Prevention of enamel erosion through CO_2 laser irradiation. An *in situ* study

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An *in situ* study

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“I dedicate this study to God, my Family, the Volunteers, and all the people who contributed to the accomplishment of this study”

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ABBREVIATIONS LIST (ABKÜRZUNGSVERZEICHNIS)

CO_2	Carbon dioxide
%	percentage
J	joule
cm^2	square centimeter
µm	micrometer
ms	milisecond
µs	microsecond
ʎ	Lambda (wavelength)
^oC	Degree Celsius
p.p.m	Part per million
min	minutes
<	Less of
NaF	Sodium Fluoride
APF	acidulated phosphate fluoride
SnF_2	stannous fluoride
TiF_4	titanium tetrafluoride
KNO_3	Potassium nitrate
CaF_2	Calcium Fluoride
$SnCl_2$	Stannous Cloride
AmF	Amine Fluoride
Er:YAG	erbium:yttrium-aluminum garnet
Nd:YAG	neodymium: yttrium aluminium garnet
Hz	Hertz
APF	Acidulated phosphate fluoride
J/cm^2	Joule per square centimeter
mJ	milijoule
W	watts
α	alpha
β	beta
TCP	Tri-calcium-phosphate
OH^-	Hidroxil ion
TET	Tetra-calcium-phosphate
α -TCP	Alpha-tri-calcium-phosphate
β -TCP	Beta-tri-calcium-phosphate
SIMS	secondary mass spectrometry
HA	hydroxyapatite
FA	fluorapatite

1. INTRODUCTION (EINLEITUNG)

Tooth wear is becoming increasingly significant in the 20th century in the long term health of the dentition due to the decline in tooth loss due to infectious diseases and the increasing longevity of teeth (Zero & Lussi 2005). Consequently a more demanding ahead the preventive and restorative skills of the practitioner will take place (Zero & Lussi 2005).

Tooth surface loss or tooth wear refers to the pathological loss of tissue by a disease process other than caries (Eccles 1982). Tooth wear can be separated into attrition, erosion, and abrasion. Attrition is defined as the loss of enamel, dentin, or restoration by tooth-to-tooth contact (Bartlett & Shah 2006). Erosion is defined as a surface dissolution of dental hard tissues by acids without the involvement of micro-organisms (Zipkin & Mc 1949). Abrasion is the loss of tooth substance from factors other than tooth contact (Bartlett & Shah 2006). There is some suggestion that the shape of the lesion is related to its etiology (Sognnaes et al. 1972). One group of authors suggested, in a literature review, those lesions with sharply defined margins could be caused by abrasive factors, whereas erosion produces broader, dish-shaped but shallower lesions (Levitch et al. 1994).

The prevalence of cervical wear has been reported to vary between 5 – 85% (Bergstrom & Lavstedt 1979; Levitch et al. 1994; Piotrowski et al. 2001; Aw et al. 2002; Oginni et al. 2003; Borcic et al. 2004). This large variation reflects the relatively few studies reporting the prevalence of cervical wear alone, as well as the different populations analyzed in the studies (Bartlett & Shah 2006). In *Diadema*, a city in São Paulo state, 967 childrens were analyzed and it was verified that 51,6% presented the teeth erosion, and 82,5% of them was restricted to enamel (Murakami et al. 2009).From these studies, it is accepted that tooth wear is an almost universal condition, but that severe dentin exposure on non-cervical sites is relatively uncommon, at 2-4% (Lussi et al. 1991; Dugmore & Rock 2004). All studies showed a tendency for prevalence to increase with age, which goes some way to explain the disparity in their findings (Wood et al. 2008).

One of the first papers to introduce the concept that abrasion is accelerated with acid-softening or mineral dissolution was a classic laboratory

investigation by Davis and Winter (1980) (Davis & Winter 1980). This work has been supported by numerous laboratory studies showing that the combined effect of erosion and abrasion is greater that the effect of either operating on its own (Davis & Winter 1980; Azzopardi et al. 2001; Eisenburger et al. 2003).

Nowadays, due to the changing habits of population, the erosion diagnosis in dental offices has been more common. Dental erosion may be caused by a series of extrinsic and intrinsic factors (Zero & Lussi 2000). Extrinsic factors largely include the consumption of acidic foods and carbonated beverages, sports drinks, red and white wines, citrus fruits and, to a lesser degree, occupational exposure to acidic environments (Zero & Lussi 2005). Soft drink consumption in the USA increased by 300% in 20 years (Calvadini et al. 2000). Nowadays there is a consensus that four or more acid intake per day are associated with high risk of dental erosion (Lussi & Schaffner 2000). The most common intrinsic factors include chronic gastro-intestinal disorders such as gastro-oesophageal disease as well as health issues like anorexia and bulimia, where regurgitation and frequent vomiting are common (Zero & Lussi 2005; Aranha et al. 2008).

Chemical *events in tooth erosion*

The erosive potential of a substance is not exclusively dependent on pH value and type of acid, but is also strongly influenced by its titratable acidity (buffering capacity), calcium-chelation properties, mineral contend, temperature of the beverages and by adhesion to the dental surface (Barbour et al. 2006) (Lussi et al. 2004; Lussi & Jaeggi 2008).

Zero & Lussi (2005) described the chemical properties of different beverages and foodstuffs (Table 1A). The pH, the amount of titratable acid required to raise the pH to 7.0, phosphate and calcium concentration, fluoride content and the extent of enamel softening as measured by surface microhardness (SMH) are given. Measurements of SMH were performed before and after immersion for six minutes in the foodstuffs or beverages using and the change in SMH was calculated. A positive value denotes a hardening of the surface while a negative value represents softening (Zero & Lussi 2005).

Table 1A. Baseline pH, amount of base needed to raise pH to 7.0, phosphate, calcium, fluoride concentration and surface microhardness (SMH) of different beverages (Zero & Lussi 2005)

Beverages Foodstuff	pH	OH^- to pH 7 mmol/l	P nmol/l	Ca Mmol/l	Fluoride ppm	SMH before immersion	SMH after immersion (6min)	Change in SMH
Apple juice	3.00	102	1.7	2.3	0.220	352	151	- 201
Squeezed orange juice	3.64	136	5.7	2.1	0.030	353	209	- 144
Orange juice	3.74	124	2.9	1.9	0.125	348	289	-59
Orange Yoghurt	4.08	101	43.0	32.0	0.050	354	355	+ 1
Ice Tea	3.00	26	0.1	0.6	0.825	338	187	- 151
Coca-Cola (degassed)	2.60	34	5.4	0.8	0.131	349	186	- 163

The chemical events leading to erosion are complex. When a solution comes in contact with the enamel surface of a tooth, it has to first diffuse through the acquired pellicle, which is an organic film derived mainly from salivary proteins and glycoproteins which cover the surface of teeth and only thereafter can it interact with the mineral phase of the tooth (Zero & Lussi 2005). Once in contact with enamel, the acid with its hydrogen ion (or with its chelating capacity) will start to dissolve the crystal. The unionized form of the acid will then diffuse into the interprismatic areas of enamel and dissolve mineral in the subsurface region (Featherstone & Rodgers 1981). This will lead to an outflow of tooth mineral ions (calcium and phosphate) and subsequently to a local pH rise in the tooth structure in close proximity to the enamel surface.

This process is stopped when no new acids and/or chelating substances are provided. An increase in agitation (when a patient is swishing a drink in the mouth) will enhance the dissolution process, because the solution on the surface layer adjacent to enamel will be readily renewed. Furthermore, the amount of drink in the mouth in relation to the amount of saliva present will modify the dissolution process. Citric acid common in many soft drinks may act as a chelator capable of binding minerals (calcium) of enamel or dentine, thus increasing the degree of undersaturation and favoring more demineralization (Zero & Lussi 2005). Meurman & Frank (1991) verified that between malic

acid and phosphoric acid, the citric acid was the first to cause erosion in bovine enamel after 15 minutes in solution in vitro.

The pH value, the calcium, and phosphate and to a lesser extent the fluoride content of a drink or foodstuff are important factors explaining the erosive attack (Lussi et al. 1993; Lussi et al. 1995). They determine the degree of saturation with respect to the tooth mineral, which is the driving force for dissolution of the tooth mineral (Larsen 1973). Solutions supersaturated with respect to dental hard tissue, for example yogurt, will not dissolve tooth mineral. The deposition of salivary calcium and phosphate may lead to rehardening (remineralization) of the initially acid softened enamel (Gedalia et al. 1991; Amaechi & Higham 2001).

Other factors, as temperature of an acidic drink also influences its erosive potential (Amaechi & Higham 2005). Taking the drink ice-cold (4°C) reduces its erosive effect (Barbour et al. 2006). The adhesive and displacement of the liquid are other factors to be considered in the erosive process. There appear to be differences in the ability of beverages to adhere to enamel based on their thermodynamic properties, e.g. the thermodynamic work of adhesion (Ireland et al. 1995).

Biological modifying factors

The biological modifying factors affecting the erosion process include saliva, tooth composition and structure, dental anatomy and occlusion, the anatomy of oral soft tissues in relationship to the teeth and physiological soft tissue movements such as swallowing pattern (Zero 1996; Lussi et al. 2008). The interplay of all these factors is crucial and helps explain why some individual exhibit more erosion than others, even if they are exposed to the same acid challenge (Lussi 2006; Lussi & Jaeggi 2008). Of these, the natural protective properties of saliva and its contribution to pellicle formation can be considered of greatest importance. The erosion protective functions of saliva include: dilution and clearance of erosive substances from the mouth; neutralization and buffering of acids; maintaining a supersaturated state next to the tooth surface due to the presence of calcium and phosphate; providing

calcium, phosphate and possibly fluoride necessary for remineralization. Both the quantity and quality of saliva may be responsible for some of the observed differences in the susceptibility of different patients to erosion (Zero & Lussi 2005).

Some special constituents of saliva, such mucin, seem to provide enamel protection against acid challenge. Mucin is the most common salivary proteins (Levine 1993), an important constituent of the salivary pellicle, and the main lubricant component of saliva (Levine et al. 1987; Aguirre et al. 1989; Van Nieuw Amerongen et al. 2004). Hara et al., (2008) tested the effect of human saliva substitutes, specially the mucin, in an erosion–abrasion cycling model designed for enamel and root dentin. Specimens were assigned into the groups (n = 8): artificial saliva (AS), artificial saliva + mucin (AS+M), deionized water (DIW, negative control), and pooled human saliva (HS). Each group was submitted to a cycle of 5 min in 1% citric acid, 30 minutes in the testing solutions, and toothbrushing (enamel, 500 strokes; dentin, 150 strokes, 200 g load) in fluoridated dentifrice (1,100 ppm NaF) slurry. This cycle was repeated three times each day, for 3 days. Substrate loss was measured through profilometry. Enamel wear for each group was ranked as: (AS)<(AS+M) and (HS)<(DIW), with AS+M not differing from HS. For dentin, groups AS and AS+M did not differ from each other or from DIW, but showed significantly higher wear than HS. The artificial saliva with mucin showed promise as a potential substitute for human saliva in the enamel erosion–abrasion cycling model. For dentin, none of the artificial saliva performed similarly to human saliva.

Another important role of saliva is related to the formation of the acquired pellicle (Zahradnik et al. 1976; Nieuw Amerongen et al. 1987; Meurman & Frank 1991; Amaechi et al. 1999; Hannig & Balz 1999; Hannig et al. 2004; Nekrashevych et al. 2004). This protein-based pellicle may behave as a diffusion barrier or a perm-selective membrane, preventing direct contact between acids and the tooth surface and thus inhibiting its demineralization (Amaechi et al. 1999; Hannig & Balz 1999). Any procedure that removes or reduces the thickness of the pellicle may compromise its protective properties and accelerate the erosion process. Procedures such as tooth brushing with abrasive dentifrice products, professional cleaning with prophylaxis paste, and

tooth whitening will remove the pellicle and may render teeth more susceptible to erosion (Zero 1996). The time of developed pellicle is one important factor relating to its capacity to protect dental surface against acid attack. A developing young pellicle will hardly be a diffusion barrier to an erosive agent. Only when the pellicle has matured and has achieved a certain thickness can it slow down the diffusion process. (Zero & Lussi 2005). The acquired pellicle becomes detectable on dental surfaces after few minutes of exposure to the oral environment (Skjorland et al. 1995; Hannig 1999; Attin et al. 2001). The time required for the pellicle to reform to provide optimal protection is still the subject of debate with studies reporting protective effects with pellicles formed for as little as 3 minutes (Hannig et al. 2004) to as long as 7 days (Meurman & Frank 1991). Skjorland et al., (1995), found in his in vivo study that the pellicle reached an initial thickness in about 2-3 minutes, at which level it stayed for about 30 minutes. The thickness of the acquired pellicle then increased to about three times the initial thickness and stayed at that level for the rest of the experimental periods (10 hours) (Skjorland et al. 1995). What is more common to find in literature is that the pellicle growth until reaching equilibrium between protein adsorption and desorption with 2 hours (Lendenmann et al. 2000).

Hara et al. (2006) verified *in situ* whether a two-hour pellicle protects against different erosive challenges by orange juice with different times (0, 10, 20 and 30 minutes). The results showed that enamel specimens with the pellicle had a significantly lower percentage surface microhardness, only after 10 minutes challenge. No protection was found for dentin. It was concluded that this effect was limited to the less severe erosive challenge on enamel surfaces. The author suggested that greater protection to enamel might be found if pellicle formation times, longer than 2 hours were adopted (Lendenmann et al. 2000). The maturation process may enhance the acid-resistance of the pellicle, due to structural remodeling by enzymes (Yao et al. 2001; Hannig et al. 2005). The author speculates about the clinical significance of the results, considering that the intake of 400ml of orange juice with 10 min of acid exposure and 20 min of total challenge may represent the ingestion of a regular cup of beverage in real conditions. The author also added that the repetition of this protocol for 2 and 3 times consecutively, mimicking the consumption of 2 and 3 cups of acid beverage, and not allowing either pellicle re-organization or remineralization to

occur, is thought to be representative of subjects at high risk of erosion. No pellicle interference in the development of dental erosion should be expected in such situations (Hara et al. 2006).

So, the acquired pellicle will show a more effective erosive protection when it presented a mature state. The frequent consume of acid beverages or intrinsic factors, as vomiting or regurgitation with a short interval, will not provide time enough for the reorganization of the pellicle. In the present study, the methodology used provided time enough for the development of a mature acquired pellicle until the next acid challenge were done. The volunteers were instructed for not to touch or brush the samples in any moment, in order to maintain the acquired pellicle intact.

Additionally, the greater the buffering capacity of the acidic drink or food, the longer it will take for saliva to neutralize the acid. For squeezed orange juice, 124mmol/l of base is required to raise the pH to 7, whereas for degassed Coca Cola only 34mmol/l is required. Nevertheless, once demineralized, the tooth substrate has shown limited ability to remineralize in a short period of time of exposure to saliva (Jaeggi & Lussi 1999; Attin et al. 2001; Hara et al. 2003).

Methods of erosion control

The strategies to control erosive tooth wear include the early diagnosis of hard tissue defects and the evaluation of the different etiological factors to identify persons at risk (Lussi & Hellwig 2006). The elimination of the causative factors may be difficult (Amaechi & Higham 2005), since they are associated to habits or lifestyle, and depend on nutritional, medical, psychological and professional factors, that predispose individual to dental erosion (Serra et al. 2009). Erosive lesions frequently require preventive and restorative treatments (Young et al. 2008). However, restorative procedures do not prevent erosive/abrasive wear (Turssi et al. 2008).

Clearly the most effective way of preventing erosion is to eliminate the etiological factors, whether they are of intrinsic or extrinsic origin or a combination of the two. A careful review of the patient's medical history and dietary/behavioral predisposing factors is essential (Wiegand & Attin 2003). Evaluating patients' salivary gland function is also important, due to the strong

association between decreased salivary flow and susceptibility to erosive tooth wear (Zero & Lussi 2005). In many cases it may prove to be difficult to isolate one factor due to the multifactorial nature of tooth wear. Education is the first line of defense as most patients are unaware of how their behaviors are contributing to the destruction of their teeth (Zero & Lussi 2005).

The most common patient recommendations are the following (Zero & Lussi 2005):

• Refer patients or advise them to seek appropriate medical attention when intrinsic factors, such as anorexia/bulimia or gastro-oesophageal reflux disease are involved.

• Reduce or eliminate frequent exposure to acidic soft drinks and juices.

• Avoid erosive-inducing habits such as sipping, swishing or holding drinks in the mouth and drink with a straw, ensuring the flow is not aimed directly at any individual tooth surface (if acid drinks are consumed)

• Avoid tooth brushing immediately before an erosive challenge (vomiting, consumption of acid beverages). As discussed above the acquired pellicle provides protection against erosion, and tooth brushing, especially with high abrasive (whitening) toothpaste, will remove the pellicle (Lussi et al. 2004). Prefer washing the mouth with water as brushing it immediately after an erosive challenge

• Avoid tooth brushing immediately after an erosive challenge. Enamel remains softened and susceptible to mechanical tooth wear (abrasion, attrition) for at least one hour after an erosive challenge (Jaeggi & Lussi 1999; Attin et al. 2001; Attin et al. 2004).

• Use a soft toothbrush and low abrasion dentifrice to minimize any additional tooth wear (Imfeld 1996).

• Avoid dentifrices with a low pH (Wiegand et al. 2004).

• Use a remineralising/neutralising fluoride rinse, sodium bicarbonate (baking soda) solution, milk or food such as cheese or sugar-free yoghurt after an erosive exposure.

• Stimulate saliva flow with, for example, a sugar-free chewing gum, or lozenge designed for such purpose. The use of a sugar-free lozenge may be more advisable, since gum chewing may have an abrasive effect on softened tooth structure.

• Avoid acidic drinks or foods last thing at night.

• Finish meal with something rich in calcium and phosphate such as cheese.

• Consider using modified acid beverages with reduced erosive potential instead of regular products.

Based on an understanding that the chemical properties of soft drinks, such as pH, type of acid, pKa, and titratable acidity affect their erosive potential, modification of product has been proposed over the past few years. Addition of calcium and phosphate salts to erosive drinks has shown promising results. Several calcium enriched soft drinks are currently on the market and may offer some benefit to patients at risk of dental erosion. Calcium can be found in beverages as sources calcium gluconate, calcium lactate, calcium malate, and calcium phosphate, calcium chloride and calcium citrate (Hara & Zero 2008). The selection for a specific salt will affect its solubility (Grenby 1996), as well as its effect on the taste and color of the beverages (Hara & Zero 2008). Therefore, the addition of different salts at different concentrations may not only reduce the erosive potential but also alter the original characteristics of the beverages.

Hara & Zero (2008) tested ten commercially available beverages, five with and five without calcium supplementation, in two phases. In the first phase, the pH, titratable acidity, and concentrations of calcium (total and ionic), phosphorus and fluoride, were analyzed. In the second phase, the ability of the test products to erode enamel was measured, at different time-points (0, 5, 10, 30, 60, and 120 min). Within the chemical properties tested, pH, calcium-ion concentration, and total calcium showed a strong correlation with enamel demineralization and enamel wear. Lower levels of enamel demineralization and wear were found for most of the calcium-containing beverages than for those without calcium. Generally, beverages supplemented with calcium had a reduced capacity to demineralize enamel (Hara & Zero 2008).

Only a relatively small change in the degree of saturation by adding calcium and/or phosphate, without changing the pH, may reduce the erosive potential *in vitro* (Attin et al. 2003; Barbour et al. 2005). There are, however, some clinical implications for the patient and the clinician, in that, while erosion was retarded, it was not completely prevented by the mineral additives *in vivo.* These drinks may still have the potential to slightly soften the enamel surface (Hughes et al. 1999; Hughes et al. 1999).

Yoghurt is another example of a food with a low pH (≤ 4.0), yet it has very little erosive potential due to its high calcium and phosphate content, which makes it supersaturated with respect to enamel (Lussi et al. 2004).

The fluoride added in beverages has also been described as a potential means of reducing the erosive potential of acid drinks (Grenby 1996; Attin et al. 2005). Its ability to reduce the demineralization of enamel has been shown in some studies, but the effect was limited in extent and was concentration dependent (Larsen & Nyvad 1999; Larsen 2001). Mahoney et al. (2003) found an inverse correlation of the erosive potential with the fluoride content of different beverages. However, given the low pH in many drinks and health concerns, adding fluoride to drinks is not practical. Even though fluoride may not totally prevent dental erosion, it has been shown that small amounts of fluoride, in addition to calcium and phosphate salts, can be effective in reducing erosion development (Larsen 2001; Attin et al. 2005).

It has been also demonstrated *in situ* that food with a high concentration of calcium, as cheese, can show a significant reharden eroded enamel (Gedalia et al. 1992). The intake of milk has also shown positive results (Gedalia et al. 1991).

Role of high concentrations of fluoride in dental erosion

While the benefits of topical fluorides for caries prevention are beyond refute, the ability of fluoride to prevent erosion cannot be presumed, since the acidic challenge in erosion is often stronger. Different forms of providing fluoride in the prevention of erosion have been described, such as: topical application, gel, varnish, solutions.

Recent evidence from *in vitro* and *in situ* studies suggests that high concentrations of topical fluoride are able to limit progress of erosion and enhance remineralization (Wiegand & Attin 2003; Ganss et al. 2004; Fowler et al. 2006; Newby et al. 2006; Lussi et al. 2008), however, these findings need to be confirmed in clinical studies. Even though the dental surface lost by an erosive attack cannot be recovered, a partially demineralized surface (surface-softened lesion) can be reharden in the presence of fluoride, preventing or reducing further surface loss (Ganss et al. 2004). Fluoride has not been conclusively proven to prevent erosion (Larsen & Richards 2002).

As dentifrices are excellent vehicles for the delivery of fluoride because of their widespread availability and use (Hara et al. 2009), some studies were proposed in order to verify different toothpastes components in the prevention of erosion lesions. Although some studies showed a limited beneficial effect of commercially available fluoridated toothpastes on erosion and abrasion (Davis & Winter 1977; Magalhaes et al. 2007), other studies did not (Lagerweij et al. 2006; Ganss et al. 2007). Some studies were performed *in vitro* and some others *in situ*. Different fluoride compounds: NaF, SMFP, SnF_2, TiF_4, KNO_3, AmF, have shown different levels of protection against erosion (Hove et al. 2006; Schlueter et al. 2007). Hara et al (2009) tested different toothpastes with different components, but with the same fluoride contend (1,450ppm NaF, with or without 5% KNO_3) and concluded that both fluoride-containing dentifrices provided statistically superior remineralization and erosion resistance than control group (dentifrice without fluoride). Between the two fluoride dentifrices there were a significant greater surface microhardness recovery with dentifrice with KNO_3, but there was no significant difference between the two dentifrices in relative resistance to further erosive challenge (Hara et al. 2009). The less hardness reduction in fluoride treated group can be due to the formation of a

less soluble substrate, by the formation of fluorapatite-like minerals. Additionally, CaF_2 deposits can be formed on the enamel surface (Christoffersen et al. 1988), which may disassociate during a pH drop releasing fluoride (Lagerlof et al. 1988). The presence of fluoride ions in solution can also decrease the demineralization by reducing the acid solubility of the tooth surface (Wiegand & Attin 2003). However in this study (Hara et al. 2009) the results reflect the remineralizing effect of one application of dentifrice slurry on enamel eroded by dietary acid. The mechanical action of toothbrush could wear away part of the demineralized enamel layer, not allowing enough time for fluoride remineralization. Zero et al., (2006) tested in an *in situ* remineralization model, the ability of a low abrasion fluoride dentifrice containing potassium nitrate to enhance the remineralization of enamel that was previously subjected to an *in vitro* dietary erosion challenge. Thirteen subjects completed a crossover design study with four randomly assigned dentifrice treatments: placebo dentifrice (0 ppm F; PD); dose response control dentifrice (250 ppm F; DD); clinically tested fluoride dentifrice (1100 ppm F; FD); and test dentifrice (1150 ppm F + 5% KNO_3; TD). Each subject wore a palatal appliance holding eight bovine enamel blocks that were previously exposed for 25 minutes to an *in vitro* erosive challenge with grapefruit juice. Surface microhardness (SMH) was determined prior to the erosive challenge (baseline), after the *in vitro* erosive challenge, after *in situ* remineralization. The test dentifrice was shown to significantly enhance the remineralization of enamel previously subjected to an erosion challenge.

Rios et al., (2008) conducted a double-blind, crossover *in situ* study consisting of three phases (seven days each). In each phase, the authors tested one of the dentifrices (5.000 ppm fluoride [F]; 1.100 ppm F; no F). They performed erosive challenges with the use of cola drink (60 seconds, four times/day) and abrasive challenges via tooth brushing (30 seconds, four times/day). The authors determined the enamel loss via profilometry. For the erosion-plus-abrasion condition, the study results showed that enamel wear was significantly higher than that with erosion alone. The findings showed no significant differences between the dentifrices regarding enamel wear. Within the *in situ*, ex vivo conditions of this study, the authors concluded that the highly

concentrated fluoride dentifrice did not have a protective effect on enamel against erosion and erosion plus tooth brushing abrasion.

Although tooth fluoride can minimize the effects of erosion demineralization, even the highly concentrated fluoride dentifrice does not appear to prevent totally enamel erosion (Serra et al. 2009).

In the toothpaste, beyond the fluoride, the abrasives seem also to interfere the tooth erosion/abrasion Hara et al., (2006) tested *in vitro* the hypothesis that fluoride and abrasivity of dentifrices can interact, modulating the development of erosive–abrasive lesions. Human enamel and root dentin specimens were submitted to cycles of demineralization (120 ml of 1% citric acid for 2min, static), remineralization (120 ml of artificial saliva (1.45 mM, for 60 min, 100 rpm) and tooth brushing (500 and 150 brushing strokes for enamel and dentin, respectively) using six dentifrices formulated with three different abrasivity levels: low (L), medium (M) and high (H); with (+F) and without (-F) fluoride. Surface loss was quantified by optical profilometry. The protective effect provided by the salivary pellicle against abrasion (Joiner et al. 2008) and erosion (Hara et al. 2006) was partially reproduced, by using mucin-based artificial saliva. In dentin, it was ranked: L < M < H, for both +F and _F dentifrices. In enamel, +F dentifrices had similar results; however for _F formulations, M and H did not differ. Fluoride reduced surface loss in enamel, at all abrasive levels. In dentin, the same fluoride effect was observed but only for the low abrasive formulation. Both fluoride and abrasivity were important modulators of enamel surface loss, while abrasivity had a higher impact than fluoride on dentin. The author concluded that both fluoride and abrasivity were important modulators of enamel surface loss, while abrasivity had a higher impact than fluoride on dentin.

High-concentrated fluoride applications, such as oral rinses, gels or varnishes, have been considered as most effective in reducing the development of enamel erosion (Ganss et al. 2004). The fluoride agents that have been investigated in most *in vitro* studies about dental erosion are those that have been used over years for caries prevention: sodium fluoride (NaF), acidulated phosphate fluoride (APF), stannous fluoride (SnF_2) and amine fluoride (AmF). More recently, the preventive effect of other fluoride agents, such as 4%

titanium tetrafluoride (TiF_4) solution, have been investigated in erosion tests (Tveit et al. 1983; Buyukyilmaz et al. 1997; Tezel et al. 2002; van Rijkom et al. 2003; Vieira et al. 2005; Hove et al. 2006; Vieira et al. 2006; Hove et al. 2007; Schlueter et al. 2007; Magalhaes et al. 2008). While some studies showed an inhibitory effect of TiF_4 on erosion (Tveit et al. 1983; Buyukyilmaz et al. 1997; Tezel et al. 2002; van Rijkom et al. 2003; Hove et al. 2006; Hove et al. 2007; Schlueter et al. 2007), other studies did not found a protective effect (Vieira et al. 2005; Vieira et al. 2006; Magalhaes et al. 2008). The low pH of the TiF_4 solution (around 1.2), favors the linking between titanium and oxygen of the group phosphate, leading to the formation of a titanium dioxide glaze-like layer on the surface (Tveit et al. 1983; Buyukyilmaz et al. 1997). This glaze-like layer might be associated with a decreased softening of the enamel surface. It is speculated that the titanium ions might play an important role as they might substitute calcium in the apatite lattice (Mundorff et al. 1972; Ribeiro et al. 2006). Moreover, it is suggested that titanium interacts with the enamel surface because of the low pH of the agent, thus leading to an increased fluoride uptake by enamel (Magalhaes et al. 2009).

Besides common fluoride preparation (NaF and AmF), fluoride compounds containing polyvalent metal cations have recently been investigated with respect to their erosion-inhibition potential. In this context, tin (stannous chloride) containing preparations showed, for enamel a promising reduction in erosive mineral loss of 50-90% (Willumsen et al. 2004; Hove et al. 2006; Hooper et al. 2007; Ganss et al. 2008; Schlueter et al. 2009; Schlueter et al. 2009). Some *in situ* studies also verified these effects (Young et al. 2006; Hooper et al. 2007; Hove et al. 2008). One serious limitation of most of these solutions was that they were not pursuant to the directives for dental hygiene products and cosmetics (high concentrations of active agents or high acidity of the preparations) and therefore were not suitable as over the counter products. Schlueter et al., (Schlueter et al.) tested in an *in situ* study one tin-containing fluoride solution, that was conformed to the directives of oral hygiene products in both concentration and pH. Three solutions were tested: placebo (negative control); a commercially available tin- and fluoride-containing (SnF_2) mouth rinse (positive control, 409 ppm Sn2+, 250ppm F-, pH 4.2) and an experimentally solution (pH 4.5) containing 1,900 ppm Sn2+ (SnCl2) and 1,000

ppm F- (AmF/NaF). Tissue loss was determined profilometrically. The experimental solution was notably effective for enamel but was less effective for dentin (averages in enamel: placebo 54.8 µm; positive control 24.5 µm; positive control 9.7µm).

A summarize from the studies developed with fluoride compounds in erosion prevention after 2007 is described below in Table 1B:

Table 1B – Studies that verified the effect of different fluoride compounds – after 2007.

Author	Year	Model	Compound and concentration	Acid challenge and abrasion	Remineralization	Measurement	Result + positive prevention - = negative result
AS = Artificial Saliva PA = Profile Analysis APF = Acidulated Phosphate-Fluoride NF = Neutral Fluoride FV = Fluoride Varnish IS = In situ IV = In vitro							
Schlueter	2010	IS	Fluoride solution (250ppm; 1000ppm) and tin (409ppm ; 1900ppm) 30 sec. after demineralization	Citric acid 5min 6x/day 7 days		PA	(+) for both, but the higher concentrations showed better results.
Wiegand	2009	IV	TiF_4 (1% F) AmF (1% F) Associated or not with CO_2 laser	Sprite Zero® 4/day 90sec 5 days *Without abrasion*	AS	PA	(+) AmF + AmF + CO_2 laser (-)TiF_4 (+)TiF_4 + CO_2 laser
Wiegand	2009	IV	TiF_4 (1,5%) – pH 3.5 NaF (2,02%) – pH 3.5 TiF_4 (1,5%) – pH 1.2 NaF (2,02%) – pH 1.2	HCl 10 X 60 sec *Without abrasion*	AS	PA Release of calcium	(+) only for ph 1.2 substances
Steiner-Oliveira	2009	IV	FFA (1,23%) pH 3.5 Associated or not with CO_2 laser	Citric acid 3/dia - 5min 3 dias *Without abrasion*	AS	PA + Ca, F, P analysis	(+) FFA (+) FFA + CO_2 laser
Sobral	2009	IV	FFA (1,23%) pH 5.3 Associated or not with Nd:YAG laser	Citric acid 30 min. or 90 min. *Without abrasion*		Hardness and loss of weight	(+) for APF + Nd:YAG laser
Hara	2009	IS	1450ppm NaF 1450ppNaF +5%KNO_3	Grapefruit juice 25min *Intraoral PhaseI – 4 hourss*		Hardness	(+) for all groups with NaF. NaF +5%KNO3 presented the best result
Hara	2009	IV	Dentifrice 0ppm NaF 1100ppm NaF	Citric Acid (2min) 5000 cycles of brushing for enamel. Dentifrice was	AS	PA	(+) for dentifrice with NaF.

				used during the cycles.			
Magalhães	2009	IS	TiF_4 (4%)	Coca cola® 5min – 4x/day 4 days *Without abrasion*		Hardness	(+)
Rios	2009	IV	FFA (1,23%) VF (2,26%) Associated or not with Nd:YAG laser	Sprite Light® 1min - 4/day 5 e 10 days *Without abrasion*	AS	PA	(+) for all groups Better results with APF + Laser
Rios	2008	IS	Dentifrices -placebo -5000ppm F -1100ppm F	Coca cola® 60 sec - 4x/day 7 days Abrasion 30 sec brushing, 4/dia		PA	(-) for all dentifrices
Wiegand	2008	IV	Tif_4; ZrF4; HfF4 (0.401% AmF (1.25%)	HCl (25min)		Calcium Release	(-) AmF (enamel) (+) only Tif_4 1%
Hove	2008	IS	TiF_4- SnF_2- NaF	HCl – 2min – 2x/day 9 days		PA	(+)TiF_4- SnF_2
Lussi	2008	IV	Dentifrices 1100ppm – 1450ppm NaF ou SnF (3min – with agitation)	Citric Acid 3 min	AS	Hardness	(+) All dentifrices when applied before the acid.
Magalhães	2008		TiF_4 (4%)	Coca cola® 5min - 4x/day - 4 days		Hardness	(-)
Vlacic	2007	IV	NF (1,23%) Associated or not with different lasers	HCl (1M) 5 min *Without abrasion*		Hardness	(+) all groups with NF and laser
Magalhães	2007	IS	Dentifrices - 1098ppm F - placebo	Coca cola® 5 min - 4x/day 7 days Brushing abrasion (30 cycles)		Hardness	(+) Samples brushed with fluoride dentifrices
Vieira	2007	IS	Fluoride Varnish (0.1%F)	Sprite ® 5min - 3x/day Brushing abrasion 5sec after each acid challenge		PA	(+)
Ganss	2007	IS	Dentifrices with fluoride (0.125%F) or without fluoride + Topic application of fluoride (FFA - 1.25%) + Fluoride solution (0.025%F) (Intense fluoridation)	Citric acid 20 min – 2x/day 5 days Brushing abrasion 30 sec.		PA	(+)Intense fluoridation

Relevant considerations about erosion/abrasion process

Although it is known that the acids can soft the enamel leading to a tissue more prone to abrasion, the exactly time that this tissue stays softened is not completely known. Attin et. al. (2001) evaluated the effect of different periods of intraoral remineralization to decrease the susceptibility of previously demineralized enamel against tooth brushing abrasion. Six human enamel specimens (A-F) were recessed in the buccal aspects of each of eight intraoral appliances which were worn for 21 days by 8 volunteers. Demineralization of the samples was performed twice a day extra orally in the acidic beverage Sprite Light® for 90 seconds. Subsequently, the enamel specimens were brushed at different times. Specimen A was brushed immediately after the demineralization. The remaining samples B-E were brushed after the intraoral appliances had been worn for various periods of remineralization: specimen B, 10 min; C, 20 min; D, 30 min and E, 60 min, respectively. Specimen F was only demineralized and remineralized, but not brushed. After 21 days, enamel wear was measured with a laser profilometer. The following values (mean +/- standard deviation) were obtained: specimen A, 6.78+/-2.71 microm; B, 5.47+/-3.39 microm; C, 6.06+/-3.18 microm; D, 5.43+/-2.58 microm; E 4.78+/-2.57 microm, and F 0.66+/-1.11 micro;m. Analysis of variance revealed a significant influence of remineralization period on abrasive wear. However, even after a remineralization period of 60 min the wear was significantly increased as compared to the demineralized, but not brushed control. It is concluded that the abrasion resistance of softened enamel increases with remineralization period and at least 60 min should elapse before tooth brushing after an erosive attack (Attin et al. 2001).

High power lasers in demineralization prevention.

Several studies have already shown the benefic effect of high power lasers in the field of caries prevention (Featherstone et al. 1998; Ana et al. 2007; Castellan et al. 2007; de Freitas et al. 2008; Zezell et al. 2009; de Freitas et al. 2010). The possibility of increasing the enamel resistance to

demineralization after laser irradiation was first demonstrated in 1965 with a ruby laser (Stern & Sognnaes 1972; Stern et al. 1972). Over time and with the increasing knowledge about the interaction of laser with hard tissue, the effect of CO_2 laser was tested. As good results relating to the inhibition of incipient caries were observed with this laser and as it provides a very efficient interaction with the tissue, this laser has been preferentially studied (Fox et al. 1992; Fried et al. 1997; Featherstone et al. 1998; Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2009).

Interaction of CO_2 laser with Dental Enamel

The interaction between laser and tissue is extremely dependent on optical properties of tissue; accordingly the composition of the tissue is of great importance to find the wavelengths that lead to the acid resistance of enamel. The enamel is composed in volume by 85% mineral, 12% water and 3% organic constituents and the mineral part consists largely by carbonated hydroxyapatite crystals arranged in prisms of approximately 5 mm diameter (Dibdin 1993; Fried et al. 1997). Because of the enamel composition, it has a high absorption to wavelengths in the infrared region between 2.7 and 3 µm emitted by erbium lasers and the wavelengths between 9-11 µm emitted by CO_2 lasers. In these regions of the spectrum the spreading is insignificant and the deposition of energy is determined by the coefficient of absorption and reflection (Niemz 1996; Fried et al. 1998; Zuerlein et al. 1999; Zuerlein et al. 1999; Eduardo et al. 2002). The two regions of the spectrum mentioned had, therefore, a great potential to interact with enamel and dentin. However, the four main wavelengths emitted by the CO_2 laser (9.3, 9.6, 10.3, 10.6 µm) have an absorption coefficient greater in enamel than the erbium lasers which makes the interaction more efficient relating to energy.

The absorption of CO_2 lasers by enamel is associated to the absorption of the phosphate mineral bands of the structure. Among the four main wavelengths, the two shorter, 9.3 and 9.6 µm have the greater absorption and coincide with the absorption phosphate band. With the longest wavelengths (10.3 and 10.6 µm), the absorption is smaller and the degree of coincidence with the band of phosphate (Zuerlein et al. 1999).

The caries preventive effect achieved by laser irradiation described in literature has been related to the temperature increase in tissue (Kuroda & Fowler 1984). The increase of temperature occurs only when the light from the laser beam is largely absorbed. Therefore, the higher the absorption coefficient of a wavelength, the greater is the potential cause the heating of the tissue (Seka et al. 1995). The absorption coefficient is inversely proportional of the penetration of laser light, as described by Beer-Lambert (Niemz 1996). Thus, as more electromagnetic radiation is absorbed by a given tissue, the lower the depth of penetration occurs. Therefore when the time of the interaction laser-tissue occurs near from the time of thermal relaxation of the structure, the thickness of the heated layer must be near to the profound of absorption from the wavelength used. It can be inferred, for CO_2 lasers, which for a same amount of energy and an interaction time near the time of thermal relaxation of tissue, the irradiation with 9.6 μm cause the greatest increase of temperature, but in the 1 μm from the outer surface. With the 10.6 μm wavelength, it will cause the smallest increase in temperature, but in a more profound tissue (12 μm).

Prevention of demineralization with a CO_2 laser

The preventive effect of tooth demineralization can be achieved either with the CO_2 laser operating in continuous mode as well as in pulsed mode. Irradiation of enamel in continuous mode with 120 J/cm^2 can reduce mineral loss observed in microradiographic and also converts the mineral phase of enamel to a hydroxyapatite phase significantly less soluble than the non-irradiated enamel (Wong et al. 1990) (Wong et al. 1990; Fox et al. 1992). Also, several studies have demonstrated significant reduction of the solubility of enamel in acidic medium (Hossain et al. 1999; Hossain et al. 2002; Tepper et al. 2004). However, the great disadvantage of radiation in continuous mode is that it can cause a big increase in surface temperature and also the most spread into the tissue. Considering that an increase in pulp temperature of only 5.5°C could cause irreversible damage to the pulp, the continuously presents greater risks for in vivo use (Zach & Cohen 1965). For this reason, the most recent researches, studying the effects of CO_2 in prevention of demineralization,

has been conducted using the pulsed emission. Therefore, considering the interval between a pulse and the next, it is possible that the thermal relaxation happens and therefore the spread of heat to the inner layers can be reduced (Seka et al. 1995).

As in continuous mode, the irradiation of enamel with pulsed CO_2 laser mode is also able to significantly inhibit the demineralization of enamel. This effect has been observed with very different parameters and different conditions of irradiation (Featherstone et al. 1998; Hsu et al. 2000; Klein et al. 2005; Steiner-Oliveira et al. 2006). More systematic evaluations for irradiation with CO_2 lasers emitting at 10.6 µm can be find from the group of Featherstone et al. According to this group, for the same duration pulse (100 µs), the same number of pulses overlapping (25) and the same repetition rate (10 Hz), the highest percentages of inhibition of mineral loss are observed in energy density around 12 J/cm^2 (Featherstone et al. 1995; Featherstone et al. 1998; Featherstone et al. 1998; Kantorowitz et al. 1998). A quite high percentage of inhibition of progression of caries was also observed after irradiation with lower energy density (3.4 J/cm^2), but with a repetition rate of 20 Hz and a pulse duration 50 times (Hsu et al. 2000; Hsu et al. 2001). The reductions of caries progression so far in literature are truly inspiring and show the potential of CO_2 lasers for the preventive treatment. However, along with the positive aspect of the treatment, also occurring side effects, which refer generally to thermal damage in structure. Most of the parameters that showed a reduction of mineral loss around 90% have also shown to lead to excessive heat damage in surface (seen in the form of cracks and microcracks) and excessive temperature increase inside the tissue (Featherstone et al. 1995; McCormack et al. 1995; Hsu et al. 2000). Evident cracks in scanning electron microscopy have also been observed for irradiation with 6, 10 and 11.5 J/cm^2 with 10 ms pulse duration but also with a higher energy than 83.3 J/cm2 and pulses of 667 µs (Tsai et al. 2002; Tagliaferro et al. 2007). The width of the laser pulse has a direct influence on increasing the temperature on the enamel surface and also in the propagation of heat into the deeper dental layers. With shorter pulses, a greater rise in surface temperature can be expected and with longer a minor increase in the surface, but with greater spread of heat into the interior of the tissue (Fried et al. 1996).

In the case of irradiation of enamel with CO_2 laser emitting at 10.6 μm the thermal relaxation time of the structure is 90 μs, so pulse durations of the same order of magnitude or smaller than this value have a high potential to optimize the increase in surface temperature and while minimizing the diffusion of heat into the deeper layers (Fried et al. 2001; Apel et al. 2002). With a shorter pulse width, it decreases the amount of energy required to modify the tissue and promote both the increase in acid resistance of enamel as well the threshold of ablation. Therefore, the smaller the pulse width used, the smaller should also be the energy density to allow sub-ablative effects (Fried et al. 1999; Zuerlein et al. 1999; Zuerlein et al. 1999; Fried et al. 2001).

Despite the advantages, in terms of heat generation in hard dental tissues observed by irradiation with pulse durations around 10 μs (which are smaller than the thermal relaxation time of tissue) with the wavelength of 10.6 μm there are not a lot of investigations with respect to its potential of inhibition of demineralization.

With the length of 9.6 μm on the other hand, shorter pulse durations and smaller energy densities have recently been tested and results showed that these parameters are capable of causing a large inhibition of mineral loss. For pulses of 100 μs duration, the greater inhibition mineral solubility is observed in irradiation with 3 to 5 J/cm^2 (Featherstone et al. 1998). For pulses of 8 μs, with only 1 J/cm^2 a reduction rate of solubility of 50% can be achieved (Gerard et al. 2005). The application of acidulated phosphate fluoride gel on the enamel surface prior to irradiation promotes a greater reduction (62%) in terms of mineral loss, for the same 1 J/cm^2, with pulses of 5μs (Santos et al. 2001). The quality and quantity of information about the inhibition of caries promoted by CO_2 lasers are considerably higher for the length of wavelength of 9.6μm.

The emission at 9.6 μm has a higher absorption coefficient in tissue, so it is less absorbed and penetrates the enamel. However, although less absorption can be find with 10.6μm irradiation, it can cause the modification of a thicker layer of enamel, and therefore contain the demineralization for more time, one very interesting thing regarding to teeth erosion process (Fox et al. 1992; Fox et al. 1992). With the wavelength of 10.6 μm, some longer duration of pulses is already tested (100-50.000 μs). The single study with very short pulses (0.1 to

0.2 μs) was performed by Nelson et al still in the early 1980s, but only with a density of very high energy (50 J/cm^2), a 50% inhibition of mineral loss was observed. The combination of low energies, around 1 J/cm^2 with pulse durations short (5 to 50 μs) for the wavelength of 10.6 μm has not been studied. Esteves-Oliveira et al., 2009 (Esteves-Oliveira et al. 2009) did the first study testing *in vitro* with bovine enamel samples, low fluence CO_2 (10.6 μm) parameters in the caries preventive effect with the least thermal damage. The results showed that the parameter of 0.3J/cm^2; 5μs; 226Hz for 9 seconds increased caries resistance by up 81% compared to the control and even significantly better than fluoride application (25%). The scanning electron microscopy examination did not reveal any obvious damage caused by laser irradiation.

Considering that: the depth of penetration tissue is approximately 10 mm greater at 10.6 μm (Fox et al. 1992); the short pulse lead to a higher surface temperature, but with lower heat propagation to the deeper layers, thus decreasing the risk of pulpal damage (Fried et al. 1999); the promising results in caries prevention with low fluence of CO_2 laser (10.6 μm) (Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2009), this wavelength should also provide some preventive effects in erosion prevention.

Prevention of dental erosion with CO_2 laser (10.6 μm)

Up to now only two studies verified the effect to CO_2 in the prevention of dental erosion. Wiegand et al., 2009 (Wiegand et al. 2009) tested in enamel and dentin if the CO_2 laser (10.6 μm; 10μs; 50Hz) could increase the effect of titanium tetrafluoride (TiF_4) and amine fluoride (AmF) in protecting enamel and dentin against erosion *in vitro*. The samples were submitted to a 5 day de- and remineralization cycle using Sprite Zero (pH 2.6) four times daily for 90 seconds (4 cycles/day). After demineralization the samples were rinsed with tap water and transferred into artificial saliva (20ml/sample) for 2 hours. After the last erosion challenge of the day, the samples were stored overnight in artificial saliva. The enamel and dentin loss were quantitatively analyzed using profilometry. The results showed that the AmF decreased enamel and dentin erosion, but CO_2 laser irradiation did not improve its efficacy. TiF_4 showed only a limited capacity to prevent erosion, but CO_2 laser irradiation significantly

enhanced its ability to reduce enamel erosion. But, with the parameters tested laser alone were not capable of prevent enamel and dentin erosion.

Steiner-Oliveira et al., 2009 evaluated *in vitro* with bovine enamel samples if CO_2 laser (10.6 μm; enamel: 1J/cm^2; 3W; 5ms pulse duration; 10Hz – dentin: 0.6J/cm^2; 2W; 5ms pulse duration. 2Hz) irradiation with previous fluoride gel application could increase the resistance of enamel and dentin to erosion. The erosion challenge was performed with demineralization cycles with 0.3% citric acid, pH 2.45 for 5 min) and remineralization was performed with artificial saliva for 60 min). The cycles were performed 3x/day during 3 days. Dental surface losses as well as the concentration of calcium, phosphorous and fluoride in the demineralization solutions were determined after each cycling. The results showed that laser alone was not able to prevent enamel or dentin surface losses due to erosion. Its combination with fluoride showed some protective effect, but according to the authors, this effect was mostly due to the fluoride effect.

It's important to point out that both studies made use different parameters suggested in the present study. Despite the usage of artificial saliva in the studies during remineralization period, it do not reproduce the behavior of oral cavity, where the saliva is continuously been secreted. Considering that CO_2 laser has shown promising results in caries prevention, it could also be consider that CO_2 laser should present good results in erosion prevention to.

2. OBJECTIVE (ZIELSETZUNG)

This *in situ* study aims to verify, through enamel surface loss and fluoride uptake analysis, the effect of low-fluence CO_2 laser (10,6 µm) irradiation and fluoride in the prevention enamel erosion caused by citric acid in different times (1, 3 and 5 days). The study aims to detect any protective effect of laser as wells as the duration of this protective effect. A complementary *in vitro* model was developed and the results of *in situ* model were also compared to the results of *in vitro* to verify the particularities of each methodology.

3. MATERIAL AND METHODS (MATERIAL UND METHODEN)

The research comprises *in situ* and complementary *in vitro* experiments.

IN SITU MODEL

The study was totally developed in RWTH – Aachen, Germany. 10 volunteers (4 males and 6 females) took part in the study which was performed according to the Declaration of Helsinki and was approved by Ethical Committee of UNIKLINIK – RWTH Aachen. Subjects were volunteers who had given informed written consent. The average ages of volunteers were 33.6 (±7.3).

Exclusion criteria were general/systemic illness and medication likely to interfere with saliva secretion.

Inclusion criteria were good oral health (no frank cavities or significant gingivitis/periodontitis, no visible plaque); no removable dentures or orthodontics devices; stimulated saliva flow rates >1ml/min (Birkhed & Heintze 1989), adequate saliva pH, consistency and buffer capacity (Ganss et al. 2007). The methodology of saliva test used is described below.

<u>Salivary tests</u> (Saliva-Check Test; GC Corporation; Tokyo, Japan) (Figure 1)

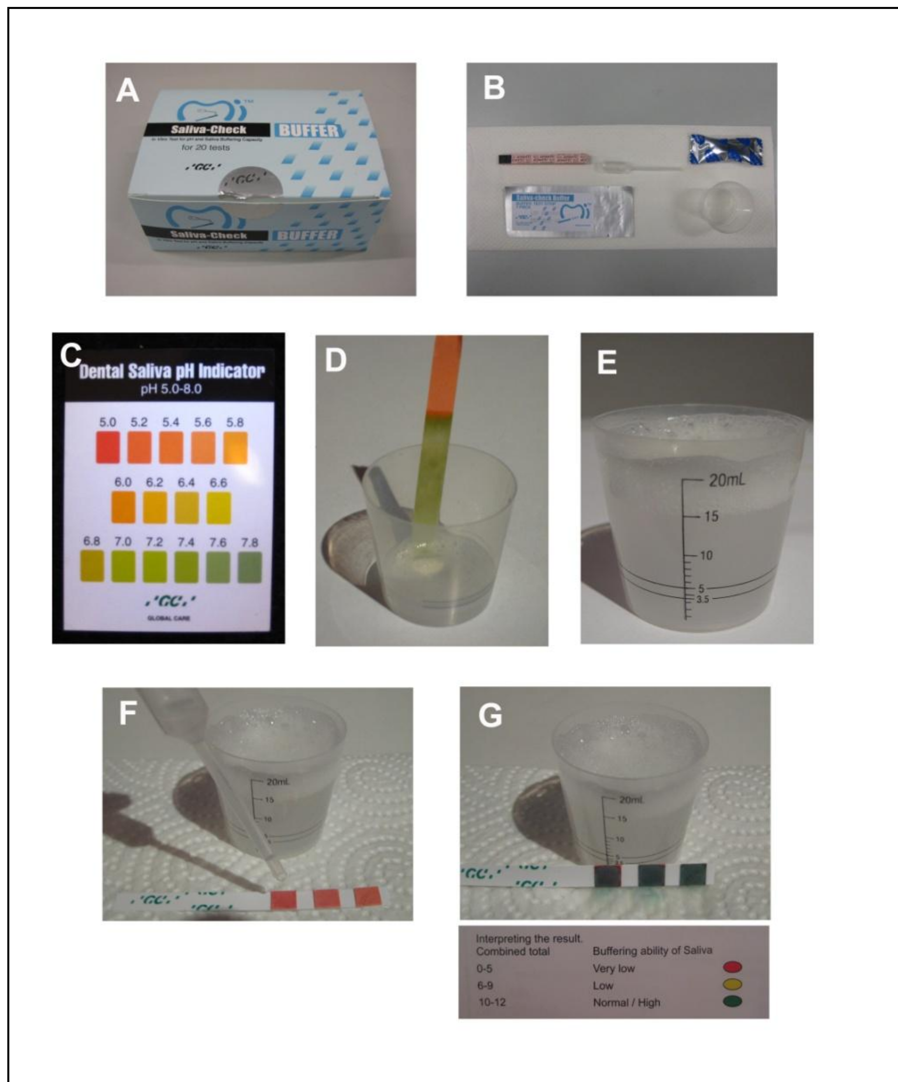
- *Salivary consistency:* Visually the resting salivary consistency was assessed. If it was presented as sticky frothy saliva or frothy bubbly saliva, than it was considered increased viscosity saliva. If it was presented as watery clear, in was considered normal viscosity saliva.
- *pH measurement:* The patient was instructed to expectorate any pooled saliva into the collection cup. A pH test strip was then inserted into the sample of resting saliva for 10 seconds, and then checked the color of the strip. The color was compared with the testing chart available by the salivary test. The pH of saliva could be classified into highly or moderate acidic saliva and healthy saliva.
- *Testing of stimulated saliva:* Instructions to the patient to chew a piece of wax was given, in order to stimulate salivary flow. After 30 seconds, the patient was let to expectorate into the spittoon. Continue chewing

for a further 5 minutes, collecting all the saliva into the collection cup at regular intervals was done. The quantity of saliva could be measured by checking the mL markings on the side of the cups. If the volume collected was less than 3.5mL, it was considered very low salivary flow rate. If it was between 5.0 – 3.5mL, the salivary flow rate was considered low and if it was bigger than 5.0mL it was considered normal salivary flow rate.

- *Buffering capacity of saliva:* A buffer test form the salivary test was removed from the foil package and placed onto an absorbent tissue with the test side up. Using a pipette, sufficient saliva from the collection cup was drawn and dispensed one drop onto each of the 3 test pads of the salivary kit. Immediately the strip was turned 90° to soak up excess saliva on the absorbent tissue. The test pads began to change color immediately and after 2 minutes the final result could be calculated by adding the points according to the final colour of each pad. The conversion table was present in the test. According the value the saliva buffer capacity could be classified as very low, low and normal/high.

Figure 1

Salivary test procedures



A- Salivary test: Saliva-Check Test, GC Corporation®, Tokyo, Japan; **B-** Components of the test: plastic pipette plastic cup with checking volume marking, wax, pH test strip, test pads for buffer capacity; **C-** Reference guide for pH analysis supplied in the kit.; **D-** Result from pH analysis; **E-** Result from stimulated salivary flow; **F-** Buffer capacity analysis; **G-** Result from buffer capacity and reference guide for analysis of buffer capacity supplied by the manufacturer.

The *in situ* study used a total of 240 samples in a crossover design with 4 treatments (4 Groups) and periods of 1, 3 and 5 days each (some samples were removed in day 1, 3, and 5). The samples distribution is described above (**Table 2**).

Table 2: Groups and samples distribution (*in situ*)

Day	**Group Control** Erosion only	**Group CO_2 Laser** Rofin SC x 30, Rofin-Sinar *Laser,* GmbH, Hamburg, Germany	**Group Fluoride** Topical Amine-Fluoride for 3 min (Elmex® Gel, Switzerland, 1.25% AmF, ph 4.8-6.0,	**Group Fluoride + CO_2 Laser**
Day 1	n= 20	n= 20	n= 20	n= 20
Day 3	n= 20	n= 20	n= 20	n= 20
Day 5	n= 20	n= 20	n= 20	n= 20
Total	n= 60	n= 60	n= 60	n= 60

The volunteers received written instructions and a schedule. The palatal appliances were worn during day and night expect for meals. After meals or drinks, 15 minutes elapsed before reinsertion. For erosive demineralization the mounth appliances were immersed extra orally in 40 ml 0.05M citric acid (Citric acid monohydrate – $C_6H_8O_7 \cdot H_2O$; M=210,14g/mol - E. Merck, Darmstadt, Germany); pH 2.3; room temperature; for 20 minutes twice daily (between 6.00 and 9.00 a.m and between 6.00 and 9.00 p.m.) and thoroughly rinsed with tap water before reinsertion (**Figure 2**). The demineralization solution was renewed after each treatment period and its pH monitored. After erosion treatment, the samples were reinserted.

Part of the samples were removed in day 1 (n=2/volunteer) after 2 times submitted to acid challenge, some samples were removed in day 3 (n=2/volunteer) after 6 times submitted to acid challenge, and the rest in day 5 (n=2/volunteer) (10 times submitted to acid challenge).

The illustration of one day example of the study can be seen in figure below:

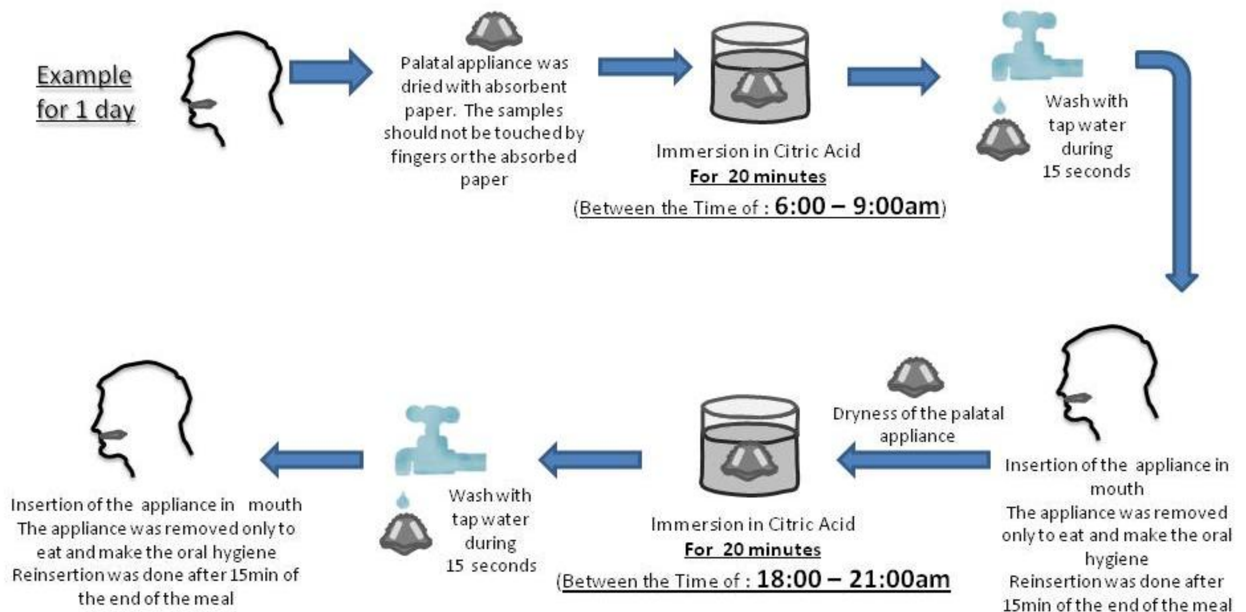


Figure 2: Daily procedures of the volunteers

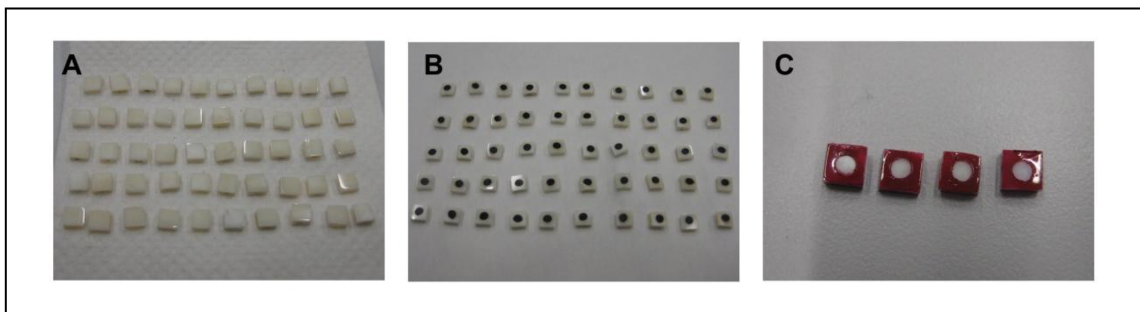
Individual oral hygiene was performed with fluoride-free products (Calendula – Zahncreme; Weleda®; D-Schwäbisch Gmünd; A-Wien) and without appliances *in situ*. Before and between the treatment periods, a minimum 7 days lead in and wash out period with fluoride-free oral hygiene procedures was included.

Sample Preparation

Enamel blocks were cut from bovine incisor teeth (Isomet, Buehler, Ollinois, USA) presenting the dimensions of: 5 x 5 x 2mm. The enamel surface was polished with grit silicon carbide granulation of 800, 1200 and 4000 (Buehler, Illinois, USA) followed by polishing the enamel surface with 1μm diamond solution (Buehler, Illinois, USA). Between each series of polishing, the

samples were washed in deionized water for 3 minutes. The blocks were cleaned properly and observed in a stereomicroscope to ensure the absence of structural defects and stored in deionized water. A disc of adhesive tape (Scotch 3M) with a diameter of 2.5mm was glued on the center of the enamel surface. All the rest surfaces of the samples were painted with red nail polish (**Figure 3**). After nail polish drying, the piece of tape was removed and the surface was cleaned with cotton soaked with deionized water to remove any adhered adhesive.

Figure 3: Preparation of the samples



A – Samples after being cut and polished; **B –** A disco f adhesive tape with a diameter of 25mm was glued on the center of the enamel surface; **C –** The surfaces of the samples were painted with nail polish. After nail polished drying, the piece of tape was removed. As superfícies das amostras foram cobertas com esmalte após secagem, o adesivo foi então removido.

Palatal appliance

Palatal appliances from resin hard elastic transparent blanks, bond to acrylic (2mm x 125mm - catalog ≠ 3419 –Duran, Iserlohn, Germany), capable of fitting two pieces of plastic holders were made individually for each patient, after prior luting with alginate. The polycarbonate units have a thickness of 1.45mm, with dimensions of 5 x 28mm and present four chambers to help the

samples fixation. The enamel samples were arrested in the polycarbonate units by orthodontic wire and wax and the polycarbonate units were fixed in the palatal appliance with resin. The palatal appliance can be seen in details in the figure below (**Figure 4**):

Figure 4: Mounting of Palatal appliances

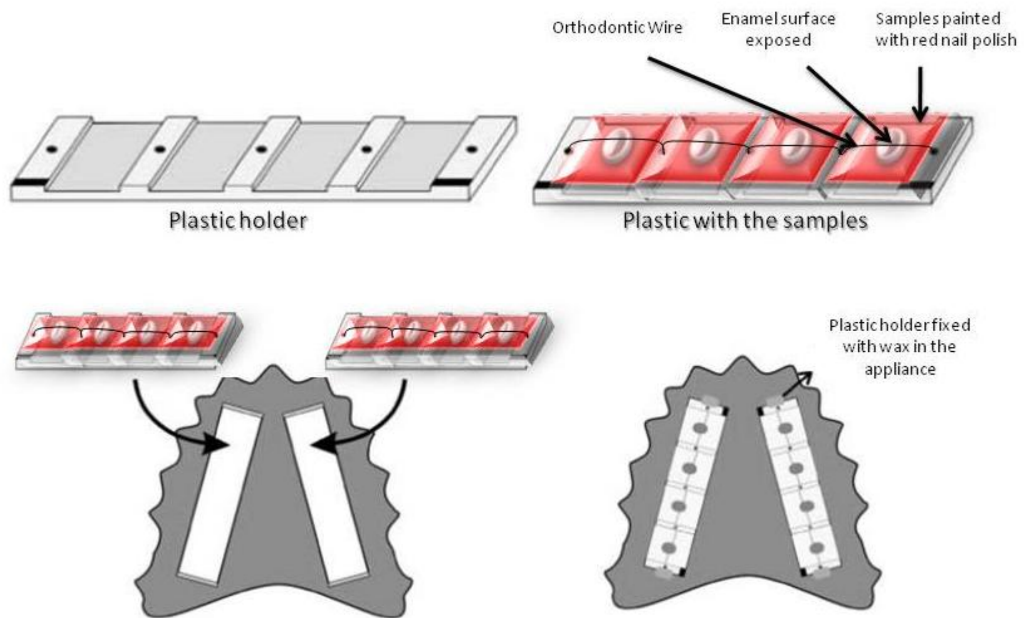


Figure 5: Picture of Palatal appliance

Fluoride application

Samples from group 2 and 4 included fluoride treatment. So the samples were dried with absorbent paper and submitted to topical application of 3 min (Elmex® Gelée, Wybert, Lörrach, Germany; 1.25% fluoride in the form of aminefluoride, ph 4.8-6.0) and then washed with distilled water during 10 seconds and dried with absorbent paper.

Laser Irradiation

The irradiation was performed using a pulsed CO_2 laser emitting at 10.6µm (Rofin model SC x 30, Rofin-Sinar Laser GmbH, Hamburg, Germany from the Department of Conservative, Periodontology and Preventive Dentistry, RWTH, Aachen). The irradiation distance was 19.8 cm, the determined that a beam diameter of 2.5 mm on the sample surface. The energy emitted by the laser was controlled prior to irradiation of each group and later in a range between two samples, using a power meter (Coherent Detector FieldMaster GS + LM45, Coherent, USA). Prior to irradiation, samples are dried. The parameters used were: $0.3J/cm^2$, 226Hz, 5µs/pulse.

Figure 6: CO_2 laser (Rofin model SC x 30, Rofin-Sinar Laser GmbH, Hamburg, Germany).

IN VITRO MODEL

For the *in vitro* study, 144 bovine enamel samples were prepared the same way as *in situ* samples, described above. Samples were stored in deionized water in 4^oC and were divided into the groups according to **table 3**:

Table 3: Groups and samples distribution (*in vitro*)

Day	**Group Control** Erosion only	**Group CO_2 Laser** Rofin SC x 30, Rofin-Sinar *Laser,* GmbH, Hamburg, Germany	**Group Fluoride** Topical Amine-Fluoride for 3 min (Elmex® Gel, Switzerland, 1.25% AmF, ph 4.8-6.0,	**Group Fluoride + CO_2 Laser**
Day 1	n= 12	n= 12	n= 12	n= 12
Day 3	n= 12	n= 12	n= 12	n= 12
Day 5	n= 12	n= 12	n= 12	n= 12
Total	n= 36	n= 36	n= 36	n= 36

For erosive demineralization of *in vitro* study, the samples were immersed in 40 ml 0.05M citric acid (Citric acid monohydrate – $C_6H_8O_7 \cdot H_2O$; M=210,14g/mol - E. Merck, Darmstadt, Germany); pH 2.3; room temperature; for 20 minutes twice daily and after acid exposure, rinsed with tap water and stored in deionized water 4^oC. Some samples were analyzed in day 1 (2 times submitted to acid challenge), some in day 3 (6 times submitted to acid challenge), and the rest in day 5 (10 times submitted to acid challenge), according to table 2. The demineralization solution was renewed after each treatment period and its pH monitored.

Each treatment (laser irradiation and fluoride application) submitted previously to acid challenge in specific groups were done in the same way of *in situ* samples.

Samples Picture

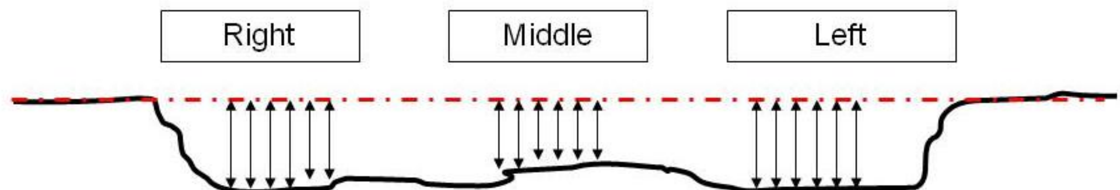
Pictures with 4x magnification from the *in situ* samples was taken after the treatment using a Estereomikrosocp (MZ6, Leica Microsystems GmbHm Wetzlar, Germany, from the Department of Conservative, Periodontology and Preventive Dentistry, RWTH, Aachen), a digital camera (Hitachi HV – C2A – Wetzlar, Germany), and the programm Diskus 4.2 (Hilgers, Königswinter, Germany)

Profilometric Measures

After the treatments, samples were stored in deionized water ($4^{o}C$). The samples were glued on a plastic layer of 50 x 100 x 2 mm (Exakt GmbH, Norderstedt, Germany) using transparent adhesive for fixation (Technovit 7230VLC, Heraeus Kulzer GmbH, Wehrheim, Germany) and keeping the enamel face uppermost. The nail polish was removed from the enamel surface with nail polish remover. The enamel surface was analyzed through a digital profilometer (Konturenmessgerät MarSurf XC2, Hersteller Firma Mahr GmbH, Mahr GmbH, Göttingen, Deutschland) and the software - MarSurf XC2 (GmbH, Göttingen, Deutschland). The scanning was provided by 0,7 mN strength, by a tungsten carbide point with 25um ray. Three lines were scanned in each sample with a distance of 100µm each, and in each line 6 measures in the left periphery were taken, as well as 6 measures in the middle and 6 measures in the right periphery, according to the **Figure 7**.

Figure 7: Standart of profilometric analysis

In each line 6 measures in each place (right, middle, left) were made



3D Profilometer Analysis

After the treatments, 1 sample/group was reserved for the 3D Profilometer analysis. The samples were stored in deionized water ($4^{o}C$). The samples were glued on a plastic layer of 50 x 100 x 2 mm (Exakt GmbH, Norderstedt, Germany) using transparent adhesive for fixation (Technovit 7230VLC, Heraeus Kulzer GmbH, Wehrheim, Germany) and keeping the enamel face uppermost. The acid-resistant varnishwas removed from the enamel surface with varnish remover. The enamel surface was analyzed through a 3D digital profilometer (Hommeltester T8000, Hersteller Hommel-Etamic GmbH, Hommel-Etamic GmbH, VS-Schwenningen, Deutschland Germany from the WZL Institute – *Werkzeugmaschinenlabor* – Aachen) and the software Turbo Wave und Hommel Map Expert (Hersteller Hommel-Etamic GmbH, Hommel-Etamic GmbH, VS-Schwenningen, Deutschland, Germany). The machine determined the deepest parallel line in the lesion and quantified the distance over and above the line, building the measures into a 3D image. The scanning was provided by a diamond point, with a conic shape with 2 µm ray.

Fluoride Analysis

After surface wear measurements made by the digital profilometer, 15 samples/group were selected and a disc of adhesive tape (Scotch 3M) with a diameter of just 2.5mm was again glued exactly on the demineralized surface of the sample. All the rest surfaces of the samples were painted with red nail polish. After nail polish drying, the piece of tape was removed and the surface was cleaned with cotton soaked with deionized water to remove any adhered adhesive in surface. Then the samples were placed individually in plastic tubes, to which was added 0.5ml of HCl 0.5 M. Then, the bottles containing the blocks were shacked during 90 seconds on a vortex. After agitation, it was added 0.5ml of TISAB II (containing 20.0g NaOH/L), with the purpose of immediate neutralization of reaction (Peres 1997). Then the total solution was divided into two plastic tubes containing 0.5ml each one. The amount of fluoride in solution was analyzed using a specific electrode (Orion 920A, Research Inc. - Boston, USA). Calibration curves were prepared from 0,019 to 3,8ppm of fluoride. Each sample has 2 measurements, and an average as taken between them.

Scanning electron microcopy

In order to observe the surface changes caused by the proposed treatments, 4 additional *in situ* samples for each group were subjected to scanning electron microscopy in University Hospital Aachen (UNIKLINIK), RWTH, Aachen. Samples were washed three times of five minutes each with phosphate buffer 0.1 M. The dehydration of the samples were proceed in sequence of alcohols: two washes of 30 minutes in 70% ethanol, two washes of 30 minutes in 96% ethanol, four washes of 30 minutes in 100% alcohol. The samples were glued to metal stubs, identified, covered by a thin layer of gold and observed under a scanning electron microscope. The pictures were taken under magnification of 300, 2000 and 6000.

Energy Dispersive X-ray analysis (EDX)

The electron microscope was equipped also with an EDX-microanalysis system (Si/Li semi-conductor detector with a super ATM Window for the detection of the light elements atomic number Z>4) The equipment belongs to University Hospital Aachen (UNIKLINIK), RWTH, Aachen. The EDX was used for line scans of the intensity lines of F, P and Ca. The depth of your measurements is nearly 3-5 µm in the surface because of the penetrating primary electron beam used (accelerating voltage 20 kV). Two samples of each group (Control, Laser, Fluoride + Laser and Fluoride) were analyzed after the respective treatment (no Treatment, Laser, fluoride + Laser and Fluoride). A total of 810 points were taken from each line. Only the decimal points (10, 20, 30, 40, 50,….800) were chosen and used to provide the graphic and the statistics.

Light Polarized Microscopy

The blocks were sectioned longitudinally in the center of the lesions using a diamond tape (E 300, Exakt GmbH, Norderstedt, Germany) under water cooling. One of the halves was stored and the other was dehydrated in an alcohol sequence (70%, 80%, 90%, 96% and 100%) and soaked in special histological acrylic resin (K-Plast, Medim Histotechnologie GmbH, Giessen, Germany). The resin blocks were obtained and then glued on a plastic microscope slide (Exakt GmbH, Norderstedt, Germany) of 50 x 100 x 2 mm using a transparent adhesive (Technovit 7230VLC, Heraeus Kulzer GmbH, Wehrheim, Germany). The surface was polished until the enamel was exposed and then polished with sandpaper aluminum oxide with granulation of 800 and 4000, under cooling with water. It was then glued on a plastic microscope slide 25 x 75 x 1.5 mm (Exakt GmbH, Norderstedt, Germany) using a transparent precision (Technovit 7210 VLC, Heraeus Kulzer GmbH, Wehrheim, Germany). In order to maintain the parallelism between the two blades, the polymerization of the adhesive was made in a device that contains a parallel support for the two slides (Exakt 402, Exakt GmbH, Norderstedt, Germany). After polymerization, a diamond wire refrigerated cut the samples glued in the blade in order to obtain a slice of 500 µm thickness. To reduce them to 100µm (± 10

mm), the blades were polished in polishing equipment for special histological cuts containing a digital controller of thickness (model CS401 Exakt 400, Exakt GmbH, Norderstedt, Germany). After being stored in distilled water (pH = 7) for 24 hours, the slides were soaked in distilled water and covered with glass cover slip for observation in polarized light microscope Axio Imager A1 (Carl Zeiss, NY, EUA Zeiss fom Patology Deparment, University of São Paulo, Brazil) with magnification lens of 10x and 25x. Images were transmitted to a computer connected to the microscope through a digital camera (AX10 cam HRC, Carl Zeiss Microimaging, Thornwood, NY, EUA) and the software Axio Vision (Carl Zeiss, NY, EUA)

Statistical Analysis

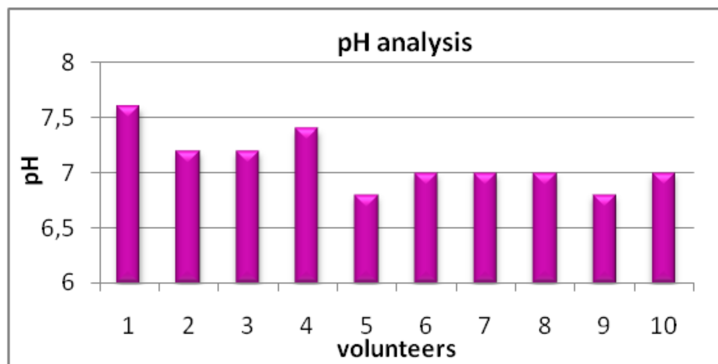
Continuous variables are presented as mean and standard deviation (SD). Categorical data are presented by frequencies and percentages. For the analysis of enamel surface loss and fluoride uptake in enamel a repeated measures ANOVA was conducted to asses differences between treatments. The ANOVA model included the values of surface loss or uptake at different time points as repeated factor, the different treatment as grouping factor and a time by treatment interaction term. To compare values on two time points for one treatment or two treatments on one time point, suitable contrast were formulated and tested. All tests were two-sided and assessed at the 5% significance level. Because of the exploratory nature of this study, no adjustment to the significance level to account for multiple testing was made. All analyses were done with SAS version 9.1.3 (SAS Institute, Cary, NC, USA).

4. RESULTS (ERGEBNISSE)

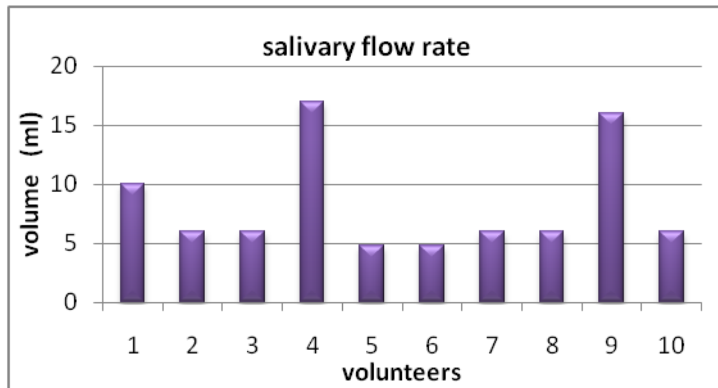
Salivary Test Results

All the volunteers selected in the study presented normal salivary values. The results from each volunteer are described below. Graphic 1 shows the pH of the saliva of each volunteer. The pH values were between 6.8 and 7.6. Graphic 2 shows the individual stimulated salivary flow rate from the volunteers. All the values were inside the normality window, higher than 1ml/minute. Graphic 3 show the buffer capacity from the volunteers. All volunteers showed the value 12, considered normal/high by the salivary test used.

Graphic 1: pH analysis from de saliva from the 10 volunteers. All the volunteers presented normal salivary pH.



Graphic 2: Analysis from de salivary flow rate from the 10 volunteers. All the volunteers presented normal flow rate from saliva.



Graphic 3: Analysis from the saliva buffer capacity from 10 volunteers. All the volunteers presented normal buffer capacity from saliva.

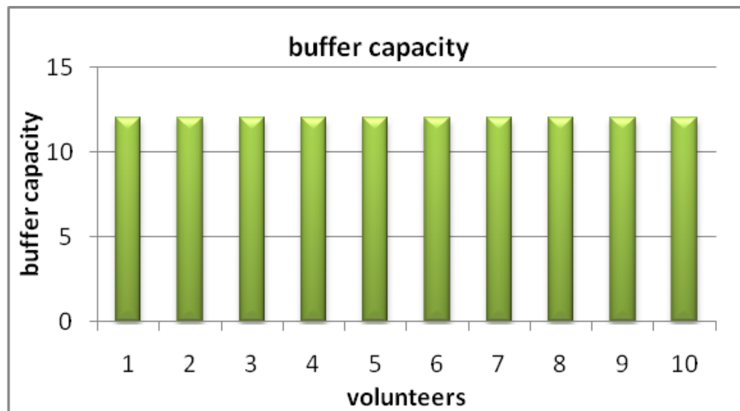
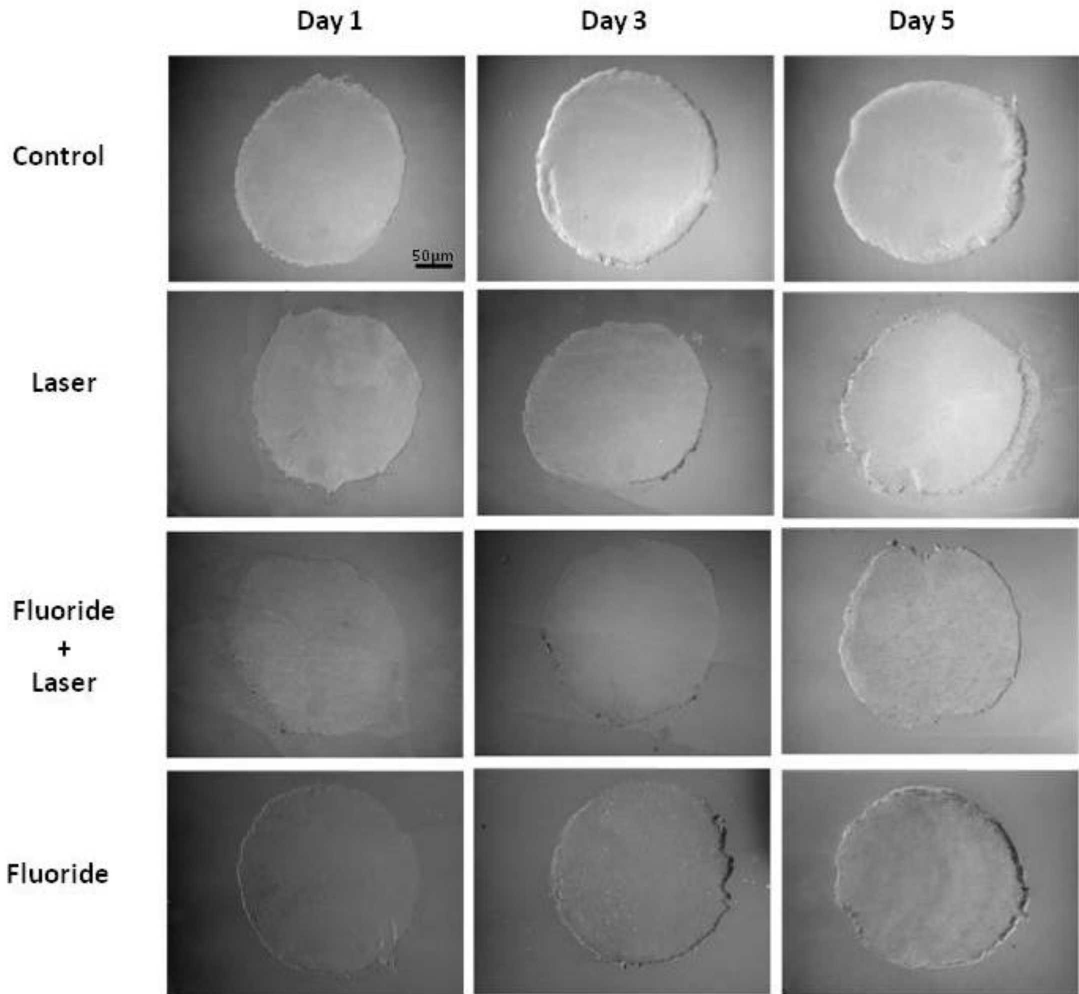


Image of the *in situ* samples after the treatments.

In figure 8, pictures of the *in situ* samples after 1, 3 and 5 days of treatments showed that the erosion lesion can be seen in all of them, with different depths depending on the time and group. Visually, the control group always presented the deepest lesion. The group Fluoride + Laser in day 1 seems to present the shallowest lesion (Figure 8).

Figure 8: Images of the in situ samples after the treatments



Analysis of lesion depth through Profilometric Measurement

The *in situ* and *in vitro* model samples were collected after 1, 3 and 5 days and the depth of the lesion was obtained through profilometry. The lesion depth increased with the time, in both models (*in situ* and *in vitro*) (Graphics 4 and 5). The *in vitro* model showed in all the times a significant difference, indicated through an asterisk (*) between the experimental groups and control group (Graphic 5, Table 7).

In the *in situ* model, the groups Laser **(L)** and Fluoride + Laser **(FL)** showed a significant difference between Control group **(C)** in the days 1, 3 and 5, showed through an asterisk (*) (Graphic 4, Table 6). The group Fluoride +

Laser **(FL)** presented in all times significant difference between Fluoride **(F)** group (Graphic 4, Table 6), showed through a cross (+).

Graphic 4: Profilometric analysis of lesion depth in the *in situ model*. (**C** – Control; **L** – Laser; **FL** – Fluoride + Laser; **F** – Fluoride). (*****) indicates significant difference between Control Group; (+) indicates significant difference between Fluoride Group; (++) indicates significant difference between Laser Group (Results are detailed in Table 5).

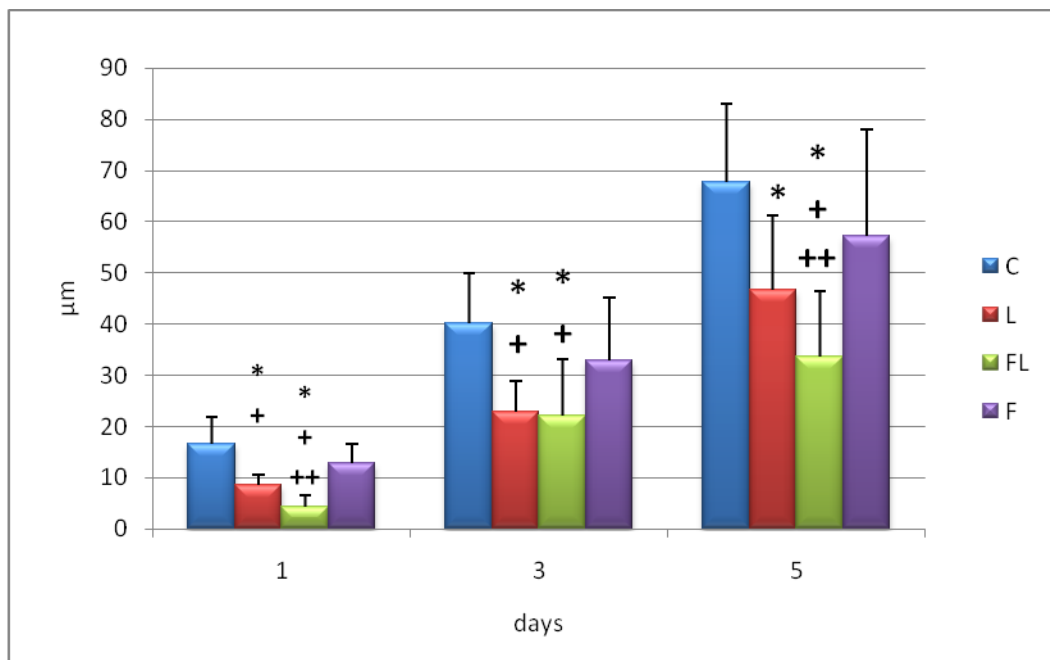


Table 4: Average and standard deviation from the measurements of the lesion depth in the in *situ* model.

Group	Average (± standard deviation)
DAY 1	
C	16.52 (± 5.31)
L	8.56 (± 2.07)
FL	4.34 (±2.15)
F	12.82 (±3.79)
DAY 3	
C	40.18 (±9.87)
L	22.73 (±6.19)
FL	22.07 (±11.05)
F	32.87 (±12.3)
DAY 5	
C	67.79 (±15.20)
L	46.70 (±14.48)
FL	33.60 (±12.77)
F	57.14 (±20.86)

Table 5: Significant difference between groups and *p* values in the *in situ* model.

DAY 1				
	C	**L**	**FL**	**F**
C		(p<0.01)	(p<0.01)	
L	(p<0.01)		(p<0.01)	(p<0.05)
FL	(p<0.01)	(p<0.01)		(p<0.01)
F		(p<0.05)	(p<0.01)	
DAY 3				
	C	**L**	**FL**	**F**
C		(p<0.01)	(p<0.01)	
L	(p<0.01)			(p<0.05)
FL	(p<0.01)			(p<0.05)
F		(p<0.05)	(p<0.05)	
DAY 5				
	C	**L**	**FL**	**F**
C		(p<0.05)	(p<0.01)	
L	(p<0.05)		(p<0.01)	
FL	(p<0.01)	(p<0.01)		(p<0.01)
F			(p<0.01)	

Graphic 5: Profilometric analysis of the lesion depth in the *in vitro model*. (**C** – Control; **L** – Laser; **FL** – Fluoride + Laser; **F** – Fluoride). Between all groups in all times there are significant differences (Results are detailed in Table 7).

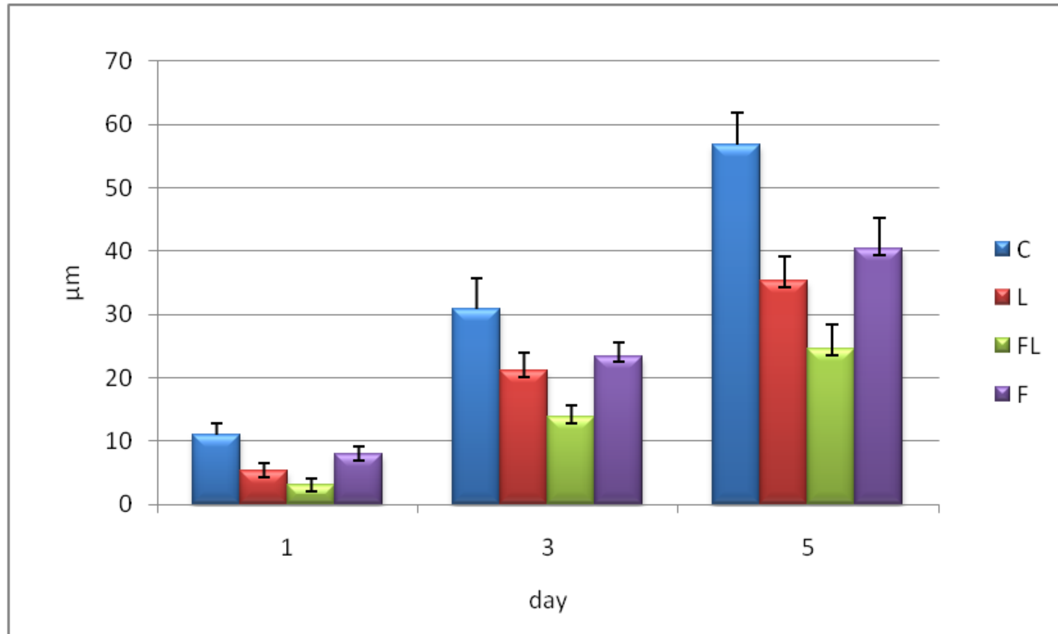


Table 6: Average and standard deviation from the measurements of the lesion depth in the in *vitro* model.

Group	Average (± standard deviation)
DAY 1	
C	11.02 (±1.85)
L	5.26(±1.38)
FL	3.03(±1.03)
F	7.99(±1.20)
DAY 3	
C	30.82(±4.98)
L	21.05 (±2.88)
FL	13.84(±1.91)
F	23.45(±2.06)
DAY 5	
C	56.75(±5.10)
L	35.21(±4.01)
FL	24.64 (±3.84)
F	40.43(±4.79)

Table 7: Significant difference between groups and *p* values in the *in vitro* model.

DAY 1				
	C	**L**	**FL**	**F**
C		($p<0.01$)	($p<0.01$)	($p<0.01$)
L	($p<0.01$)		($p<0.01$)	($p<0.01$)
FL	($p<0.01$)	($p<0.01$)		($p<0.01$)
F	($p<0.01$)	($p<0.01$)	($p<0.01$)	
DAY 3				
	C	**L**	**FL**	**F**
C		($p<0.01$)	($p<0.01$)	($p<0.01$)
L	($p<0.01$)		($p<0.01$)	($p<0.05$)
FL	($p<0.01$)	($p<0.01$)		($p<0.01$)
F	($p<0.01$)	($p<0.05$)	($p<0.01$)	
DAY 5				
	C	**L**	**FL**	**F**
C		($p<0.01$)	($p<0.01$)	($p<0.01$)
L	($p<0.01$)		($p<0.01$)	($p<0.01$)
FL	($p<0.01$)	($p<0.01$)		($p<0.01$)
F	($p<0.01$)	($p<0.01$)	($p<0.01$)	

Analysis of fluoride content

The fluoride content in the *in situ* samples were analyzed after 1, 3 and 5 days of treatment and *in situ* placement of the samples (Graphic 6, Table 8 and 9). The groups Fluoride + Laser (**FL**) and Fluoride (**F**) presented the highest level of fluoride content in days 1 and 3, indicated with an asterisk (*). Laser group **(L)** presented always the lowest content of fluoride, and presented a significant difference with Fluoride group (**F**), showed with a cross (+).

Graphic 6: Fluoride analysis in the samples in the *in situ* model (**C** – Control; **L** – Laser; **FL** – Fluoride + Laser; **F** – Fluoride). (*****) indicates significant difference between Control Group; (+) indicates significant difference between Fluoride Group; (++) indicates significant difference between Laser Group (Results are detailed in Table 9).

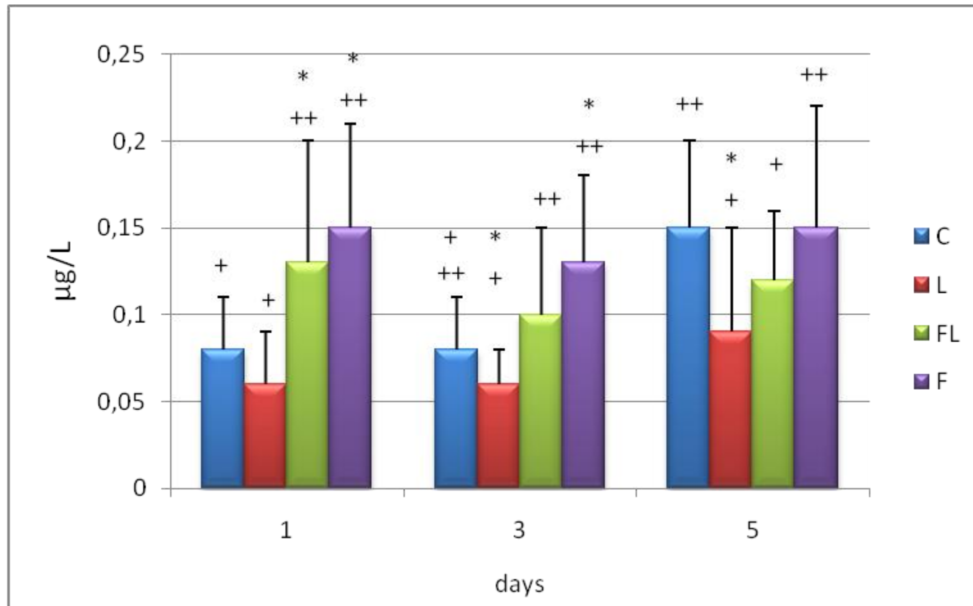


Table 8: Average and standard deviation from the fluoride measurements in the in *situ* model.

Group	Average (+ standard deviation)
DAY 1	
C	0.08 (+0.03)
L	0.06 (+0.03)
FL	0.13 (+0.07)
F	0.15 (+0.06)
DAY 3	
C	0.08 (+0.03)
L	0.06 (+0.02)
FL	0.10 (+0.05)
F	0.13 (+0.06)
DAY 5	
C	0.15 (+0.05)
L	0.09 (+0.06)
FL	0.12 (+0.04)
F	0.15 (+0.05)

Table 9: Significant difference between groups in fluoride analysis and *p* values in the *in situ* model.

DAY 1				
	C	**L**	**FL**	**F**
C			(p<0.01)	(p<0.01)
L			(p<0.01)	(p<0.01)
FL	(p<0.01)	(p<0.01)		
F	(p<0.01)	(p<0.01)		
DAY 3				
	C	**L**	**FL**	**F**
C		(p<0.01)		(p<0.01)
L	(p<0.01)		(p<0.01)	(p<0.01)
FL		(p<0.01)		
F	(p<0.01)	(p<0.01)		
DAY 5				
	C	**L**	**FL**	**F**
C		(p<0.05)		
L	(p<0.05)			(p<0.01)
FL				(p<0.05)
F		(p<0.01)	(p<0.05)	

<u>Analysis with 3D Digital Profilometer</u>

In the 3D Digital Profilometer images, it can be seen that in all groups an erosion lesion was formed (Figures 9A, B, C). According to the color scale, the zero point is referred to the yellow color and the highest the presence of a blue color, the deeper the lesion is. Lesions tending to be more yellow in color and therefore shallower can be seen in all days, in groups Laser and Fluoride + Laser especially in the middle of the lesion. This confirms the 2D measurement, where the groups Laser (L) and Fluoride + Laser (FL) presented the shallowest lesions.

Figure 9A: Images obtained with the 3D profilometer for all groups in Day 1

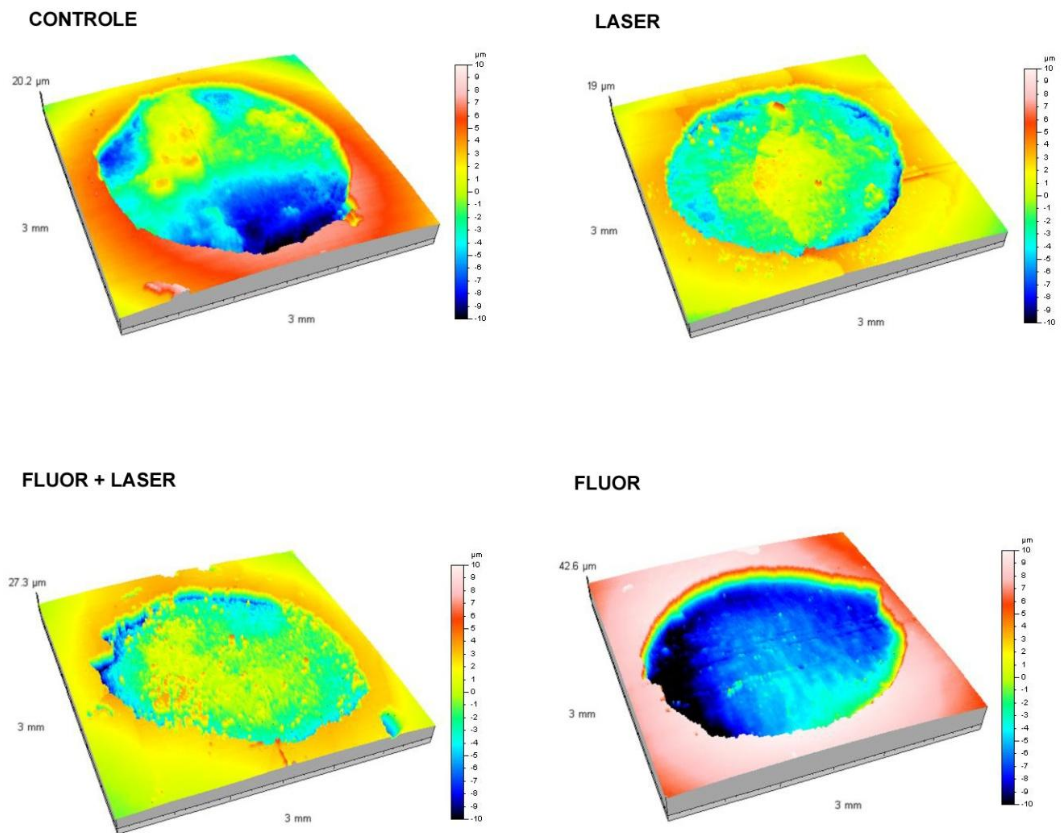


Figure 9B: Images obtained with the 3D Profilometer for all groups in Day 3

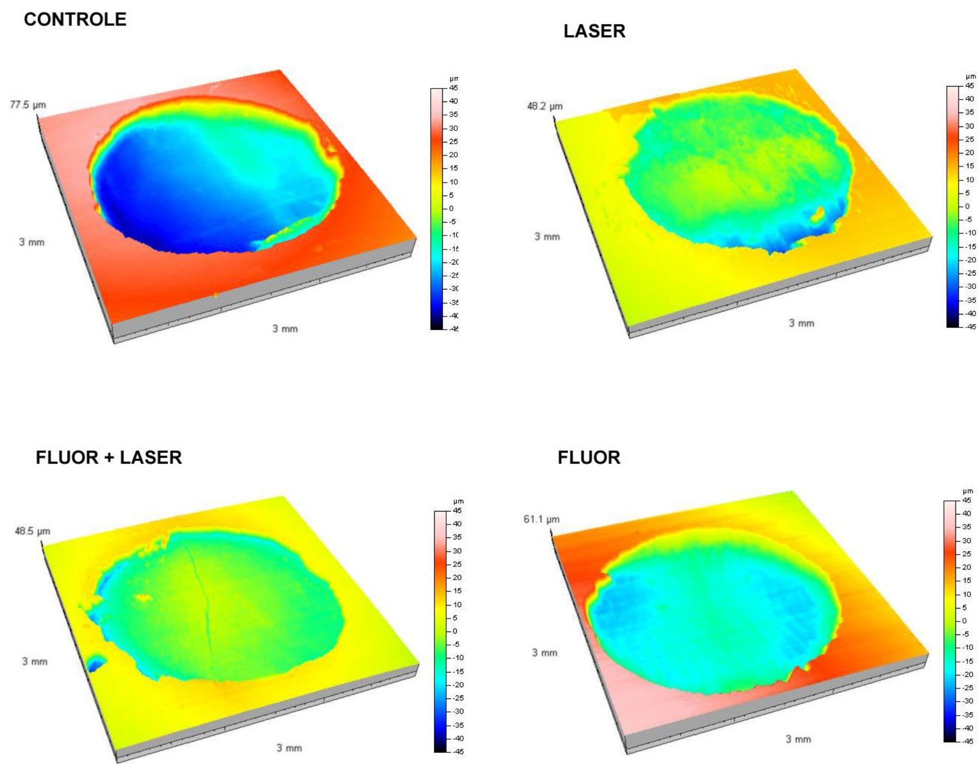
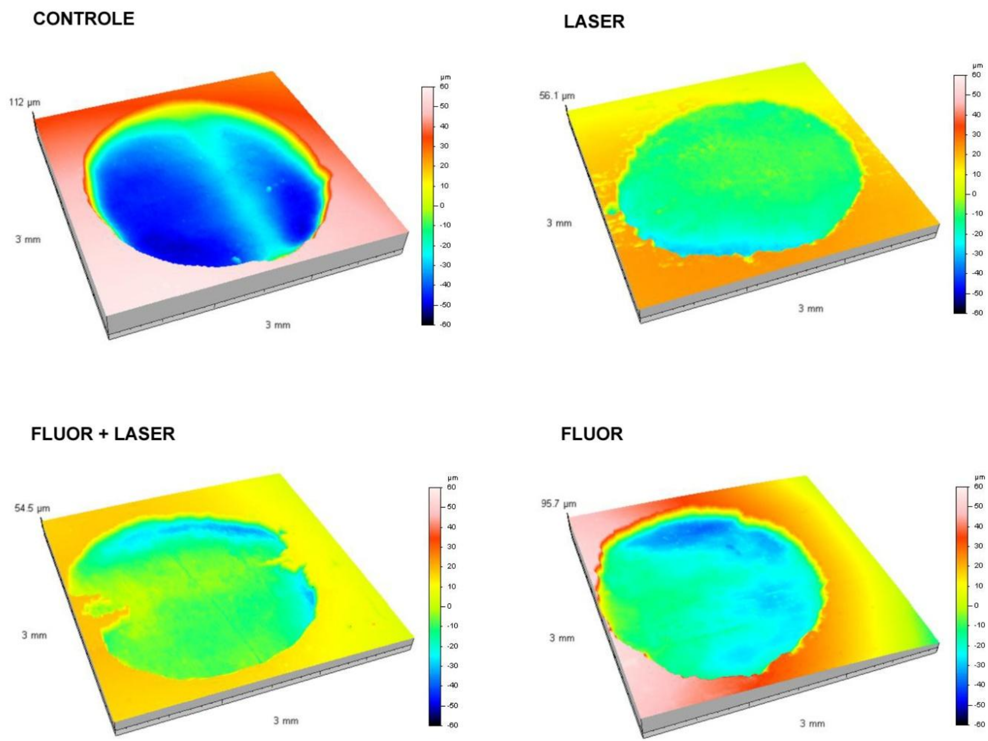


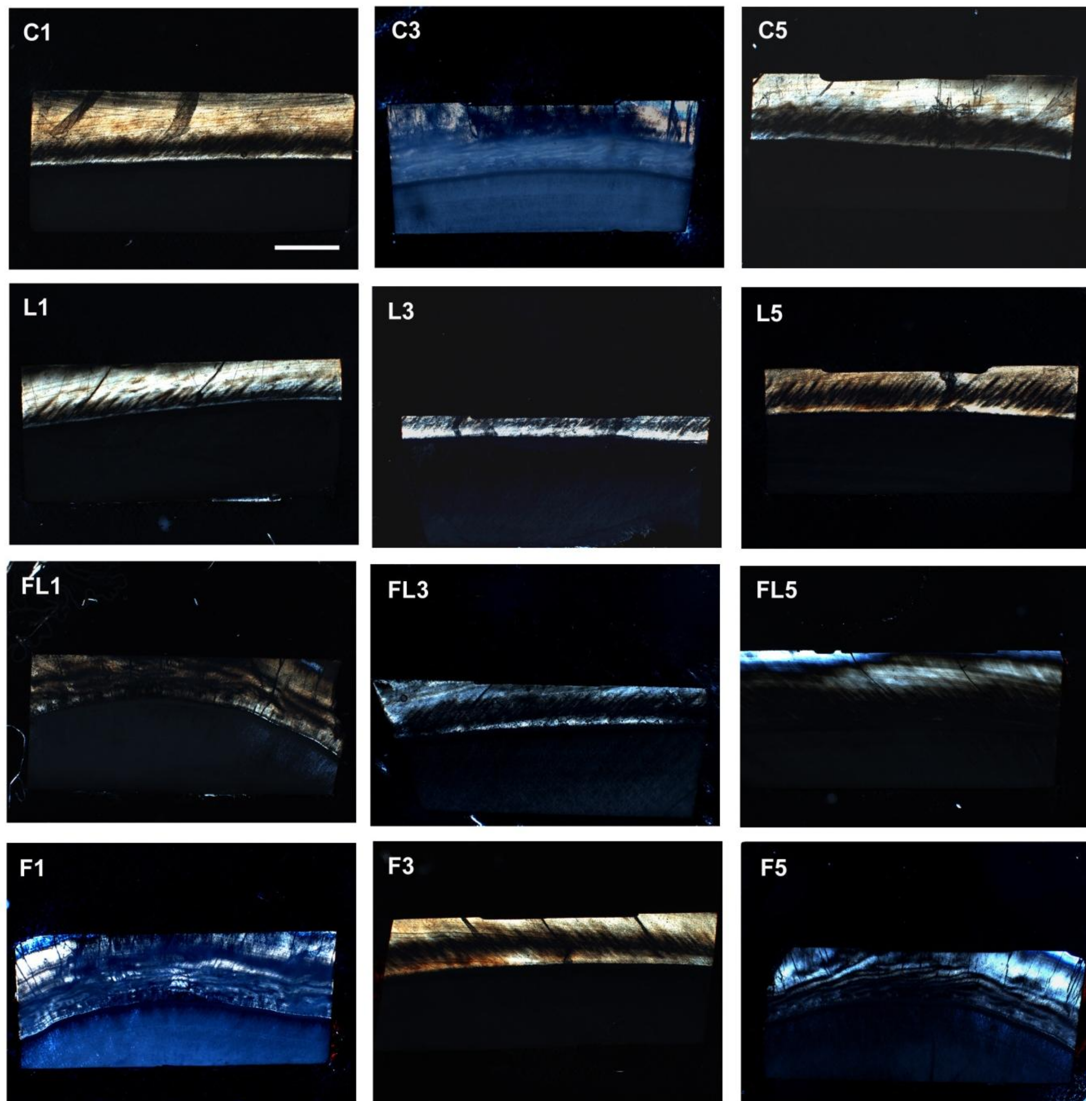
Figure 9C: Images obtained with the 3D Profilometer for all groups in Day 5



Morphological evaluations under Polarized Light Microscope

The transversal slices of the samples observed under a light polarized microscope clearly show the progression of the erosion lesions from day 1 to 5.

Figure 10: Transversal Images of the erosion lesions in different times in the four groups.

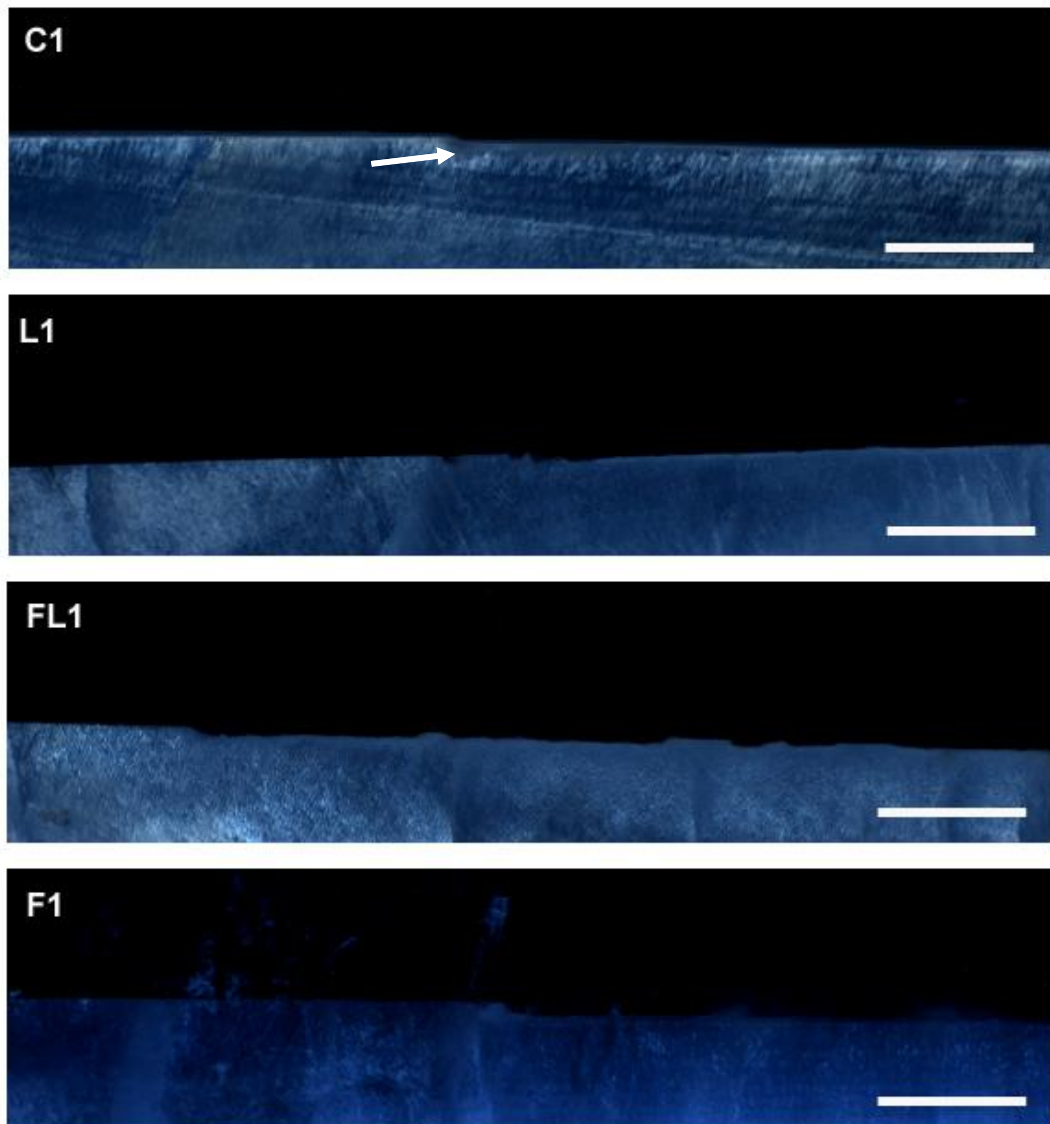


(**C1** – Control Group Day 1; **C3** – Control Group Day 3; **C5** – Control Group Day 5; **L1** – Laser Group Day 1; **L3** – Laser Group Day 3; **L5** – Laser Group Day 5; **FL1** – Group Fluoride + Laser Day 1; **FL3** – Group Fluoride + Laser Day 3; **FL5** – Group Fluoride + Laser Day 5; **F1** – Group Fluoride Day 1; **F3** – Group Fluoride Day 1; **F5** – Group Fluoride Day 1; **magnification bar = 1000µm**)

Polarized Light Microscopy

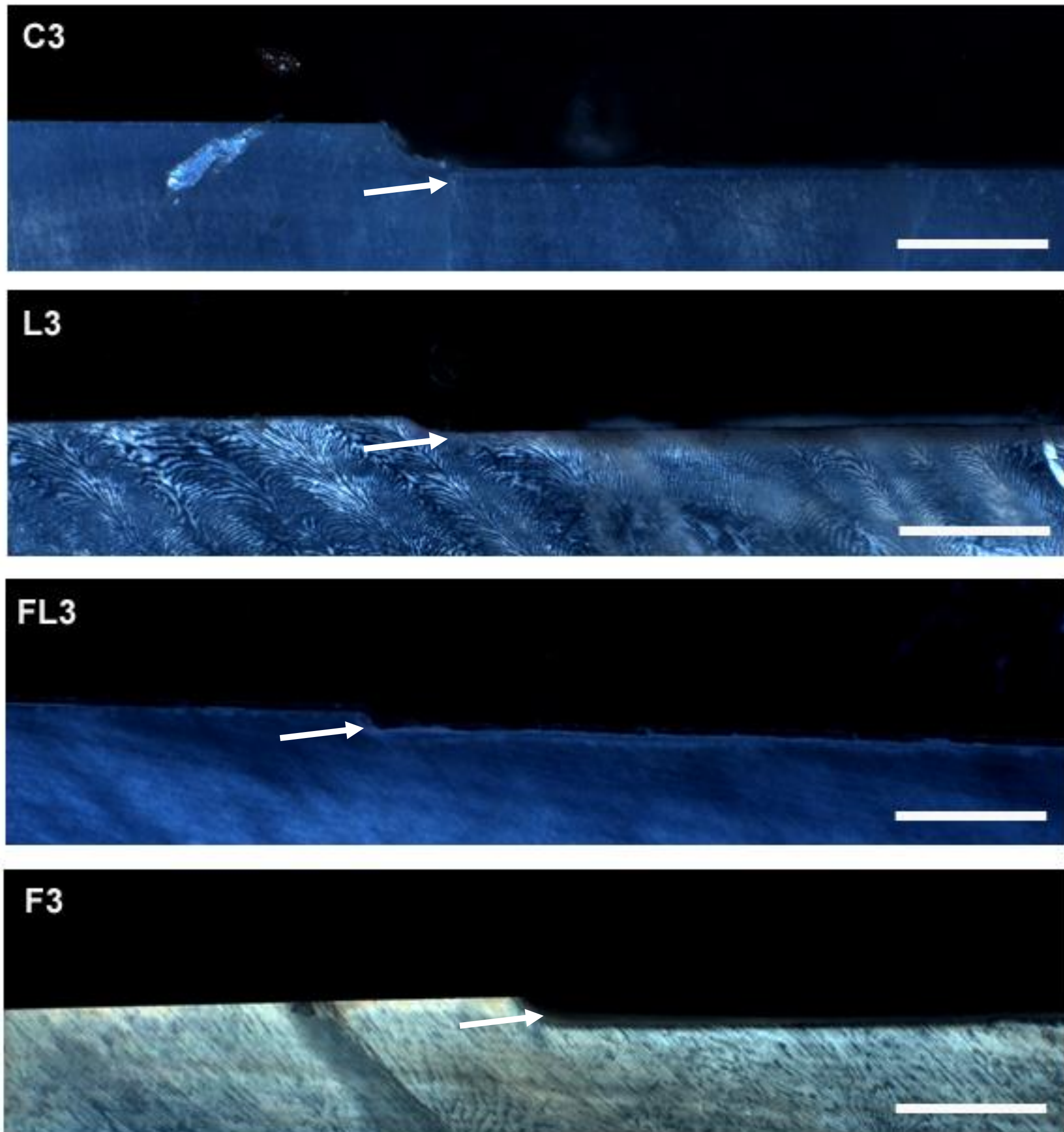
The polarized light microscopy of the *in situ* samples show the demineralization depths of the samples in all the days (Figures 11 A, B, C). On day 1 (Figure 11A), the control group presented the deepest demineralization depths. On day 3 (Figure 11B), no evident difference could be seen between the groups, as well as on day 5 (Figure 11C). The demineralization areas are shown through an arrow.

Figure 11A: Polarized light microscopy of the groups in day 1.



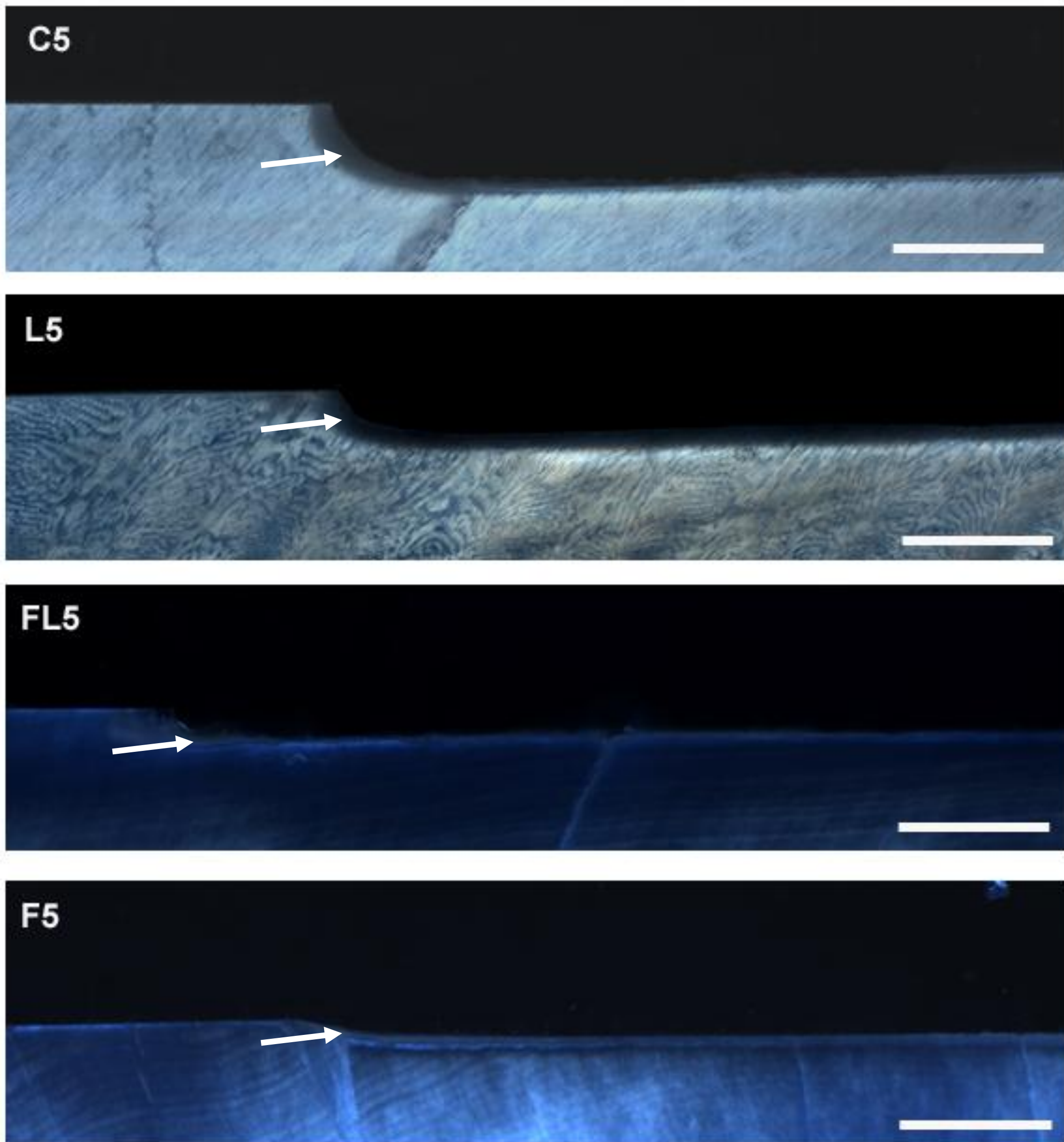
(***C1*** *– Control Group Day 1;* ***L1*** *– Laser Group Day 1;* ***FL1*** *– Fluoride + Laser Group Day 1;* ***F1*** *- Fluoride Group Day 1;* ***magnification bar = 200µm****)*

Figure 11B: Polarized Light microscopy of the groups in day 3.



*(**C3** – Control Group Day 3; **L3** – Laser Group Day 3; **FL3** – Fluoride + Laser Group Day 3; **F3** - Fluoride Group Day 3; **magnification bar = 200µm**)*

Figure 11C: Polarized Light microscopy of the groups in day 5

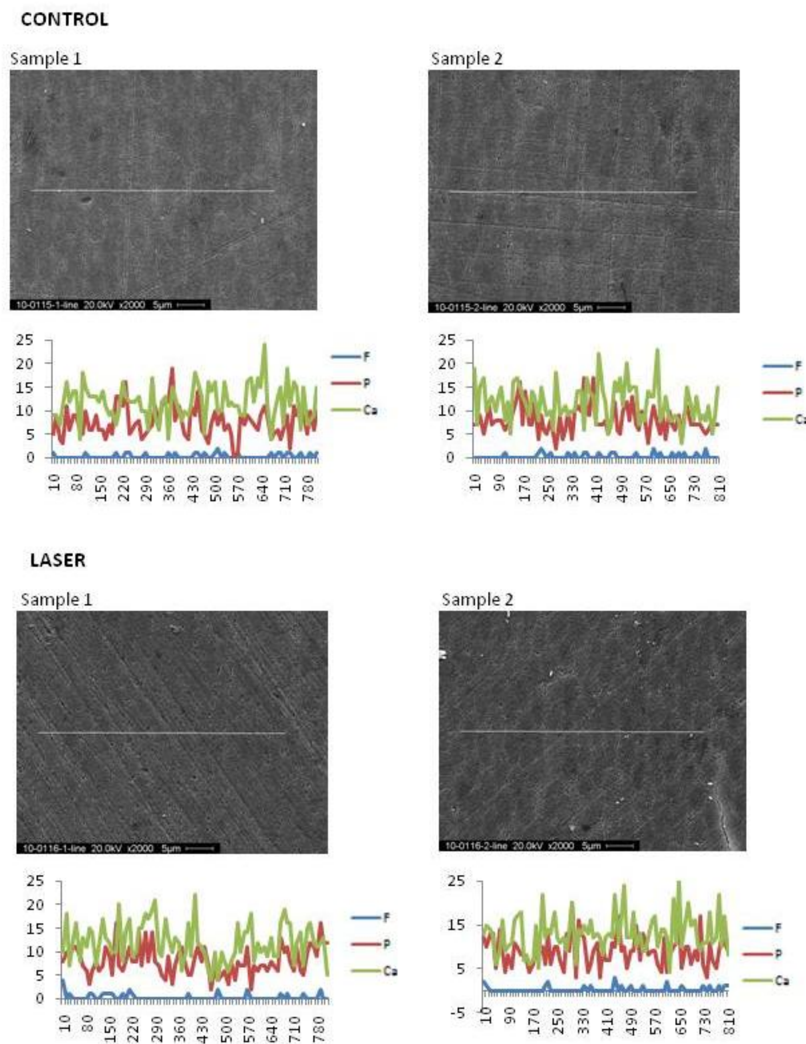


*(**C5** – Control Group Day 5; **L5** – Laser Group Day 5; **FL5** – Fluoride + Laser Group Day 5; **F5** - Fluoride Group Day 5; **magnification bar = 200µm**)*

Scanning Electron Microscopy with EDX Analysis after Surface Treatment

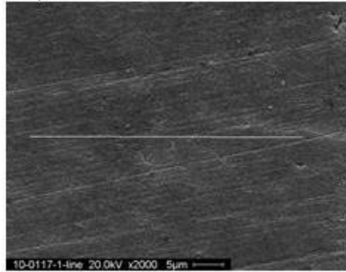
Scanning electron microscopy after the respective treatment of two samples from control, laser, fluoride + laser and fluoride group (Figure 12) showed no evidence of ablation, cracks or any irradiation damage to the enamel in the groups where laser was applied. The line where the fluoride (F), calcium (Ca) and phosphate (P) were measured in the samples is marked in the micrograph as a white line. The correspondent graphic of the substance analysis of each micrograph are illustrated underneath the micrograph.

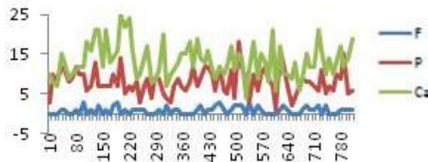
Figure 12: SEM pictures and correspondent substances (F, Ca, P) analysis of each sample. Pictures taken at 2000x magnification.



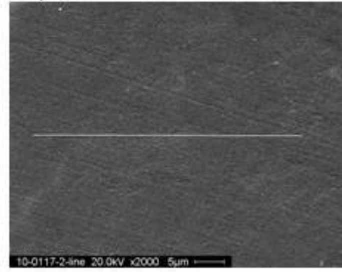
FLUORIDE + LASER

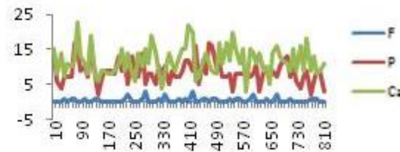
Sample 1





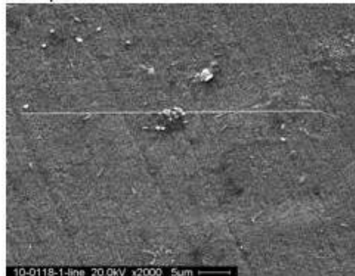
Sample 2

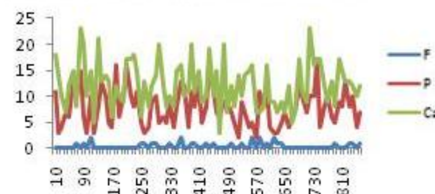




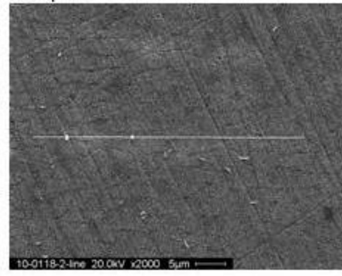
FLUORIDE

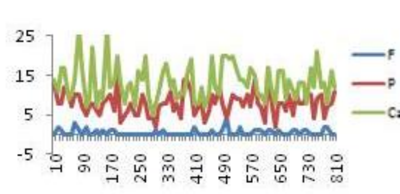
Sample 1





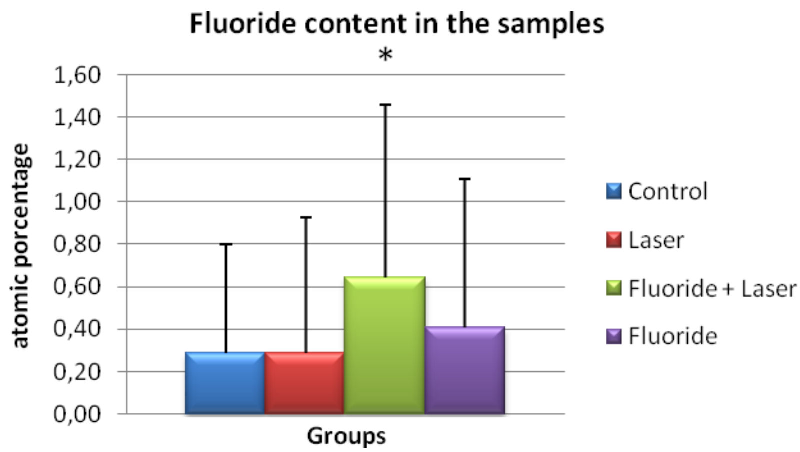
Sample 2



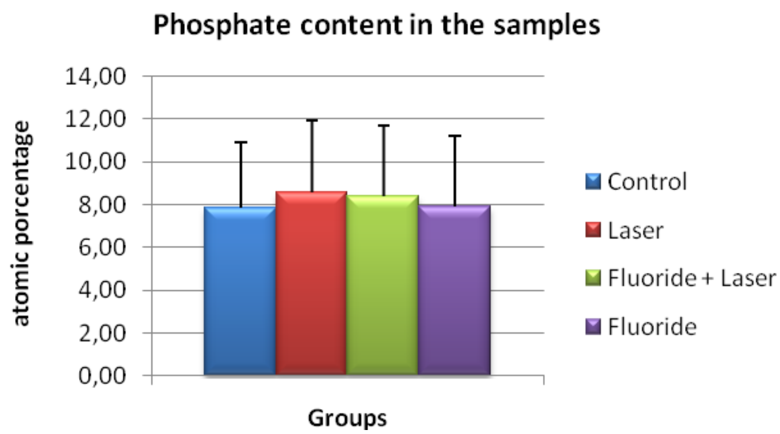


The average of Fluoride, Calcium and Phosphate content in the analyzed samples are shown in Graphic 7, 8 and 9. The analysis of Calcium and Phosphate did not show any significant difference between the groups (Graphic 8 and 9). The statistical analysis showed that the Fluoride content of the group Fluoride + Laser was significantly higher than all the others groups.

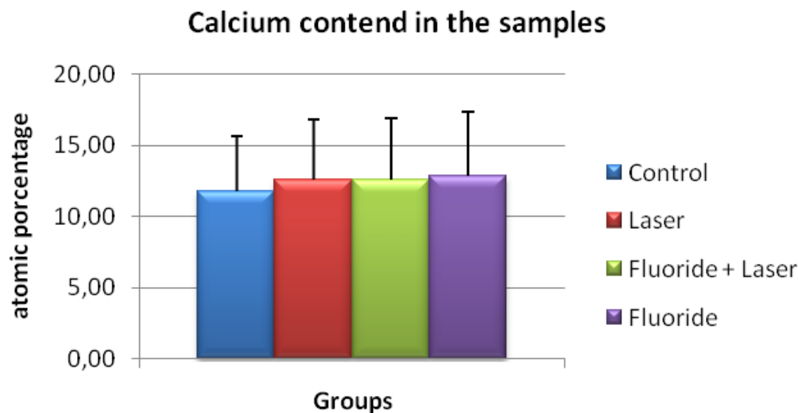
Graphic 7: Fluoride content in the groups analyzed through EDX. Asterisk (*) indicates significant difference between control group.



Graphic 8: Phosphate content in the groups analyzed through EDX



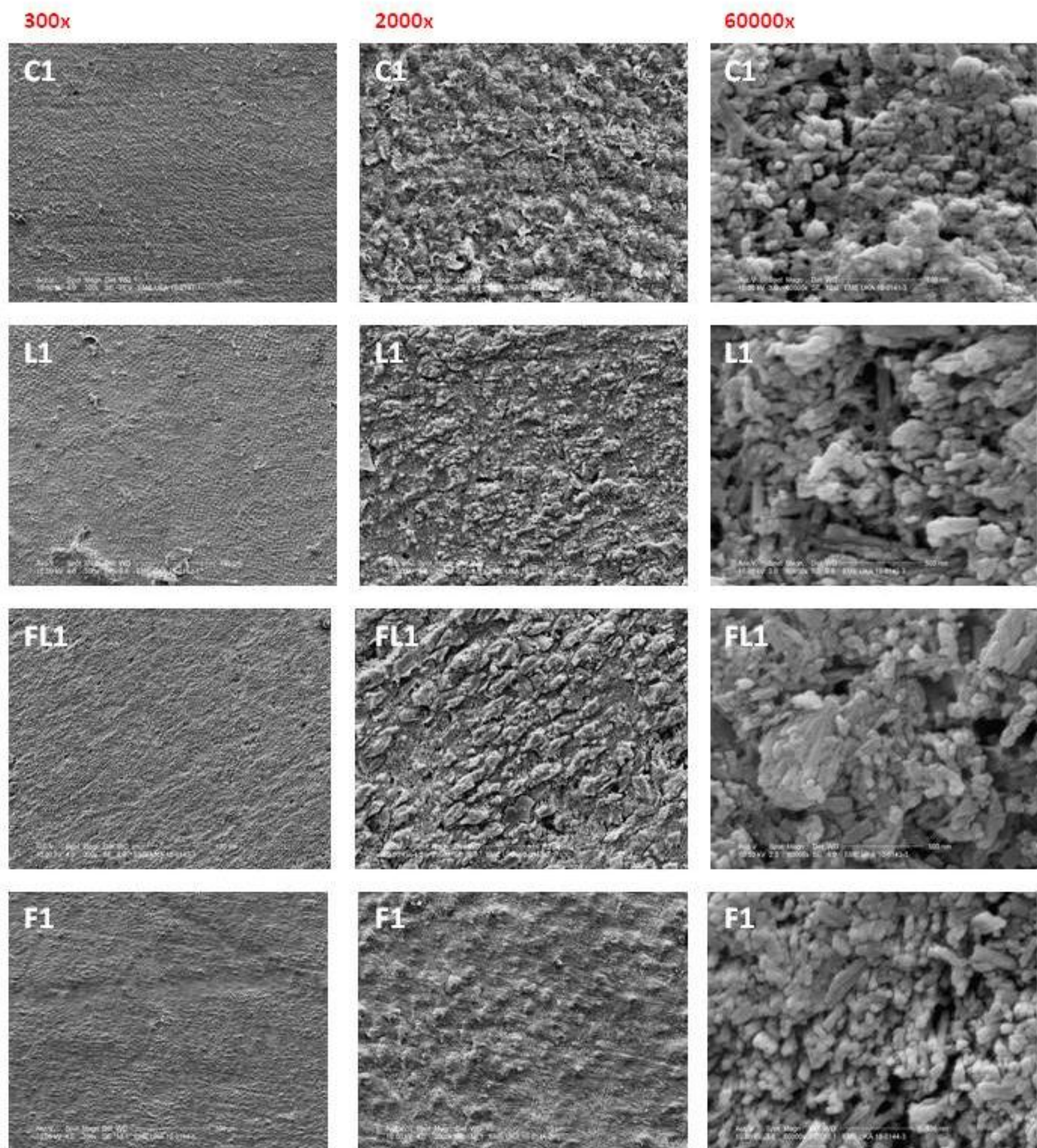
Graphic 9: Calcium contend in the groups analyzed through EDX



Scanning electron Microscopy after *In Situ* Erosive Challenge

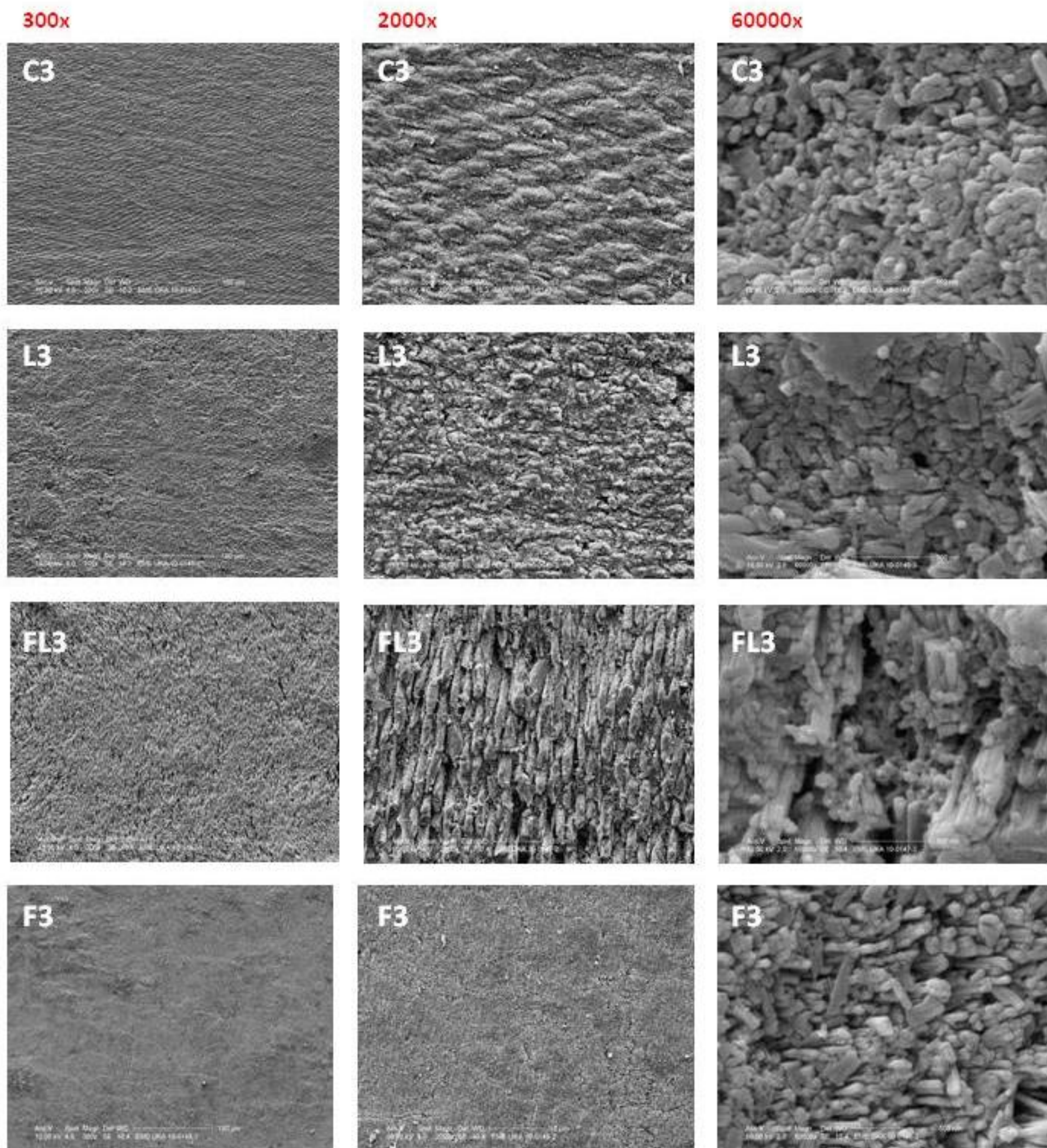
The *in situ* samples after the 1, 3 and 5 days of treatments were analyzed through scanning electron microscopy in the magnification 300, 2,000 and 60,000X. In Figures 13, 14 and 15 the SEM pictures of the groups in day 1, 3 and 5 respectively can be seen. The morphology of the samples showed characteristically aspect of eroded surfaces. It is known that in prismatic human enamel, the progression of erosion affects the prism sheath first and thereafter, with a more severe challenge, also the prisms cores.

Figure 13: SEM pictures from in situ samples in day 1



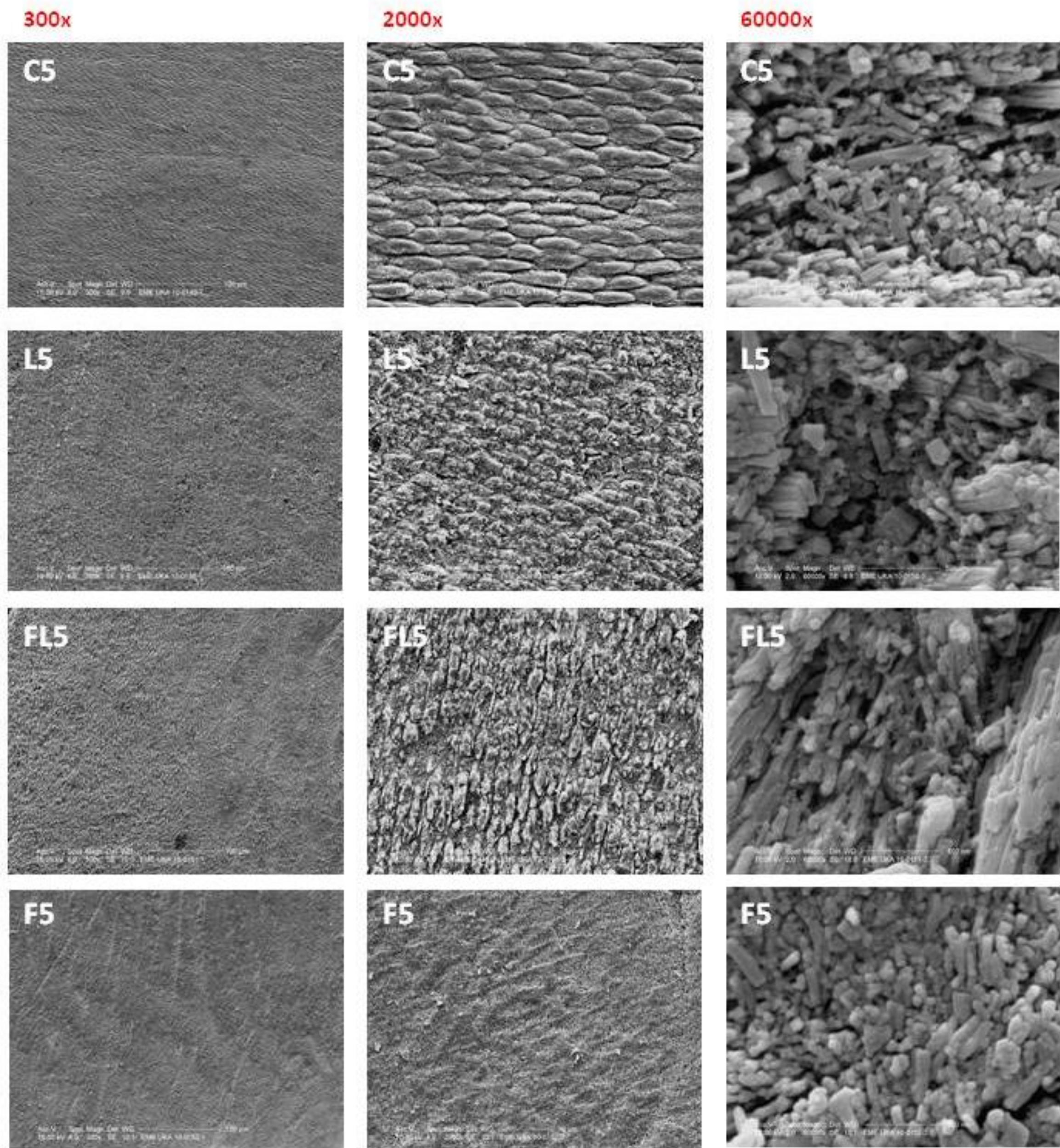
*(**C1** – Control Group Day 1; **L1** – Laser Group Day 1; **FL1** – Fluoride + Laser Group Day 1; **F1** – Fluoride Group Day 1).*

Figure 14: SEM pictures from in situ samples in day 3



***(C3** – Control Group Day 3; **L3** – Laser Group Day 3; **FL3** – Fluoride + Laser Group Day 3; **F3** – Fluoride Group Day 3).*

Figure 15: SEM pictures from in situ samples in day 5



***(C5** – Control Group Day 5; **L5** – Laser Group Day 5; **FL5** – Fluoride + Laser Group Day 5; **F5** – Fluoride Group Day 5).*

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5. DISCUSSION (DISKUSSION)

There is evidences that the presence of dental erosion is growing (Lussi et al. 2008), and in parallel, studying concerning to dental erosion has increased considerably. In the seventies, less than five studies per year were published; this number was still below ten in the eighties and has now risen to about 50 studies per year (Young et al. 2008).

Soft drink consumption is one of the factors related to the erosion increase. Soft drinks consumption in the USA increased by 300% in 20 years (Calvadini et al. 2000). Around the year 1995, between 56 and 85% of children at school in the USA consumed at least one soft drink daily, with the highest amounts ingested by adolescent males. Nowadays there is a consensus that four or mode acid intake per day are associated with high risk of dental erosion (Lussi & Schaffner 2000).

Among the big variety of acid beverages found commercially, the citric acid from juices are doubly harmful to the tooth surface, once it has a double action. Besides the effect of hydrogen ions, acid anions (citrate) may complex with calcium, reducing the supersaturation of saliva and dissolving the crystal surface (Lussi et al. 2004; Lussi & Jaeggi 2008). In the present study the erosion was promoted by immersion of palatal appliance in the citric acid twice daily during 20 minutes as already described in the study from Ganss et al., (2007). According to the literature, the erosion time of 20 minutes in citric acid is used to achieve a maximum of surface softening (Stösser & Nekrashevych 1998). Consequently, the effective of any preventive method used in a study developed with 20 minutes of acid challenge can show promising clinical results, considering the severity of acid challenge during 20 minutes with citric acid. In the study of Ganss et al. 2007, it was investigated the effectiveness of both waiting periods between acid exposure and tooth brushing and fluoride applications in preventing tooth brushing abrasion of acid softened enamel surfaces. The authors used human enamel samples derived from extracted third molars. The experimental period of the study was 5 days, and the enamel loss was determined profilometrically. The enamel samples recessed in the buccal aspects of the appliance, differently from the present study that fixed the bovine enamel samples in palatal side of the appliance. In the study from Ganss

et al., (2007) the control group, that was submitted only to erosion, without brushing abrasion, as the present study, showed around 45 μm of enamel loss after 5 days. In the present study it could be verified after the same 5 days of treatment (2 citric acid challenges of 20 minutes per day), the average of enamel loss close to 68 μm. Probably the difference in the enamel (bovine enamel in this study and human enamel in the study from Ganss et al., 2007), as well as the position of the samples in the mouth, could be the factors responsible for the difference in the enamel loss. Bovine enamel has been used widely used in dental research as a model for human enamel. The advantages of using bovine teeth are the possibility of obtaining bigger areas of plane enamel and their better availability compared to human enamel. Besides, these teeth present composition optical properties similar to human teeth (Arends et al. 1980). Nevertheless the rate of demineralization progression in these teeth is higher. Rios et al. (2006), verified that human enamel presented less wear compared to bovine enamel, when samples were submitted *in situ* to erosion (5 minutes in 150 ml of cola drink, 4 times per day), followed by brushing abrasion. Featherstone & Mellberg (1981) have already described that artificial caries progression was 3 times higher in bovine than in human enamel. Human enamel is compact than the bovine one, which is more porous and has less mineral contend (Featherstone & Mellberg 1981; Meurman & Frank 1991).

Dental erosion is influenced by several biological factors described in the literature as: quantity and quality of saliva, tooth composition and structure, dental anatomy and occlusion, the anatomy of oral soft tissues in relationship to the teeth and physiological soft tissue movements such as swallowing pattern (Zero 1996; Lussi et al. 2008; Barbour et al. 2006; Lussi et al. 1993; Lussi et al. 1995; Lussi et al. 2004; Lussi & Jaeggi 2008; Ireland et al. 1995; Lussi 2006; Lussi & Jaeggi 2008). So the researches should take these factors in account, to get the most credible results. Recently several studies related to dental erosion are developed through the *in situ* model (Schlueter et al. ; Hughes et al. 1999; Jaeggi & Lussi 1999; Hara et al. 2006; Rodrigues et al. 2006; Zero et al. 2006; Hooper et al. 2007; Magalhaes et al. 2007; Rios et al. 2008; Hara et al. 2009; Magalhaes et al. 2009). This model provides that the sample stays in the oral conditions, specially being affected by salivary properties.

In this study, the volunteers stayed with the palatal appliance with the fixed samples during 1, 3 and 5 days. All the volunteers presented normal pH, buffer capacity and stimulated salivary flow rate. The samples were fixed in the palatal regions of the appliance. Despite some controversial results in the literature concerning about the real role of saliva in erosion progression, studies corroborate that the *in situ* model is the nearest condition to what truly occurs clinically. The role of saliva in the erosion process is not fully understood. Saliva has been mostly considered the most important biological factor influencing dental erosion (Hara et al. 2006). Several salivary protective mechanisms come into play during an erosive challenge: dilution and clearance of erosive agent from the mouth, neutralization and buffering of acids, and slowing down the rate of enamel dissolution through the ion effect by salivary calcium and phosphate (Lussi & Jaeggi 2008). In the *in situ* methodology adopted in this study and also by other studies, some of these aspects cannot be taken account, once the acid challenge were done extra orally. In this case the saliva would act by the remineralization process.

In this study the rehardness role of saliva in the acidic-softened enamel surface should also be taken account for the obtained results (Feagin et al. 1969; Gedalia et al. 1991; Amaechi & Higham 2001). This role was also confirmed in the study from Rios et al., (2006) where the stimulated saliva could prevent or reduce the wear and the percentage change in microhardness of bovine and human enamel submitted to erosion followed brushing abrasion immediately or after 1 hour. Nevertheless, *in situ* other investigations could not find a significant rehardening effect of saliva (Garberoglio & Cozzani 1979; Gedalia et al. 1991; Collys et al. 1993; Jaeggi & Lussi 1999; Attin et al. 2001).

Despite the controversial result found in literature, one relevant evidence of the importance of saliva in dental erosion development is that erosion may be associated with low salivary flow rate and low buffering capacity (Lussi & Jaeggi 2008). Additionally, saliva substitutes containing calcium and phosphate may be relevant to remineralize erosive altered enamel (Amaechi & Higham 2001).

In the *in situ* model developed in this study, the acquired pellicle played an important role in the results, bringing the results next to what occur clinically. There is a consensus in literature about the importance of the salivary pellicle in erosion prevention (Zahradnik et al. 1976; Nieuw Amerongen et al. 1987;

Meurman & Frank 1991; Amaechi et al. 1999; Hannig & Balz 1999; Hannig et al. 2004; Nekrashevych et al. 2004). The pellicle may behave as a diffusion barrier or a perm-selective membrane, preventing direct contact between acids and the tooth surface and thus inhibiting its demineralization (Amaechi et al. 1999; Hannig & Balz 1999). As the volunteers of this study were instructed to use the palatal appliance during day and night, there was enough time for the formation of a mature pellicle. The volunteers could put the palatal appliance in the citric acid between 6:00 - 9:00 am in the morning and between 18:00 - 21:00 pm in the afternoon/night. So there was a minimum of 15 hours for the developed of a mature pellicle, between the acid challenges. The time of developed pellicle is one important factor relating to its capacity to protect dental surface against acid attack. A developing young pellicle will hardly be a diffusion barrier to an erosive agent. Only when the pellicle has matured and has achieved a certain thickness can it slow down the diffusion process (Zero & Lussi 2005). The studies developed *in vitro*, loose this important biological factor.

In this study, the same protocol was performed in two different models: *in situ* and *in vitro*. During the time between the acid challenges, the samples were maintained in the oral cavity of the volunteers in the *in situ* model, and in the *in vitro model,* samples were stored in deionized water in ambient temperature. Curiously, despite the saliva effect on the samples regarding about remineralization, the *in situ* model always presented more tissue loss compared to the *in vitro* in all the times with all the groups (Graphic 4 and 5). These occurred probably due to the abrasion of the tongue. As the samples were fixed in the lateral sides of the palatal appliance, the tongue exert direct abrasion in the samples. These finding was in accordance with other studies that speculated that the tongue could be a possible biological factor, which modified tooth wear process (Holst & Lange 1939; Dugmore & Rock 2004; Lussi & Jaeggi 2008). Holst & Lange (1939), considered mechanical abrasion caused by the tongue to be a contributing factor in erosion caused by vomiting. Observations from animal studies also provide support in that beverages produced erosion mainly on the lingual surfaces teeth (Stephan 1966).

It could also be noted that standard deviation of the values of tissue loss (Graphic 4 and 5) in the *in situ* model are bigger that *in vitro* model. Despite the

fact that the same acid challenge was performed for both models for the same time, the *in situ* model presented assorted variations of the volunteers. All the volunteers presented salivary properties (pH, buffer capacity, flow rate) under the normal conditions, however, under the normality, there is a variation among them. Additionally there is a variation between the tongue strength of the volunteers. Some of them presented a bigger force in this muscle than others, so the abrasion in the samples were higher, consequently the tissue loss. So the samples of the *in situ* model in this study were also submitted to an abrasion force, differently from the *in vitro* model that was only submitted to erosion, and suffered no abrasion force.

Nowadays, efforts from researches all over the world are being made in order to develop a treatment that can convert the enamel more resistance to erosion. Fluoride has been the main field of the researches that aim to prevent dental erosion. As high intensity lasers are known to prevent caries, recently studies were performed verifying the effect of high intensity lasers in the prevention of dental erosion (Vlacic et al. 2007; Magalhaes et al. 2008; Rios et al. 2009; Sobral et al. 2009; Wiegand et al. 2009). As it has been demonstrated that CO_2 laser (λ= 10.6µm) is effective in changing the chemical composition and morphology of enamel and dentine, inhibition demineralization in both *in vitro* and *in situ* conditions (Nelson et al. 1987; Nammour et al. 1992; Featherstone et al. 1998; Hsu et al. 2000; Klein et al. 2005; Rodrigues et al. 2006; Steiner-Oliveira et al. 2006; Tagliaferro et al. 2007; Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2009) and as CO_2 seem appropriate for using in dental tissues, due to its high absorption in enamel and dentin substrates, this study tested a low-fluence of CO_2 laser irradiation parameters in erosion prevention *in situ* and *in vitro*.

In this study, in the *in situ* model, the treatment with laser with the parameters: 0.3J/cm^2; 5µs; 226Hz showed **48%** , **43%** and **31%** of enamel loss compared to control group (100%) in 1, 3 and 5 days respectively. Fluoride + Laser showed **74**%; **45**% and **50**% in 1, 3 and 5 days respectively and Fluoride **23**%, **18%** and **15**% in 1, 3 and 5 days respectively. The Laser and Fluoride + Laser groups presented a significant reduction as compared to control group. The Fluoride group showed the smallest capacity of enamel loss prevention, but this difference was not significant.

In the *in vitro* model developed, although the enamel loss was always reduced in comparison with the *in situ* model, due to several factors described below, the behavior of the groups maintain almost the same aspect of the is situ model. The treatment with laser *in vitro* showed **52,%** , **31%** and **37%** enamel loss compared to control group (100%) in 1, 3 and 5 days respectively. Fluoride + Laser showed **73**%; **55**% and **57**% in 1, 3 and 5 days respectively and Fluoride **28**%, **24%** and **29**% in 1, 3 and 5 days respectively. In this model, all treatments showed significant difference compared to control. The group Fluoride + Laser presented the best result *in vitro* to and the Fluoride group showed the smallest capacity of enamel loss prevention between all treatments, but in the *in vitro* model this difference was also significant. These results can also be seen in the 3D Profilometer images (Figure 9), according to the color scale, the zero point is referred to the yellow color and the highest the presence of a blue color, the deeper the lesion is. Lesions tending to be more yellow in color and therefore shallower can be seen in all days, in groups Laser and Fluoride + Laser especially in the middle of the lesion. The control group presented always the most blue cavity, followed by fluoride group.

So, it could be observed that *in situ*, where the conditions of the model are close to what happen clinically, laser can significant prevent enamel erosion in all times analyzed. When laser is associated with fluoride, the rate of prevention increased. Many studies have demonstrated the effect of irradiation with several lasers in the decrease of the solubility of tooth enamel, but until today the way this decrease occurs is not completely understood.

Three different theories have been proposed to explain the changes in enamel that result in this effect, and they are all related to the temperature increase in tissue. So far, it is not clear which of them has the predominant effect, but it seems that more than one of these changes happen at the same time and that the sum of its effects results in inhibition of demineralization. The first theory associates the reduction of the solubility of dental enamel with the melting and fusion of enamel. As in several studies the fusion and recrystalization of the surface of enamel was observed after irradiation, it was created the theory that the melted layer would lead to a decrease in the permeability of the acid ions and therefore the reduction of mineral dissolution (Stern & Sognnaes 1972; Stern et al. 1972; Lenz et al. 1982; Walsh & Perham

1991). But, the currently available evidence indicates that the fusion of enamel is not necessarily needed to have the inhibition of demineralization and that the changes in the fused enamel are not the most interesting to reduce its solubility.

Borggreven et al. (1980) by measuring the penetration of ionic and non ionic marker in enamel, showed an increase in the permeability of enamel in regions where fusion occurred after irradiation with CO_2 laser. Nelson et al. (1987) observed in infrared spectroscopy the formation of phosphate-tetra-calcium, which is a more soluble phase than enamel, after irradiation with 50 J/cm^2 and surface fusion (Nelson et al. 1987). In this study, with the parameters adopted, no melting areas could be seen in the scanning microscope images at 2000x magnification (Figure 12).

Moreover, it is known that the temperature in which the enamel surface begins to melt is around 1100°C, and fusion, around 1280°C, and that the changes of hydroxyapatite phase and changes in enamel composition result in increased solubility (Kuroda & Fowler 1984; Fowler & Kuroda 1986; Nelson et al. 1986; Nelson et al. 1987; Ellies et al. 1988).

The other two theories proposed to reduce the demineralization enamel are related to: crystallographic changes that make the mineral less soluble and the theory of blocking the ions diffusion from the decomposition of the organic matrix. The heating of the enamel causes changes in its composition and structure, and changes of hydroxyapatite phase. These changes are different at different temperatures and also the changes dissolution is different according to the increase of temperature. According to Fowler & Kuroda (1986) in the range of 100-650°C the heating of the enamel leads to decreased of water content, decomposition of proteins, partial loss of carbonate and formation of pyrophosphates. Between 650-1000°C, growth of crystals, formation of α-and ß-TCP, OH-reduction and elimination of carbonate are observed. Between 1100-1600 °C, the phases α -TCP and Tet-TCP can be observed with a further reduction of OH-. In two highly temperatures, the phases of hydroxyapatite formed present higher solubility product than the natural enamel, which increases its solubility. Between 100 and 650°C, less soluble phases are not produced and reduction of carbonate formation and pyrophosphate are known to decrease solubility of enamel, so this track has been described as interesting

for the changes induced by laser. However, the same authors showed evidence that in the range of 650-1000 °C occur the formation of a second phase of ß - TCP, which is more similar to the hydroxyapatite and contains fewer impurities than natural enamel.

At the same time, the content of carbonate and water are reduced and increased of OH-occur. These changes can also make the crystals more pure and will probably lead to the reduction of enamel solubility (Evans et al. 1980; Fowler & Kuroda 1986; LeGeros 1991; Bachmann & Zezell 2005; Ana et al. 2007). The carbonated hydroxyapatite present in the enamel contains impurities and the reduction of these impurities reduces its solubility. Therefore when only the reduction of carbonate is taken into account, the ideal increase of temperature of enamel should be between 800-900 °C, range that it can be find greater removal of impurities (Zuerlein et al. 1999).

A third theory described the influence of organic matrix decomposition in enamel solubility reduction. After the heating between 400 and 500°C of enamel, it was observed the decrease of its permeability and an 8 fold reduction of its solubility comparing to natural enamel (Sato 1983; Hsu et al. 1994). Considering that the protein decomposition of the enamel occurs at 350°C, there is the hypothesis that the organic matrix decomposition products can seal the enamel porous and blocked the entrance of the acid ions (Holcomb & Young 1980; Fowler & Kuroda 1986). This effect was shown in the study of Hsu et al. (2000) where CO_2 laser irradiation was performed in enamel samples containing organic matrix and also in enamel samples where the organic matrix was removed. In the samples containing organic matrix, the inhibition in caries progression, so in the demineralization, was bigger. The birefringence analysis showed that the temperature was not higher that 400°C. In this range of temperature, the water is eliminating from the tissue, and the obstructed porous are observed. Through the spectrometric of paramagnetic resonance the presence of products of organic matrix decomposition were observed at 400°C (Bachmann & Zezell 2005). Also supporting this theory, with Er: YAG laser, the reducing pores was demonstrated after irradiation by laser samples containing organic matrix and not in samples where the organic matrix was removed (Ying et al. 2004).

As noted, there is evidence to support all the theories and is likely that the effect is the result of the combination of them. The carbonate present as an impurity of hydroxyapatite crystals in enamel can be removed from the structure after heating between 400 and 800°C. As the pulse duration has a direct influence on the increase temperature, its influence in the loss of the carbonate structure is expected. This influence exists, and with the wavelength of 10.6 μm the reducing of the pulse duration from 100 to 2 μs reduces 50% the energy necessary for the removing the carbonate from the structure. The irradiation with 100 μs, the elimination happens with 6 J/cm^2 and 2 μs with 3 J/cm^2 (Fried et al. 1996; Zuerlein et al. 1999).

For these observations it is clear that irradiation with shorter pulse widths have greater potential to promote the increase of temperature necessary to make the less soluble hydroxyapatite, but also with a smaller increase of temperature in the deep layers close to the pulp.

Several studies demonstrated that laser energy can increase the resistance of tooth structure to mineral loss from organic acids involved in dental caries, but few studies are published regarding the use of high power lasers in dental erosion prevention. The first author who tested different high power lasers in the dental erosion prevention *in vitro* was Vlacic et al., (2007). The author tested *in vitro* different wavelength at $15J/cm^{-2}$ (Argon: 488, 514nm, KTP: 532nm, Diode laser: 633, 670, 830nm and Nd:YAG laser 1064nm) after neutral sodium fluoride gel application (1.23%). The enamel samples were exposed to an erosive challenge (1.0M HCl – 5 minutes). The author verified that all wavelength of laser light examined associated with fluoride provided a significant protective effect against softening compared with the negative control surfaces, showing significant increase in enamel hardness. Nevertheless, the author did not evaluate the temperature increase or the heat damages this fluence could cause to the tissue.

More recently, some other two studies also verified the effect of Nd:YAG Laser in erosion prevention *in vitro*. Magalhaes et al. (2008) tested different parameters of Nd:YAG and fluoridated agents used separately or together in dentin erosion reduction (G1: control; G2: Acidic phosphate fluoride, APF, for 4 minutes; G3: Fluoride varnish for 6 hours; G4: 0.5W Nd:YAG laser 250μs pulse,

10Hz, 35J/cm^2, 30 sec; G5: 0.75W Nd:YAG laser 52.5 J/cm^2; G6: 1W Nd:YAG laser 70J/cm2; G7: APF + 0.75W Nd:YAG laser; G8: NaF + 0.75W Nd:YAG laser) . They were treated and stored in artificial saliva for 24 hours and submitted to four erosive cycles of 1 minute in Sprite light®. Between the erosive attacks, the samples were maintained in artificial saliva for 59 minutes for remineralization. The erosive wear was evaluated by profilometry. The results showed that laser irradiation was not able to reduce dentin erosion and fluoride application was able to increase the dentin´s resistance to erosion, and APF showed better results than fluoride varnish. Maybe the results obtained are due to the low absorption of Nd:YAG laser with dental tissue.

Rios et al (2009) tested Nd:YAG laser to with the same parameters and methodology of the study from Magalhães et al (2008) in enamel. The enamel wear was measured by profilometry. Regarding to enamel the authors concluded that the association between APF application and laser irradiation seems to be an alternative preventive measure against erosion. Maybe the results obtained are because of preventive effect of fluoride. Additionally, no abrasion were induced in the testing model.

Sobral et al., (2009) also verified the effect of Nd:YAG laser and fluoride in prevention of bovine (1W; 100mJ; 10Hz; 141.5J/cm^2) and human (1W; 100mJ; 10Hz; 125J/cm^2) enamel *in vitro*. Nevertheless, the author used a pigment to increase photo absorption. The Nd:YAG laser irradiation associated with fluoride in both human and bovine enamel was more effective and yielded statistically significant results for surface microhardness and enamel wear.

As CO_2 laser provides a very efficient interaction with hydroxiapatite (Fox et al. 1992; Fried et al. 1997; Featherstone et al. 1998; Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2008; Esteves-Oliveira et al. 2009), this laser is expected to present better results in erosion prevention, in comparison of the previous studies described.

The first study that verify the effect of CO_2 (10.6µm) laser in erosion prevention analyzed the influence of CO_2 (10.6µm) laser irradiation on the efficacy of titanium tetrafluoride (TiF_4) and amine fluoride (AmF) in protecting enamel and dentin against erosion when applied before or during laser irradiation in vitro (Wiegand et al. 2009). The author tested CO_2 laser (10.6µm,

10μs pulse duration, 50Hz frequency, energy density of 28.6J/cm^2 for enamel and 15J/cm^2 for dentin). The results showed that under the conditions tested AmF reduced enamel and dentin erosion but CO_2 laser did not enhance its efficacy. TIF_4 showed only a limited capacity to prevent erosion, but CO_2 laser irradiation enhanced its efficacy to reduce enamel loss when applied during the application of TiF_4. According to the author, based on SEM pictures, CO_2 laser irradiation induced a melting of the granular surface precipitates, which might result in greater uptake and retention of titanium, fluoride, or both, leading to greater acid resistance of enamel. In contrast CO_2 laser irradiation before TIF_4 application did not lead to visible changes of the surface precipitates. The CO_2 laser irradiation alone did not lead to any improvement of enamel or dentin acid resistance.

Steiner-Oliveira et al. (2009), also verified if CO_2 laser (10.6μm) treatment in combination (or not) with previous fluoride gel application could increase the resistance of bovine enamel and dentin to erosion, through successive erosive challenges. The authors used the following laser parameters for enamel: 1J/cm^2; 3W; 5ms pulse duration, 10Hz and for dentin: 0.6J/cm^2; 2W; 5ms pulse duration; 2Hz. The samples were submitted to the following treatments: Control (no treatment), Fluoride, Laser, Fluoride + Laser. The treated specimens were submitted to demineralization (0.3% citric acid, for 5 minutes) and remineralization in artificial saliva for 60 minutes. The cycle was repeated 3 times a day, for 3 days. The authors verified that treatment with laser alone was not able to prevent enamel or dentin surface losses due to erosion. The association between fluoride and laser showed some protection, but mostly due to the fluoride effect. No significant synergistic interaction or long-term protection could be observed for the fluoride-laser combined therapy.

Both studies cited above did not verified any benefit of CO_2 laser in erosion prevention, despite the efficient interaction of its wavelength with enamel and the previous good results published in the field of caries prevention. Probably the parameters adopted were not suitable for demineralization prevention.

The parameters adopted in the present study was based in a previous research (Esteves-Oliveira et al. 2009), where was verified that the parameter

of 0.3J/cm^2; 5µs pulse duration, 226Hz could increase enamel caries resistance in 80% without causing undesirable surface damaging and excessive temperature rise.

The present study was the first developed *in situ* with this set of parameters. *In situ*, a significant difference was obtained with both groups Laser and Fluoride + Laser in all times tested. The highest protection was obtained in association of Fluoride and Laser. The differences in the results found between the published studies regarding CO_2 laser and erosion may be especially due to the parameters used. Esteves-Oliveira et al., (2009) tested several parameters of CO_2 (10.6µm) laser in caries prevention and not necessary the highest energy or highest pulse duration were the most effective. With shorter pulses, a greater rise in surface temperature can be expected and with longer a minor increase in the surface, but with greater spread of heat into the interior of the tissue (Fried et al. 1996). The studies that did not reach satisfactory results with CO_2 laser (10.6µm) (Steiner-Oliveira et al. ; Wiegand et al. 2009) used higher fluencies and pulse durations as compared to the present study. The present study also verified that the demineralization prevention inherent of CO_2 laser irradiation, seems to be associated with low-fluence and short pulse durations, conforming the results obtained by the authors Esteves-Oliveira, et la. (2009).

In the present study the fluoride seems to increase the result obtained with laser. In the present study, both the *in vitro* and *in situ* model, the group fluoride + laser group presented the lowest erosion depth (Graphics 4 and 5). In other studies a synergism between laser and fluoride in the reduction of enamel solubility has been shown (Hsu et al. 1998). Until now, mechanisms of combined effects of laser and fluoride remained unclear. There are currently at least two speculations related to two types of fluoride formation induced by laser treatment. One was suggested by scanning electron microscopic study, which demonstrated that laser-fluoride (Nd:YAG) treatment produced numerous spherical or globular precipitates that morphologically resembled calcium fluoride-like deposits on root surfaces (Zhang et al. 1996). These fluoride precipitates may serve as reservoirs to replenish fluoride used up during periodic episodes of demineralization (Haider et al. 1999). Another speculation, supported by a few experiments, emphasized the role of lasers on enhancing fluoride uptake into the tooth crystalline structure in the form of firmly-bound

fluoride (Goodman & Kaufman 1977; Zhang et al. 1996; Meurman et al. 1997). Using synthetic hidroxyapatite, it has been shown that CO_2 laser treatment could even transform hydroxyapatite (HA) into fluorapatite (AF) in the presence of fluoride (Meurman et al. 1997). Stephen et al (2004) also concluded through secondary mass spectrometry (SIMS), that laser increased fluoride uptake into enamel *in vitro*. In the present study the ion-specific electrode was used to analyze the fluoride concentration in the solution after partially dissolution of enamel with HCL. Only in day 1 the groups: laser + fluoride and fluoride showed a significant more amount of fluoride than the other groups. In day 3 these groups present non significant higher levels of fluoride, and in day 5 the values were very similar to control group. So it could be observed that the increase fluoride found in these groups could not be maintained for longer periods.

It has been suggested that laser-induced uptake of fluoride into the hydroxyapatite structure may be energy dependent (Hattab 1987). It was already reported that for the phase transformation of synthetic hydroxyapatite to fluorapatite, the minimal energy density was reported to be $38 J/cm^2$ (Meurman et al. 1997). Nevertheless, it was showed that greater laser energy may not necessarily result in greater fluorite uptake (Chin-Ying et al. 2004). Probably the results found in the present study indicate that only one topical application did not present a longer action in demineralization prevention. The EDX analysis was performed immediately after the irradiation in all groups. This technique could only detect significant fluoride in the group fluoride + laser. So, the presence of a significant higher levels of fluoride could be detected by two methodologies in the present study in short periods after the treatments (immediately after and 1 day in situ), but after 3 days this difference is not significant.

In the present study, the fluoride alone could prevent enamel loss only in the *in vitro* model (Graphic 5). The same result was obtained in the study of Wiegand et al. (2009), with amine fluoride. Both studies were developed *in vitro*, without any abrasion of the surface. It can be speculated that amine fluoride could present a protective effect in erosion, but only when no abrasion occur. In the present study, in the *in situ* model, where the abrasion of the tongue exists, fluoride presented no significant protection against erosion.

Fluoride is the main agent used to enhance remineralization. However, in literature, to control mineral loss caused by erosion, high concentrations and frequencies seem to be needed (Serra et al. 2009). The impact of highly fluoride concentration treatment on the progression of enamel erosion has been analyzed in several studies. The action of fluoride is mainly attributed to a precipitation of CaF_2 on eroded dental surfaces (Ganss et al. 2004; Ganss et al. 2004). The formation of the CaF_2 and its protective effect on demineralization depend on the pH, F concentration and type of F salt of the agent (Saxegaard & Rolla 1988).

However, the role of fluoride application on the prevention of dental erosion is still controversially discussed (Saxegaard & Rolla 1988). Using different approaches, several groups demonstrated increased wear resistance after the application of a highly fluoridated gel with a pH of 4.75 on erosive damages enamel (Attin, Deifuss et al. 1999; Ganss, Klimek et al. 2001; Lussi, Jaeggi et al. 2004). It has also been shown *in vitro* that fluoride treatment was unlikely to provide a preventive effect against erosion, because an acidic drink will rapidly dissolve accessible CaF_2 ad remove traces of previous topical fluoride treatment (Larsen & Richards 2002; Ganss et al. 2007). In the present study, in the *in situ* model fluoride alone did not show any significant prevention in erosion in any evaluated time. It could be speculated that, in the experimental model developed in this study, fluoride could not prevent enamel loss, when it was submitted to abrasion forces as the tongue. When no abrasion force was induced in the samples, as in the *in vitro* model, fluoride presented a significant protection against enamel erosion. In literature a big range of *in vitro* studies tested the capability of different fluoride compounds and concentrations in erosion prevention with significant results (Magalhães et al., 2009; Wiegand et al., 2009; Steiner-Oliveira et al., 2009; Rio et al., 2009), but when abrasion is incorporate the results seems not to be so good (Rios et al., 2008). In literature, fluoride has not been conclusively proven to prevent erosion (Larsen & Richards 2002).

The morphology of the samples seen in Figures 13, 14 and 15 showed characteristically aspect of eroded surfaces already described in literature (Meurman & Frank 1991). It is known that in prismatic human enamel, the progression of erosion follow the order where the prism sheath areas are

affected first and thereafter, with a more severe challenge, also the prisms cores (Meurman & Frank 1991). In day 1 (Figure 13), all the groups presented irregular enamel surface, due to citric acid demineralization. The group Fluoride + Laser seems to preserve to some extent the enamel prisms cores more than the other groups (2000 x magnification). In day 3, the groups also presented enamel irregular surfaces with porosities, typical from erosion. It's more apparent, than group Fluoride + Laser could preserve big amount of prism cores, whereas the prism sheats suffered dissolution. In this group it can also be noted that the enamel surface presented the highest irregular surface. Considering that these groups presented the lowest depth erosion cavity, analyzed through profilometry, the more irregular surface can be directly related to abrasion resistance. As other groups presented less capability of abrasion resistance, its surface is not so irregular, due to removal of altered superficial prism layer. In day 5, the groups: laser and fluoride + laser presented the most irregular surfaces, probably due to better capacity of abrasion resistance and also the exhibition patterns of core prisms more conserved that the other groups. Nevertheless, the majority of studies that analyzed the morphology of enamel after erosion was developed *in vitro*. No of the few in *situ studies*, developed a similar methodology tested in this study.

In vitro, Meurman & Frank (1991), (Meurman & Frank 1991) compared the pattern of eroded human and bovine enamel. Both presented the same characteristic dissolution, where initial erosion after 15 minutes or 30 minutes immersion in citric acid was seen to affect specifically the prism sheath areas, with visible dissolution. Longer immersion caused dissolution of enamel prism cores, as observed in this study to.

Lippert et al., (2004) verified a prismatic structure of enamel with grainy surface through atomic force microscopy that after 2 minutes in citric acid. The author also verified that exposure to artificial saliva led to the deposition of mineral phase. Previous research (Eisenburger et al. 2001) proposed in this context that this grainy surface appearance is due to the presence of an amorphous mineral layer of calcium phosphate phase, which is formed after enamel samples suffer an erosive attack. This could explains the surface structure of softened samples observed by the author, and the loss of this surface feature after tooth brushing, since this mineral layer is only weakly

bound to the underlying enamel surface (Eisenburger et al. 2001). In the present study no difference between the groups could be seen in higher magnification (60,000X). No mineral grainy surface structure could be seen with the scanning electron microscopy. The *in situ* study from Sauro *et al.*, (2008) verified the enamel surface after the scanning electron microscopy replica technique. The study tried to mimic what happen clinically, as the volunteers maintained 50ml of beverages containing citric acid for 4 minutes in oral cavity. The SEM pictures showed several porosities in demineralized enamel, and characterized by demineralization of the prism cores. The author linked the intense demineralization with the pronounced erosive potential of citric acid, due to the fact that citric acid acts as a chelator, binding minerals such as calcium. The study also did not allow time for the samples to stay in contact with saliva. The volunteers only maintained the beverage in oral cavity during 4 minutes, after this time the samples were direct analyzed through scanning electron microscopy replica technique. In the present study, although, the acid challenge proposed was longer, the samples could suffer saliva remineralization, as well as, influence of other biological factors.

Rios et al., (2008) developed an *in situ* study, where the volunteers immersed the device for 5 minutes in 150 ml of cola drink, 4 times per day, during 7 days. After the acid challenge some samples were brushed using a fluoride dentifrice. The SEM analysis showed enamel prism core dissolution on the surfaces submitted to erosion, while on those submitted to erosion and abrasion a more homogeneous enamel surface was observed, probably due to the removal of the altered superficial prism layer. Although the difference in acid challenge, this pattern could also be seen in the present study particularly in day 3 and 5 (Figure 14 and 15) in the group fluoride + laser.

None of SEM pictures in all magnifications could detect calcium fluoride-like deposits as other author did (Chin-Ying et al. 2004). The higher magnification (60,000X) was also provided in order to visualize any fluoride globular precipitates (Duschner et al. 1997). Probably no fluoride precipitate was observed because only one fluoride application was performed in the groups: fluoride or fluoride + laser. Precipitates were observed on the tooth surfaces in literature as a result of numerous applications, mostly described as globular entities with a diameter from 4-15 nm (Nelson et al. 1984) up to 1µm

(Saxegaard & Rolla 1989) . The distribution density of the globules on the surfaces and their size was reported to depend on the concentration of fluoride and on the pH of the applied agent (Arends et al. 1988; Saxegaard & Rolla 1988). The precipitate was characterized chemically as calcium fluoride-like material, which also consisted of surface-adsorbed phosphates (Saxegaard et al. 1988). In his study, Duschner *et al*. (1997) could verify fluoride globules, when longer time application was performed. The time used by the author (72h in contact with neutral NaF) is not viable clinically.

So, until now no treatments seem to completely preventive dental erosion. The CO_2 laser has shown promising results, especially when associated with fluoride. The low-fluence used can probably be safe for *in vivo* usage, but additional studies should be performed to confirm its safety in clinical use. Fluoride, with high concentrations, is not recommended clinically, a barrier found in this kind of therapy. So, the results found in this study could be an indicative of CO_2 laser use in erosion prevention. Other associations regarding to the frequency of fluoride application, laser irradiation, association with other fluoride compounds should also be tested for the obtaining of any improvement of the results.

6. CONCLUSIONS

Under the conditions of the *in situ* model, in which several variables act directly on the samples, it could be concluded that treatment with CO_2 laser (ʎ = 10.6 mm) associated with fluoride was able to cause a significant prevention of enamel erosion at all analyzed times both in relation to control as well as to fluoride group. The treatment with CO_2 laser alone showed significant prevention in relation to the control group only, in all investigated times. On the other hand, topic application of amine fluoride (1,25% of F^-) did not caused significant reduction of erosive enamel surface loss

In the *in vitro* model, it could be concluded that the treatment with CO_2 laser (ʎ = 10.6 mm) with or without fluoride, and fluoride alone resulted in significant erosion prevention of tooth enamel in all analyzed times.

Amine-Fluoride gel used in this study could only lead to erosion prevention *in vitro*. *In situ*, the amine-fluoride topic application was not effective. So, amine-fluoride has a potential to prevent erosion only when no abrasion is submitted to the tissue.

ABSTRACT (ZUSAMMENFASSUNG)

Dental erosion is increasing as consequence of changing habits from the population, as well as increase of prevalence gastric reflux or vomiting. The possibility of making dental enamel more resistance to erosion is the objective of this study. 10 volunteers participated in the *in situ* study, in a crossover design with 4 treatments (G1 – control, no treatment; G2 – CO_2 laser irradiation; 0.3J/cm^2-5µs-226Hz; G3 – topical fluoride treatment – 1.25% - 3 min; G4 - CO_2 laser + fluoride treatment). For each treatment the volunteers used palatal appliances containing fixed sterilized bovine enamel samples during day and night except for meals. For erosive demineralization the mouth appliances were immersed extra-orally in 80ml of 0.05M citric acid (pH 2.3) for 20 minutes twice daily. Individual oral hygiene was performed with fluoride-free products and without the appliance *in situ*. Before and between the treatment periods, a 1 week wash out period was included. Two samples were collected from the appliances for analysis on days 1, 3 and 5 (n=20/day/treatment). Surface loss was measured by digital profilometer. Additional fluoride measures, morphological analyses and EDX analyses were performed. For the *in vitro* model all the procedures were repeated, but instead of maintaining the samples in oral cavity, they were maintained for the same time in deionized water (n=12/day/treatment) and the surface loss was analyzed by digital profilometer. The results showed that he groups laser and fluoride + laser presented significant lower surface loss in all times both *in situ* and *in vitro* models. Fluoride presented significant surface loss only in the *in vitro* model. The EDX analysis, showed that fluoride + laser group presented significant more fluoride than the others groups, and the fluoride measurements of the samples showed that only in the first day the groups fluoride and fluoride + laser presented significant more fluoride that the other groups. CO_2 laser irradiation at 0.3J/cm^2 (5µs, 226Hz) associated or not with fluoride decreases enamel erosive surface loss caused by citric acid, *in situ* and *in vitro*. This effect is still observed after 5 days of repeated acid attacks.

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Hiermit erkläre ich, dass die dieser Disseration zu Grunde liegenden Originaldaten bei mir, Karen Müller Ramalho, Rua Cayowaa - 2251 ap 34, 01258-011- São Paulo, Brasilien hinterlegt sind.