Influence of the chlorhexidine application on adhesive interface stability: literature review

Carla Miranda, Gabriela Vieira Silva, Mariáh Damiani Vieira, Simone Xavier Silva Costa

Corresponding author: Carla Miranda
Rua João Pio Duarte e Silva, n. 94 – ap. 201 – Córrego Grande
CEP 88037-000 – Florianópolis – SC – Brasil
E-mail: miranda.carla@unisul.br

Abstract
Introduction: There is a consensus that dentine/resin bonding deteriorates over time, and such degradation is one of the main reasons for limiting adhesive restoration longevity. Enzymes known as matrix metalloproteinases (MMPs) are responsible for enzymatic degradation of collagen fibrils without protection, which are present in the resin-dentine interface. Therefore, these enzymes are involved in the process of adhesive interface degradation. Currently, studies point out chlorhexidine digluconate has antiproteolytic function by inhibiting the action of MMPs. Thus, it is thought this substance application prior to the use of bonding agents could slow the process of degradation of the tooth-restoration interface, resulting in longevity. Objective: To review the literature on the influence of chlorhexidine application on the stability of the adhesive interface. Literature review: Chlorhexidine digluconate proprieties and its application in Dentistry were discussed. Next, hybrid layer formation and degradation was discussed and the mechanism of action of chlorhexidine on preserving this layer was detailed. Finally, scientific studies from the last six years were analyzed on the performance of adhesive systems after chlorhexidine application. Results: Considering the results of reviewed studies, it can be concluded that chlorhexidine application did not interfere on the immediate bond strength to dentin and
hybrid layer degradation over time occurred later and/or with lower intensity. **Conclusion:** Chlorhexidine application interferes positively when incorporated into the adhesion protocols, by promoting hybrid layer stability over time.

**Introduction**

The improvement of dental adhesive materials has allowed important advancements in restorative technique, providing more conservative and esthetic treatments. Currently, scientific researches have targeted the assessment and improvement of bonding to dentine because bonding to this tooth substrate is critical. Dentine has a structural complexity because of its high organic content and intrinsic variations in mineralization and humidity. Moreover, caries removal forms the smear layer on dentinal surface. Smear layer contains debris making difficult the interaction of substances applied to dentine [53].

The adhesive systems can remove total or partially the smear layer and the mineral of underlying dentine, replacing them by resin monomers which are involved within a collagen fibrils-rich layer [17]. Thus, bonding to dentine is based on hybridization mechanisms in which the micromechanic bonding between adhesive polymers and collagen fibrils of demineralized dentine, forming the hybrid layer [40]. The literature agress that the bonding of resin to dentin deteriorates over time, and such degradation is one of the main reasons for limiting adhesive restoration longevity [41].

The mechanism of adhesive interface degradation is the result of the deterioration of both the resin components, hydrolysis, and enzymatic degradation of collagen fibrils without protection within resin/dentine bonding. With regard to these enzymes (metalloproteinases – MMPs), studies have demonstrated its effect on collagen fibril degradation and indicated the possibility of slowing this process through inhibiting its activity [3, 5, 15, 47, 49].

Chlorhexidine digluconate, usually employed as antimicrobial agent, has demonstrated antiproteolytic function by inhibiting MMP action. Thus, studies have employed this solution after acid etching and prior to bonding agents, as slowing adhesive interfaces [3, 5, 15, 47, 49].

In some studies, chlorhexidine use after acid etching and prior to bonding agent did not compromise adhesion [3, 5, 15, 47, 49]. Moreover, these studies indicated that the application of chlorhexidine solution may slow the adhesive interface degradation, and can be considered as a promising alternative in the search for long-term longevity of adhesive restorations.

This present literature review aimed to evaluate the influence of chlorhexidine on adhesive interface stability.

**Literature review**

The bibliographic search was performed through using the following databases: Scopus, Medline, Scielo, PubMed, ScienceDirect and Portal of journals sponsored by Capes. The search was not limited by the year of publication, except from the subitem “Performance of bonding agents with prior use of chlorhexidine”, from 2007 to 2013.

The following keywords were used to search the journals: chlorhexidine and dental adhesives, chlorhexidine and dentine.

**Properties and application of chlorhexidine digluconate**

Chlorhexidine digluconate solution at low concentrations acts as bacteriostatic agent, and at high concentrations it acts as bacteriolitic agent. Additionally, chlorhexidine may also inhibit bacterial adherence to surfaces through calcium competition [39].

Chlorhexidine is commercially available at different concentrations, in the form of gel or solution, and associated with other compounds, i.e., dentifrices, phosphoric acid for tooth etching [1].

Generally, chlorhexidine gluconate solution concentration ranges from 0.02 to 5% and can be employed in wound and burn treatment and as antiseptic [28]. Orally, chlorhexidine is found in mouthrinses at 0.12 % to 0.2 % [20]. Chlorhexidine gels are presented at 0.5 % to 1 % concentrations applied with toothbrushing or trays to reach all tooth surfaces, although both ways can be less effective [2]. Specifically, dentifrices contain 0.6 or 0.8 % chlorhexidine [46].

Chlorhexidine digluconate can be employed as cavity disinfectant, as solution, just after the removal of all caries tissue and prior to acid etching [13].
Other application currently proved by recent studies is chlorhexidine digluconate after acid etching. This step seems to promote and increase in restoration longevity by slowing the degradation process of adhesive interface [11].

When chlorhexidine digluconate is applied aiming to preserve the hybrid layer, used after acid etching, the concentrations should be equal or greater than 0.1% [54]. Most studies have employed concentrations of 2% [3-5,7-13, 24, 27].

Formation and degradation of hybrid layer

Hybrid layer is a zone of bonding between dentine and resin composite which is formed after dentine demineralization by phosphoric acid, exposing collagen fibrils. Accordingly, this surface receives the bonding agent which allows the organic substrate adhesion to restorative material. This adhesion is established by micromechanical phenomena between the adhesive polymers and collagen fibrils that were demineralized [40].

The mechanism of bonding to dentine is much more complex than that of enamel. Dentine has smaller content of inorganic substances and greater amount of water, making more difficult to achieve a long-lasting adhesion of resin to dentin [36].

Adhesion of resin to dentin becomes viable due to hydrophilic bonding agents capable of infiltrating and polymerizing within the collagen net exposed through dentine decalcification [4]. However, despite of significant improvements in bonding agents, adhesive interface is the area most susceptible to failure between tooth and restoration. If dentine/adhesive interface is exposed to oral cavity, marginal discoloration, loss of marginal adaptation and subsequent loss of restoration retention may occur [6].

Once hybrid layer is formed, adhesive interface stability will be based on the creation of a compact and homogenous layer. Notwithstanding, this adhesion degrades over time, due to MMPs hydrolysis and proteolytic action [4].

Mechanism of action of chlorhexidine in preserving hybrid layer

Dentine contains matrix metalloproteinases (MMPs), which are a group of zinc- and calcium-dependent enzymes regulating the physiologic and pathologic mechanism of collagen-based tissues [14]. In a study conducted by Pashley et al. [44], MMPs action was inhibited by the use of proteases, preserving the structural integrity of collagen fibrils, which would minimize hybrid layer degradation.

In vivo [27] and in vitro [10] studies have revealed that dentinal collagen degradation activities may be reduced through the use of chlorhexidine digluconate on dentine surface after phosphoric acid application and prior to adhesive application.

Metalloproteinases are a set of 23 zinc- and calcium-dependent endopeptidases having the capacity of degrading extracellular matrix components. Dentine has MMP-2 (gelatinase-A), MMP-8 (collagenase-2), MMP-9 (gelatinase-B), MMP-14 and MMP-20 (enamelysin) [38, 51].

Chlorhexidine digluconate solutions are capable of completely inhibiting MMP-2 and MMP-9 activity, even at concentrations as small as 0.03%. Thus, chlorhexidine digluconate is effective at very low concentrations of 0.02% and 0.002%, but it is not known yet which concentration is more effective and how much time is required for such application [26].

Recently, in vitro [4, 8, 10] and in vivo [11, 27] studies have demonstrated good results in inhibiting subclinical degradation after the application of chlorhexidine digluconate (2%) onto dentine etched by phosphoric acid prior to conventional single-component bonding agent.

Some authors affirmed that chlorhexidine digluconate solution application onto etched dentine did not negatively influence the immediate bond strength of adhesive systems to this substrate [4, 10, 11, 13, 25, 45].

Consequently, chlorhexidine digluconate solution application after dentine acid, etching and prior to adhesive application acts as an antimicrobial agent in addition to a more important function: the prevention or slowing of collagen fibril degradation, resulting in more stable long-term adhesive interfaces [11, 19].

Performance of adhesives after chlorhexidine use

Brackett et al. [4] conducted an in vivo study in which hybrid layer degradation in deep occlusal restorations was evaluated through transmission electron microscope (TEM). Control group was restored according to the manufacturer's instructions through one-bottle adhesive (Single Bond/3M Espe). Experimental group received the application of 2% chlorhexidine digluconate solution for 30 seconds after acid etching. After two and six months of saliva storage, microtensile tes was performed.
During the analysis of adhesive failures, the authors observed that all failures were adhesive. Both groups did not exhibit degradation after two months. A small degradation was found in control group after six months, but not in experimental group. No statistically significant differences were observed in microtensile strength between groups.

Carrilho et al. [11] tested the hypothesis that adhesive interface degradation could be prevented or slowed through application of 2% chlorhexidine digluconate, for 60 seconds, after acid etching. Single Bond (3M Espe) was applied at two moments (immediately and 14 months after saliva storage). Through microtensile test and TEM analysis, in vivo bond strength was stable in the specimens treated with - chlorhexidine digluconate, but significant decrease in control group. Test groups exhibited normal structured of collagen net. The authors concluded that the self-degradation of the collagen matrix may occur in the adhesive interface, but it can be avoided by applying an inhibitor agent.

Carrilho et al. [10] conducted an in vitro study on adhesive interface preservation with chlorhexidine digluconate through microtensile test. The authors hypothesized whether chlorhexidine digluconate would slow interface degradation through inhibiting the action of metalloproteinases. The two-step conventional adhesive (Single Bond/3M Espe) was used. The authors observed that 2% chlorhexidine digluconate used for 60 seconds significantly preserved the bond strength after six months of artificial saliva storage. Scanning electronic microscopy (SEM) analysis showed no failures in hybrid layer compared with control group after six months; cohesive failure was predominant. Therefore, the authors suggested chlorhexidine digluconate use to preserve the bond strength of adhesive interface.

Erhardt et al. [23] performed a study aiming to investigating the effect of protease inhibitors such as EDTA and 5% chlorhexidine digluconate, for 120 seconds may influence on the microtensile bond strength of an adhesive system (Adper Scothbond/3M Espe) to dentine affected by caries. SEM analysis showed predominantly mixed failures. The authors concluded that the use of inhibitors did not compromise bond strength to dentine affected by caries and suggested that further studies are necessary to discover which is the ideal MMPs inhibitor that would result in hybrid layer preservation and longevity of restorations.

Campos et al. [9] conducted a study aiming to investigate the effects of chlorhexidine digluconate at 0.2% and 2% for 60 seconds on bond strength to dentin of two adhesive systems (Single Bond/3M Espe and Clearfil Tri-S Bond/Kuraray). Three-cycle thermocycling tests were carried out immediately and after six months, at every 8 hours. The results showed that 2% chlorhexidine digluconate was capable of decreasing the loss of microtensile bond strength over time for both adhesive agents. SEM analysis found most adhesive failures. Small concentrations of chlorhexidine digluconate (0.2%) did not have the same effect when associated with self-etching adhesive.

Komori et al. [30] evaluated the effect of 2% chlorhexidine digluconate, for 60 seconds on the bond strength of two adhesive agents (Scotchbond Multipurpose and Single Bond 2, a 3M Espe) to sound and caries affect dentine, through microtensile test immediately and after six months of artificial saliva storage. The authors concluded that chlorhexidine digluconate did not affect immediate bond strength to sound and caries affected dentine. Chlorhexidine digluconate significantly decreased the bonding loss after six months in sound dentine group, but did not altered bond strength in caries affected dentine group. SEM analysis revealed that most failures were mixed, followed by interface failures.

Loguercio et al. [33] evaluated different concentrations of chlorhexidine digluconate (0.002%, 0.02%, 0.2%, 2%, and 4%) at two application times (15s and 60s) after acid etching. The following adhesive agents were used: Prime & Bond 2.1 (Dentsply) and Adper Single Bond (3M Espe). The authors concluded that 0.002% chlorhexidine digluconate, applied for 15 seconds on demineralized dentine is already capable to degrade resin/dentin adhesive interface for six months. SEM analysis showed that failure types were similar for all adhesives tested.

Stanislawczuk et al. [49] studied the effect of 2% chlorhexidine digluconate for 60 seconds, after acid etching on bond strength of resin to dentine, immediately and after six months; and evaluated the nanoinfiltration pattern when chlorhexidine digluconate was applied in aqueous solution alone or aqueous solution associated with phosphoric acid. The following adhesive agents were tested: Adper Single Bond (3M Espe) and Prime & Bond NT (Dentsply). SEM analysis revealed that most failures were mixed. The authors concluded that the use of chlorhexidine digluconate aqueous solution associated with acid was effective for reducing tooth/restoration interface degradation, immediately and six months after water storage.
Zhou et al. [54] investigated whether chlorhexidine digluconate application could preserve the interface bond strength. The following concentrations were employed: 0.05%, 0.1%, 0.5% and 1.0%, applied for 60 seconds to dentine after acid etching. The adhesive agent used for microtensile test was Clearfil SE Bond (Kuraray). Samples were analyzed in SEM showing tendency towards cohesive failures. The authors concluded that chlorhexidine digluconate may preserve bond strength since it was used at concentration equal or greater than 0.1%.

Dalli et al. [15] evaluated the effect of 1% chlorhexidine digluconate gel on bond strength to dentine in resin composite restorations using two adhesive systems (Prime & Bond NT/Dentsply and Clearfil SE Bond/Kuraray). The authors employed immediate shear bond test and the specimens were evaluated through SEM, which exhibited the predominance of adhesive failures. The authors concluded that 1% chlorhexidine digluconate gel did not adversely affect shear bond strength of adhesive agents to dentine.

De Munck et al. [16] verified the enzymatic endogenous degradation associated to self-etching bonding agents. For this purpose, the authors added MMP inhibitors: chlorhexidine, a non-specific inhibitor; and SB-3CT, a specific inhibitor of MMP-2 and MMP-9. The authors concluded that endogenous MMP-2 and MMP-9 involvement in the process of bond strength degradation is minimum for self-etching adhesive agents.

Manfro et al. [36] evaluated the use of chlorhexidine digluconate at 0.5% and 2% concentrations, for 30 seconds, after acid etching on immediate bond strength of deciduous teeth. The adhesive agent was Adper Single Bond 2 (3M Espe), and microtensile test was immediately carried out. SEM analysis found adhesive or mixed failures. The authors reported that 0.5% and 2% chlorhexidine digluconate showed similar behaviors and did not adversely affect the immediate bond strength to dentine when compared with control group. The authors affirmed that adhesion to dentinal substrate is much more complex than that of enamel because dentine has smaller inorganic content and more water amount, which makes difficult a long-lasting adhesion.

Ricci et al. [47] evaluated the mechanical stability of resin/dentine interface in the presence of 2% chlorhexidine digluconate, applied for 60 seconds, after acid etching. The authors performed the immediate microtensile test with Prime & Bond NT (Dentsply), and the specimens were analyzed in TEM. Mostly, the failures were adhesive types. The authors concluded that the use of chlorhexidine digluconate did not jeopardize the immediate adhesion and was capable to reduce the interface degradation rate at the first months after restoration.

Shafiei et al. [48] evaluated the effect of 2% chlorhexidine digluconate use, for 40 seconds, on microleakage of restorations by using four different adhesive agents (Scothbond Multipurpose/3M Espe, Excite/Ivoclar Vivadent, Clearfil SE Bond/Kuraray and Ibond/Heraeus Kulzer). The authors concluded that chlorhexidine digluconate did not affect microleakage of the four adhesive tested.

Zhou et al. [55] investigated whether chlorhexidine digluconate at the following concentrations of 0.05%, 0.1%, 0.5% and 1%, for 60 seconds, after acid etching associated with Clearfil SE Bond (Kuraray), would affect the bond strength of adhesive interface. The authors concluded that chlorhexidine digluconate associated with the adhesive did not jeopardize the immediate bond strength at concentrations equal or greater than 1%.

Leitune et al. [31] evaluated the influence of 2% chlorhexidine digluconate for 30 seconds after acid etching of dentine. The author performed shear bond strength tests immediately and after six months in deciduous teeth. The adhesive agent tested was Scothbond Multipurpose (3M Espe). The authors observed that there were no statistically significant differences between the groups evaluated.

Osorio et al. [42] conducted tests that indicating the amount of degradation suffered by the collagen exposed after acid etching and 24 hour, one and three week storage, in the presence of absence of chlorhexidine digluconate. Chlorhexidine digluconate reduced the collagen degradation in 30%. The dentine treated with self-etching adhesive, the MMPs inhibiting effect by chlorhexidine digluconate lasted for until three weeks.

Islam et al. [29] conducted an in vitro study investigating the effect of the incorporation of the extract of grape seed, hesperadin, and chlorhexidine digluconate to Clearfil SE Bond (Kuraray) on the bond strength of adhesive interface. SEM analysis showed that mostly cohesive types occurred. The authors concluded that hesperadin incorporated to primer exerted positive influence on immediate microtensile test and mechanical properties, while chlorhexidine digluconate did not affect the bond strength.

Manfro et al. [35] evaluated the effect of different concentrations of chlorhexidine digluconate at 0.5% and 2% on immediate bond strength to deciduous
dentine, immediately and after 12 months of saliva storage. Single Bond (3M Espe) adhesive was used in microtensile tests. The results confirmed the concept that chlorhexidine digluconate, at different concentrations, can prevent the degradation of the adhesive interface in deciduous teeth. Also, no significant reduction was found in bond strength values when 0.5% and 2% chlorhexidine digluconate was used.

The influence of chlorhexidine on bond of self-etching and conventional adhesive systems to dentine was evaluated through microtensile and nanoinfiltration tests after thermocycling. The results demonstrated a preservation of interface in conventional adhesives; no significant effect was found in self-etching adhesives [21].

Dutra-Correa et al. [22] clinically evaluated the hypothesis that 2% chlorhexidine digluconate use would not affect the clinical behavior of two adhesive systems: XP Bond (Dentsply) and Xeno V (Dentsply). The results demonstrated that chlorhexidine application prior to the application of adhesive systems did not exert influence on the clinical performance of the adhesive systems at six and 18 month periods.

Lin et al. [32] evaluated in vitro the influence of peripheral enamel presence, the dentinal pre-treatment with chlorhexidine digluconate, and storage time on microtensile bond strength of a two-step self-etching adhesive system and self-etching resin cement. The authors concluded that the absence of peripheral enamel and longer storage times decreased the bond strength of two-step self-etching adhesive systems self-etching resin cement. Moreover, dentinal pre-treatment with chlorhexidine improved bonding stability.

Stanisławczuk et al. [50] evaluated the effect of chlorhexidine added at concentrations ranging from 0.01 to 0.2% on two experimental adhesive systems. The authors analyzed the bond strength, conversion degree, water sorption, solubility, chlorhexidine release, microtensile, and immediate and 1-year nanoinfiltration. The results were positive for chlorhexidine addition which increased the longevity of adhesive interface, without compromising the mechanical properties evaluated.

Discussion

Despite the large number of studies on the mechanisms of resin/dentine degradation, the subject has not been completely elucidated. The last-longing adhesion to a vital and moist substrate, as dentine, is deficient. To achieve the bonding of adhesive resin to dentinal substrate, the mineral phase has to be totally or partially removed and replaced by adhesive solution. The bonding agent has to infiltrate into this collagen fibril-rich layer and polymerize in situ, forming the so-called hybrid layer [37, 40].

Currently, the use of chlorhexidine digluconate has been discussed after acid etching and prior to adhesive application because chlorhexidine digluconate inhibits MMPs, which account for the degradation of the collagen exposed on the base of the hybrid layer. Thus, its action slowed the degradation of the adhesive interface over time [33].

By evaluating studies that employed bond strength tests between dentine and restoration, immediately and with chlorhexidine digluconate use after acid etching and prior to adhesive agent application, no alteration was found in the values of control group (without chlorhexidine) [3, 5, 6, 9-11, 13, 15, 19, 27, 30, 33, 47, 49, 54]. Accordingly, this result is favorable to the use of chlorhexidine digluconate because its addition did not affect immediate bond strength, important for the initial maintenance of the restoration [17].

Other studies conducted after the specimen storage for some months aiming to simulate the restoration aging, the authors found reduction in bond strength over time. Notwithstanding, chlorhexidine digluconate group showed a significant smaller reduction than that of control group [8, 11, 32]. Similar researches pointed out to a reduction in bond strength without alteration in the values where chlorhexidine digluconate was applied [5, 10, 13, 27, 33, 54]. Based on these results, it is possible to confirm that chlorhexidine digluconate has a positive effect on slowing the adhesive interface.

Similarly, some in vivo studies have demonstrated that chlorhexidine digluconate reduced the degradation of resin/dentin interface and decreased nanoinfiltration without promoting adverse effects on the effectivity of adhesive materials [3, 5, 15, 22, 47, 49].

However, in other in vitro studies in which the storage of specimens over time was performed, a different result was observed because no significant difference between the use or not of chlorhexidine digluconate [19, 30].

The difference in results among studies possibly occur because of methodological differences and the use of different adhesive systems, which makes their comparison different.

With regard to the methodology employed, by analyzing the studies, it was verified that
microtensile test was the most used aiming to verify the bond strength between tooth/restoration [3-5, 8-11, 13, 15, 23, 24, 32]. This test has been largely employed in the literature due to accurate and safe results, because of the reduced bonding area (smaller than 2 mm²), enabling the occurrence of minor structural failures at adhesive interface. Moreover, microtensile test provides obtaining many specimens from a single tooth, performing the evaluation of bond strength on small areas, and analyzing the adhesion in clinically relevant substrates, such as sclerotic dentine or caries affected dentine [34, 41, 43, 52].

With regard the adhesive systems used, among the studies evaluated, one-step self-etching and two-step conventional adhesive systems have been the most employed. Possibly, this occurred because these systems are already simplified and the use of chlorhexidine digluconate will increased one more step for the execution of the restoration.

Additionally, conventional adhesive systems seemed to have more favorable results in relation to chlorhexidine use [4, 9-11, 30, 33, 35, 47, 49], because studies conducted with self-etching adhesive systems have shown positive effect, [32, 42], little or none influence on adhesion durability [9, 16, 21, 54]. It has been reported the need of a chlorhexidine concentration 0.1% [54] and 0.2% [9] to achieve any effect on preserving tooth/restoration interface. This fact can be explained by the involvement of endogenous MMP-2 and MMP-9 in the process of bonding degradation are minimum for self-etching adhesive systems, according to the study of De Munck et al. [16].

With regard to chlorhexidine digluconate concentration, it was observed a predominance of studies at 2%, ranging from 0.0001% to 5% [3-5, 7-13, 24, 27]. At 2%, satisfactory results were obtained with reduction and/or stabilization of adhesive interface degradation over time.

Most of researches employed the chlorhexidine application time for 60 seconds, and among the studies this time period varied from 15 to 120 seconds. Loguercio et al. [33] evaluated two application times (15 and 60 seconds) and concluded that at concentration of 0.002% of chlorhexidine digluconate for 15 seconds, it was already possible to slow the resin/dentine interface degradation for a period of six months in conventional adhesive systems.

Concerning to the storage time for posterior evaluation of the adhesive interface in in vitro studies, mostly immediate, six-, and 12-month tests were performed. The most used storage media was artificial saliva and distilled water [3, 4, 8-11, 13, 15, 24]. The storage mimics the adhesion aging, aiming to evaluate the bonding durability in in vitro studies, and the immediate analysis was performed to verify the initial resistance of the restoration [17].

In vivo studies were found in smaller number, probably because of the execution difficulty, and most studies were performed in vitro [3, 4, 11, 12, 21, 27, 50].

Based on the aforementioned discussion, it could be observed a great variation among the scientific studies regarding to methodology; however, the results seem to pint out to confirm the hybrid layer preservation with chlorhexidine digluconate use after acid etching, which would enable the increase of the longevity of restorations.

**Conclusion**

Based on the results obtained in the revised studies, it can be concluded that chlorhexidine digluconate application did not interfere in most of studies on immediate bond strength to dentin when conventional and self-etching adhesive systems were used.

Hybrid layer degradation occurred later and/or with less intensity when chlorhexidine digluconate was incorporated to adhesion protocols. Thus, it positively interfered in hybrid layer stability over time.

Therefore, changes in adhesion protocol aiming to incorporate this MMPs inhibitor agent can be considered and further investigated. However, further studies are necessary to incorporate this step to clinical restorative protocol.

**References**


