10 % for 5 days at ambient temperature, dehydrated in a battery of alcohols at rising concentrations for 24 hours in each solution and clarified in xylene. It was then embedded sequentially at ambient temperature in methyl methacrylate for 15 days, methyl methacrylate with benzoyl peroxide (1 g/100 mL) for 3 days and finally in polymethyl methacrylate and benzoyl peroxide. Polymerisation at ambient temperature took several days. The samples were cut with a WELL Precision Vertical Diamond Wire Saw (Agar Scientific); cross-sections 800 mm thick were cut on the vestibular-palatal axis. Grinding was carried out using the LaboPol-21 system (Struers), with silicon-carbide sheets and also diamond pastes of diminishing grain size to obtain sections 80 µm thick. The samples were then stained sequentially with Stevenel's blue and van Gieson's picrofuchsin. To analyse the relation between the hard and soft tissues, the samples were photographed through a Leica DM4000 B clear field microscope with a DFC420 digital camera.

The main methodological characteristics are summarised in Table V.

Cryomicrotomy. Carter *et al*. (1994) used a method developed for cryomicrotomy of non-decalcified bone on the histological preparation of teeth and related tooth tissues. Sections of mandible from Sprague-Dawley rats were frozen instantaneously in isopentane chilled to -170oC with liquid nitrogen. The sections were embedded in carboxymethyl cellulose 1.6 % and then sectioned in the parasagittal and frontal planes at -25oC in a LKB PMV 450MP high resistance cryomicrotome. The 9-µm sections were cut with a hardened-steel D-profile knife (35° angle), and mounted on cold slides covered with a pressure-sensitive adhesive. They were then fixed in buffered formalin 10 %, pH 7.0, for three minutes, stained with haematoxylin and eosin, dehydrated and covered with resin for viewing through a Zeiss Axioplan optical microscope. Additional sections were processed for immuno-histochemical analysis and sweep electron microscopy.

Petrography. Williamson (2015) presented a petrographic technique for histological preparation of thin sections of teeth that can be observed under the microscope without decalcification, allowing detailed analysis of the teeth with all the calcified and non-decalcified tissues present in a section. The sample (lower central incisors of rat) were fixed in formalin and dried overnight at 40oC. Then each tooth was fixed in a block of plastic with Epo-Tech 301 resin and left overnight at 40°C. The teeth were reduced to half their longitudinal thickness with 600-grain carborundum; each remaining half-tooth was stuck to aglass slide with Epo-Tech 301 resin and the thickness was reduced further with a

Table V. Sawing and grinding technique for the study of the dentogingival junction in human samples.

Authors	Aim of study	Fixative	Dehydration	Inclusion	Sawing-Grinding	Section thickness	Staining
Donath & Breuner, 1982	Describe a new method of sawing-grinding for the histological evaluation of maxillary bones with teeth or bones that contain implants (ceramic or metallic).	Any fixative normally used for bone and paraffin embedding.	Alcohol batteries of ascending concentrations.	Methyl Methacrylate	Band saw machine for cutting wood, with diamond band. Polishing machine (Struers Scientific Instruments, Copenhagen) with sanding disc.	5 to 10 µm	Toluidine blue stain, Masson-Goldner-slain.
Vacek *et al.,* 1994	To provide additional information on the dimensions of the dentogingival junction and its related structures using nondemineralized human sections.	Phenolformaldehyde, glycerin and alcohol.	Not mentioned	Not mentioned	EXAKT I sawing-grinding device (Exakt).	10 to 30 µm	Hematoxylin and eosin, Masson's trichrome.
Günhan *et al*., 1996	To examine the histological appearance of periodontal tissues from undecalcified teeth processed with a sawing-grinding technique.	10% neutral buffered formalin	Alcohol batteries of ascending concentrations.	Resin	Manual grinding with different grades of sandpaper.	25 ± 5 µm	Toluidine blue stain
Silva *et al.,* 2011	To present a standardized cut and wear protocol for histological evaluation of non-demineralized human teeth.	Not mentioned	Not mentioned	Crystal polyester resin (3061)	Precision saw cutting machine with diamond disc (IsoMet 1000). Motorized grinding sander machine.	30 µm	No coloring.
Calleja, 2015	Perform morphometric analysis of the dentogingival junction in human samples	Formaldehyde	Alcohol batteries of increasing concentrations and rinsing with xylene	Polymethyl methacrylate	LaboPol-21 wet grinding and polishing machine, Struers brand.	Not mentioned	1% Eosin and 1% Toluidine Blue
Agustín-Panadero *et al.,* 2020	Histologically analyze a resected human tooth with intact periodontal tissues.	10% neutral buffered formalin.	Ascending concentration alcohol batteries.	Polymethyl methacrylate	WELL Precision Vertical Diamond WirenSaw cutting machine, Agar Scientific. LaboPol-21 wet grinding and polishing machine, Struers brand.	80 µm	Stevenel`s blue and val Gieson`s picro-fuchsin.

diamond-tipped saw. Then the tooth sections were sanded to a thickness of approximately 40 mm using 600-grain carborundum. Once the desired thickness had been achieved, the samples were polished with three grades of sanding-cloth using three grades of diamond paste. Sanding eliminated approximately 10 mm, leaving tooth sections approximately 30 mm thick stuck to the glass slide. The sections can be observed with a fluorescence microscope with a blue light filter block (450-490 nm).

DISCUSSION

The histological study of periodontal tissues has always been challenging because it seeks to observe mineralized hard tissues and soft tissues at the same time. They therefore require highly specific methods to obtain sections of histological quality. Traditional techniques for histological analysis of the DGJ include the routine histological technique, CGT and cryomicrotomy. More recently, the technique of petrography has been explored (Williamson, 2015).

Before 1960, bone tissue could only be studied by routine histological techniques, involving demineralisation of the samples to obtain sufficiently soft tissues for inclusion in paraffin and cutting with a microtome (Bernick *et al*., 1951, Cano-Sánchez *et al*., 2005). To apply this technique in the teeth and periodontium, a variety of fixing and demineralising solutions have been proposed (Keklikoglu & Akinci, 2013); however, buffered neutral formalin 10 % as the fixing solution and EDTA 10 %, pH 7.3, as demineralising solution have given the most satisfactory results (Silva *et al*., 2011). Sections 6 mm thick obtained from samples embedded in paraffin and stained with haematoxylin and eosin meet the technical requirements for most morphological and morphometric evaluations of these tissues. Furthermore, this routine protocol allows the use of serial sections for more specific techniques, such as histochemical and immuno-histochemical analyses, which are suitable for evaluating the cell constituents and extracellular matrix of the teeth and periodontium (Mukai *et al*., 1986; Silva *et al*., 2011). Nevertheless, application of this technique to study the DGJ has met with some problems; for example, enamel contains 96-98 % calcium (Eimar *et al*., 2012), dentine 70 % calcium (Nanci, 2008) and cementum 50 % calcium (Nanci & Bosshardt, 2006). Thus a large proportion of basic tooth tissue is lost by decalcification; furthermore dimensional changes may occur in the soft tissues due to the retraction of the mineralised bone interface and the surrounding tissue (Vacek *et al*., 1994), as well as damage to the structure of the soft tissues, deterioration in cell integrity, and alteration in the stain properties caused by exposure to the decalcifying agents (Keklikoglu & Akinci, 2013; Savi *et al*., 2017). Moreover, to obtain histological preparations of teeth and periodontium of satisfactory quality, care must be given to specific tasks during sample processing, such as formation of access conduits for rapid penetration of the fixing solution, complete removal of the demineraliser, and additional time for dehydration, clarification and setting in paraffin (Silva *et al*., 2011).

With the arrival of hard embedding media, such as acrylic and epoxy resins, and the introduction of high resistance microtomes and special cutting systems, it has become possible to examine hard tooth tissues in conjunction with the surrounding soft tissues, providing information that was unobtainable from demineralised tissues. Thus, CGT allows the mineral phase of the tooth and the bone to be maintained intact, and a good structural relation between the organic and inorganic components can be preserved without sacrificing histological details (Donath & Breurner, 1982; Günhan *et al*., 1996). Trisi *et al*. (1991) studied the effectiveness of CGT (Exakt System) in non-demineralised hard tooth tissues, concluding that it is possible to obtain sections less than 10 mm thick from specimens like teeth, crowns, bridges, implants, and mineralised structures that cannot be cut with routine histological techniques. The following year, Rohrer & Schubert (1992) also used this technique for studies of teeth with metal fillings, crowns and implants. Nevertheless, carrying out bone histology without decalcifying the sample is a technical challenge, especially with large specimens. Due to the density and lesser permeability of bone, considerably longer fixing and processing times are required, often of several weeks (Goldschlager *et al*., 2010). Laboratories are required with more expensive, highly specialised equipment, trained staff, and more advanced installations, as well as very demanding safety standards (Karantzoulis *et al*., 2013). These procedures become even more expensive, complex and difficult when they involve the enzymatic and immuno-histochemical characterisation of sections of hard tissue processed without decalcification (Troiano *et al*., 2009).

Despite the difficulties of these two techniques (routine and CGT), Keklikoglu & Akinci (2013) state that – so long as decalcification is not a problem, due to the time it takes to prepare the tooth for histological evaluation and given the characteristics of study – the most suitable method is to make a histological preparation in paraffin of the decalcified teeth. However, if the waiting time for decalcification is a problem, or if decalcification is not a suitable technique for the study, preparations taken from samples embedded directly, using methyl methacrylate without decalcification and with haematoxylin and eosin staining, can also provide acceptable levels of histological detail.

Cryomicrotomy, using a high resistance freezing microtome, is useful for examining the structures of hard and soft tissues. Both the cell structure and the extracellular structure are well preserved, and the sections of teeth and bone appear to be suitable for examination under optical or scanning electron microscope, and for immuno-histochemical analysis. However, this technique has the disadvantage of strong, non-specific general adhesion of immuno-histochemical reagents to enamel (Carter *et al*., 1994).

Although petrographic preparation has been used for more than forty years in university geology, engineering and archaeology departments all over the world, this technique is new for dental research. It has proved useful for analysis of both hard and soft tissues in sections. It is also particularly useful for histological sections and micro-radiographies of tissues containing artificial implants (metal, ceramic, polymeric and compound) which cannot be sectioned by microtome; and it allows the use of various types of tissue staining (Williamson, 2015). Nevertheless, further studies are needed to evaluate its application to all areas of dental research.

CONCLUSION

Based on our analysis, we observed that the quality of histological sections is conditioned by the technique selected and by the care required in certain specific tasks during histological processing of the samples, to obtain samples containing tissues of both the teeth and the periodontium simultaneously. At least four factors must be considered when selecting a histological method: (a) the urgency of the case; (b) the state of mineralisation of the tissue; (c) the purpose of the investigation; and (d) the staining technique to be used (Silva *et al*., 2011).

CORREA-ARAVENA, J.; VÁSQUEZ, B.; OTZEN, T.; MANTEROLA, C. & OTTONE, N. E. Técnicas histológicas para el estudio de la unión dentogingival: Una revisión de alcance utilizando el Anatomical Quality Assurance Checklist (AQUA). *Int. J. Morphol., 41(3)*:926-936, 2023.

RESUMEN: La unión dentogingival (DGJ) es una adaptación de la mucosa oral compuesta por tejidos epitelial y conectivo íntimamente relacionados con los tejidos mineralizados del diente. La evidencia histológica disponible se basa principalmente en estudios en animales, evaluaciones separadas de tejidos duros y blandos y estudios utilizando técnicas histológicas convencionales que eliminan el esmalte de las preparaciones. El objetivo de este estudio fue realizar una revisión de la evidencia existente sobre las técnicas histológicas disponibles para el estudio del diente y el periodonto en conjunto en humanos. Se realizó un scoping review de la literatura disponible referente al estudio del diente y el periodonto en conjunto en humanos, en las bases de datos Web of Science (WoS), EMBASE, Scopus y SciELO, utilizando los términos “Histological Techniques”[Mesh]) y " Epithelial Attachment"[Mesh]. Se encontraron 159 artículos, de los cuales 54 fueron seleccionados para lectura de texto completo. Diez fueron finalmente incluidos en la síntesis cualitativa, y se aplicó la lista de verificación Anatómica Quality Assurance (AQUA) para el análisis de la calidad metodológica de los artículos seleccionados. Los resultados mostraron que los únicos artículos con bajo riesgo de sesgo en los cinco dominios según los criterios AQUA correspondían a Silva *et al*. (2011) y Agustín-Panadero *et al*. (2020). Finalmente, concluimos que la calidad de los cortes histológicos para observar los tejidos que contienen simultáneamente el diente y el periodonto, está condicionada por la técnica seleccionada y por el cuidado requerido en ciertas tareas específicas durante el procesamiento histológico de las muestras.

PALABRAS CLAVE: Técnicas histológicas; Inserción epitelial; Análisis histológico; Epitelio de unión; Unión dentogingival; Lista de verificación AQUA.

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