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EVALUATION OF THE EFFECT OF SPORTS DRINKS AND TOOTH BLEACHING IN TOOTH ENAMEL MICROHARDNESS

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LIST OF ABBREVIATIONS

CP – Carbamide Peroxide

IS – Isostar

IC – IsoCarb

KHN – Knoop Microhardness Number

NB – No Bleaching

ND – No Drink

UR – Ultra Recovery

VHN – Vickers Microhardness Number

WP – Whey Protein.

ABSTRACT

Objectives. The aim of this in vitro study was to evaluate the effect of four sports drinks and their combination with tooth bleaching in tooth enamel microhardness.

Material and Methods. Fifty samples with enamel surface were obtained from 25 human teeth. The samples were randomly assigned to 10 experimental groups (n=5) according to the several possible combinations of sports drinks (control – no drink; Isostar; Isocarb; 100% Whey Protein; Ultra Recovery) and tooth bleaching (control - no bleaching; 16% carbamide peroxide). The samples from each group were subjected to 14 immersion cycles in the respective sport drink for 60 minutes (1 cycle per day) intercalated with immersion in saliva. The bleaching agent was applied for 4 hours, after each immersion cycle. Vickers microhardness tests were performed with a microdurometer (0.98 N; 10 seconds) at 3 time points [initial measurement before the 14 cycles - baseline (T0); immediately after the 14 cycles (T1); and 24 hours after the 14 cycles (T2)].
The Vickers Microhardness Numbers (VHN) were statistically analysed using non-parametric tests of Friedman, Mann-Whitney and Kruskal-Wallis (alpha = 0.05).

Results. The Vickers Microhardness Numbers (VHN) showed a statistically significant decrease from T0 to T1 and T2 ($p<0.001$), and there were no significant changes from T1 to T2 ($p=1.000$). The VHN was statistically significant influenced ($p<0.001$) by the immersion in sports drinks, both in T1 and T2. The immersion in isotonic drinks, Isostar and Isocarb, resulted in a decrease ($p<0.05$) in VHN, when compared with the control group. The results obtained after immersion in protein supplements, 100% Whey Protein and Ultra Recovery, were statistically ($p>0.05$) similar to those obtained in the control group. Exposing enamel to 16% carbamide peroxide did not influence VHN, neither in T1 ($p=0.635$), neither in T2 ($p=0.915$).

Conclusions. Exposure to isotonic drinks had a negative impact on tooth enamel microhardness. However, tooth enamel microhardness was not affected by exposure to protein supplements. Tooth bleaching had no impact on enamel microhardness. After the 14 immersion cycles, exposing the tooth enamel 24 hours to saliva was not enough to recover tooth enamel microhardness.

Key words: “Dental Enamel”, “Hardness Tests”, “Isotonic Drinks”, “Protein”, “Tooth Bleaching Agents”, “Athletes”.

RESUMO

Objetivos. O objetivo do estudo *in vitro* foi avaliar o efeito de quatro bebidas desportivas e da sua conjugação com branqueamento dentário na microdureza do esmalte dentário.

Material e Métodos. A partir de 25 dentes humanos foram obtidos 50 espécimes com superfície em esmalte. Os espécimes foram distribuídos aleatoriamente por 10 grupos experimentais (n=5) de acordo com as várias combinações possíveis entre bebida desportiva (controlo sem bebida; Isostar; Isocarb; 100% Whey Protein; Ultra Recovery) e o branqueamento dentário (controlo sem branqueamento; branqueamento com peróxido de carbamida a 16%). Os espécimes de cada grupo foram sujeitos a 14 ciclos de imersão na respetiva bebida durante 60 minutos (1 ciclo por dia) intercalados com imersão em saliva. A exposição ao agente branqueador foi realizada após cada um dos ciclos de imersão, durante 4 horas. Os testes de microdureza Vickers foram realizados com um microdurometro (0,98 N; 10 segundos) em 3 momentos [medição inicial antes dos 14 ciclos (T0); imediatamente após o término dos 14 ciclos (T1); e 24 horas após o término dos 14 ciclos (T2)].
Os valores de microdureza Vickers foram analisados estatisticamente com testes não paramétricos de Friedman, Mann-Whitney e Kruskal-Wallis (alfa=0,05).

Resultados. Os valores de microdureza Vickers sofreram uma diminuição estatisticamente significativa de T1 para T2 ($p<0,001$), mantendo-se sem alterações significativas de T1 para T2 ($p=1,000$). Os valores de microdureza Vickers foram influenciados de forma estatisticamente significativa ($p<0,001$) pela imersão em bebida desportiva, tanto em T1 como em T2. A imersão nas bebidas isotónicas, Isostar e Isocarb, conduziu a uma diminuição ($p<0,05$) dos valores de microdureza Vickers, relativamente ao grupo de controlo. Os resultados obtidos após imersão nos suplementos proteicos, 100% Whey Protein e Ultra Recovery, foram estatisticamente ($p>0,05$) semelhantes aos obtidos no grupo controlo. A exposição ao peróxido de carbamida 16% não influenciou os valores de microdureza Vickers, nem em T1 ($p=0,635$) nem em T2 ($p=0,915$).

Conclusão. A imersão nas bebidas isotónicas teve um impacto negativo na microdureza do esmalte dentário. No entanto, a microdureza do esmalte dentário não foi afetada pela imersão nos suplementos proteicos. O branqueamento dentário não teve impacto na microdureza do esmalte. Após os 14 ciclos de imersão/ exposição à bebida/branqueamento, 24 horas de imersão do esmalte dentário em saliva não permitiu recuperar a microdureza do esmalte dentário.

Palavras-Chave: “Esmalte Dentário”, “Teste de Microdureza”, “Bebidas Isotónicas”, “Proteína”, “Produtos de Branqueamento Dentário” “Atletas”.

PREAMBLE

Nowadays, in many societies, combining whiter smiles with fitter bodies is one of the main goals of most people.

Usually, whiter smile requires a tooth bleaching treatment and a fitter body requires physical exercise, which requires hydration and supplementation. The needs to ingest isotonic drinks to ensure hydration and protein supplements to fulfil body needs is higher with the increase of the frequency and intensity of the exercises.

Many marketing strategies stress the importance of frequent fluid replacement and supplementation during and after exercising.

In the end, many people combine all that type of sports drinks with a tooth bleaching treatment.

So, evaluate the potential effect of combining tooth bleaching and the ingestion of isotonic drinks or protein supplements in tooth enamel can be of great importance. Enamel changes must be evaluated after consumption of isotonic drinks or protein supplements and after the combination of consumption those sports drinks and tooth bleaching.

1. INTRODUCTION

1.1. THEORETICAL FRAMEWORK

Dental aesthetics is of great importance for majority of the people, including tooth colour. Any discolouration or staining may impact quality of life negatively. The tooth colour reflects a combination of its intrinsic colour and the presence of extrinsic stains due to various factors such as smoking, intake of tannin rich foods and drinks, particular drinks such as tea and coffee and the use of chlorhexidine or metal salts such as tin and iron [1,2].

The increasing demand for a whiter smile, has made tooth bleaching, also referred as tooth whitening, an increasingly popular dental treatment [1,2]. It is minimally invasive and highly effective if properly used by the patients and supervised by a professional. Bleaching agents, such as carbamide peroxide, are unstable, releasing oxygen free radicals when in contact with dental tissues and oral moisture and promoting oxidation of pigments present in the tooth while making it clearer [3].

However, even when obtaining satisfactory clinical results, some clinicians have still expressed concerns about possible adverse effects produced during and after the tooth bleaching treatment on the oral tissues [3].

Scientific evidence indicate that enamel can show changes in its structure when exposed to CP, affecting its composition, its morphology and causing surface changes while decreasing teeth microhardness [3].

The desire for whiter smiles can match with the desire for a fitter body, which leads to reach a better general appearance. A fitter body requires physical exercise, which requires hydration and supplementation [4,5].

Sports researchers and nutrition specialists are stressing the importance of adding carbohydrate and salt to water and encouraging competitors to drink more during exercise. The benefits of ingesting various mixtures of water, carbohydrate and electrolytes can be expressed through improved performance and/or reduced physiological stress, on cardiovascular, central nervous and muscular systems [4,5].

Isotonic drinks are specially formulated carbohydrate–electrolyte products designed to provide fast rehydration and a better performance, with supplements and minerals which are lost through sweating during physical activity [6].

The guidelines recommend drinking small amounts of the isotonic drinks before, during and after training. As the osmotic pressure of isotonic drinks is similar to the osmotic pressure of body fluids, proper absorption of fluid is assured, resulting in a rapid quenching of thirst without burdening the stomach [7,8].

Protein supplements are also a frequent choice to athlete's diets, once physical exercise significantly increases the daily protein requirement and are beyond collectively marketed as a supplemental nurture for athletes [9-12]. Protein or amino acid supply is closely associated with muscle mass gain or loss, and oral solutions are a practical way of delivering supplements designed to promote

muscle protein anabolism in exercising subjects [13].

Apart from the benefits of sports drinks, the erosive potential is one of the main concerns [5-7,14,15]. Adding calcium [16], increasing pH [17] or adding ingredients such as casein phosphopeptide-stabilized amorphous calcium phosphate [7,18] are among the attempts to reduce de erosive potential of sports drinks.

Hence, athletes can be regarded as a group particularly prone to dental erosion and changes in tooth enamel microhardness. The aetiology of erosion lesions is complex, it is a pathological, chronic, localized loss of dental hard tissue due to chemical processes, not involving bacteria. The acids responsible for erosion are originate from intrinsic factors or diet, such as isotonic drinks and protein supplements [19,20]. Studies have identified the consumption of acidic carbonated and non-carbonated drinks as one of the main causes of dental erosion [14,21].

The reduction in the amount of saliva, which is an important buffer of acids in the mouth, during physical activity and the destructive habit of holding drink in the mouth allowing it to bath teeth or swishing between the teeth for several minutes before swallowing prolongs the sports drinks exposure, increasing the risk of erosion [20,21].

The effect of tooth bleaching and the consumption of sports drinks on dental hard tissues has long been a concern because their reaction with the organic and inorganic components of the enamel may cause chemical, structural and mechanical changes on the enamel surfaces. A decrease in enamel microhardness reflects the loss of mineral contents, or demineralization, which is a pathological process. Enamel demineralization contributes to the aetiology of caries and non-carious lesions such as dental erosion, abrasion and attrition [19-24].

1.2. LITERATURE REVIEW

To support the in vitro study, was made an electronic bibliographic research using the primary databases PubMed / MEDLINE, supplemented with cross-sectional articles. The following inclusion criteria were respected: Publications between 1980-2018, in English and Portuguese.

In the PubMed / MEDLINE database through the use of keywords combination “tooth bleaching”, “sports drinks”, “enamel microhardness”, were obtained 1451 articles, after elimination of repeated articles. By reading de titles and summaries, were selected 33 articles, to which 34 articles were added by cross-search. Making a total of 67 articles.

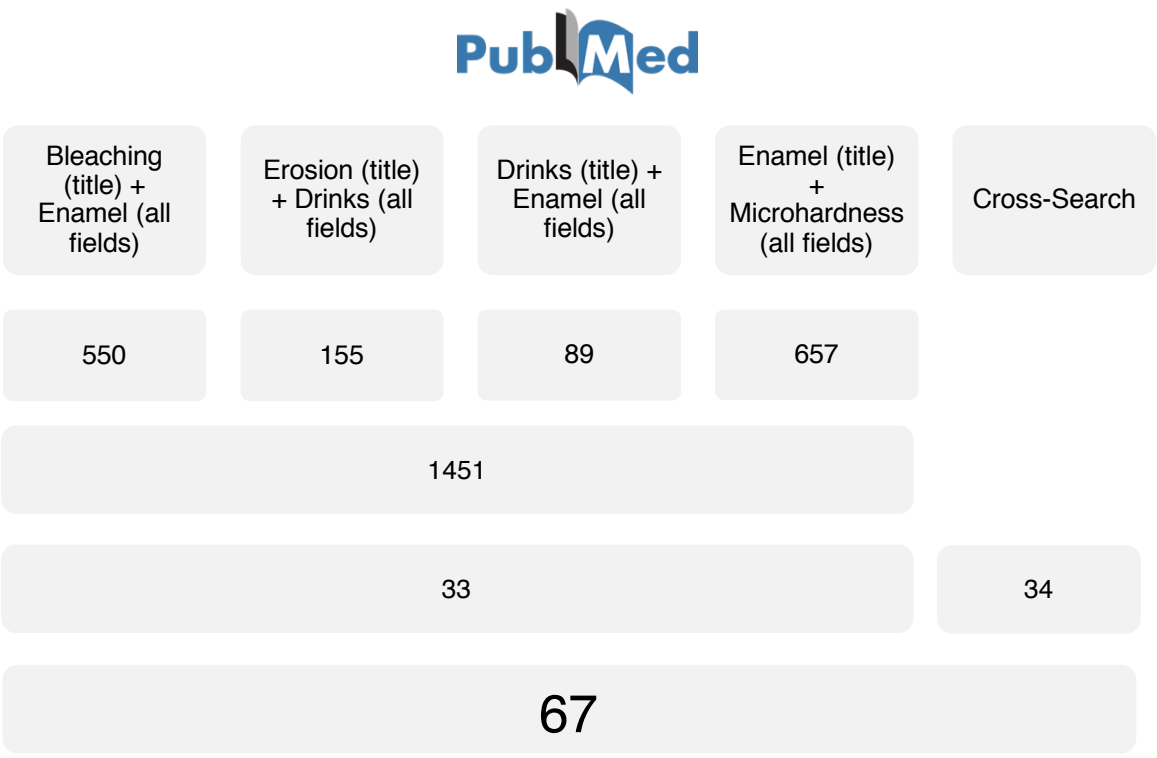

Figure 1. Electronic bibliographic research.

1.2.1. Dental Enamel

Dental enamel, as the hardest tissue in the body, can withstand a wide range of functional and non-functional loads and acts as a protective covering of teeth. Enamel is a hierarchical biocomposite made of 94–96% inorganic content, 1% organic matrix and 4–5% water [25,26].

Enamel is formed by long thin hydroxyapatite crystals (inorganic content) tightly packed together with a protein glue (hydrophobic enamelin). These rods are encapsulated by a thin protein-rich organic sheath (primarily hydrophilic ameloblastin) around 0.8–1 µm thick [27].

Organic contents play a significant role in determining the mechanical behaviour of enamel. This organic content helps to define three-dimensional cleavage planes to deflect cracks which prevent fracture from progressing through enamel and allows limited movement between the rods during stress [28,29].

The morphological alterations of enamel surface after sports drinks consumption and tooth bleaching can be seen in the decrease of surface roughness, microhardness, modulus of elasticity, fracture toughness, wear resistance, erosion, mineral content and in changes of the chemical composition of enamel [7,25]. These changes in the mechanical properties of enamel could be due to both, loss of mineral content as well as denaturation and degradation of the organic matrix by the oxidation reaction [7,25].

Microhardness tests are used to study the physical properties of materials, and they are widely used to measure tooth enamel microhardness. This method is easy, quick, and requires only a tiny area of sample surface for testing. The sample surfaces are impressed with a diamond indenter (Knoop or Vickers) at a certain load for a certain period of time. After load removal, diagonals of the indentation are measured with an optical microscope [24,30].

The microhardness number is defined by the ratio between the indentation load and the area of the impression, which depended on the indenter shape. Then the microhardness of materials is calculated using these equations: KHN=14230 (F/d^2) for Knoop microhardness or VHN=1854 (F/d^2) for Vickers microhardness. The constant value of each equation is calculated from the specific geometry of the indenter, F is the indentation load (N), and d is the diagonal of the indentation (μm) [24,31].

From the Knoop microhardness and Vickers microhardness equations, the tooth microhardness value should be constant when loads are varied, because the indentation size increases with an increase in load. However, studies of microhardness results on a wide range of loads have shown that results are not constant at very low loads. This characteristic can be attributed to elastic recovery or the viscoelastic nature, the grain size effect, indentation cracks, surface texture, or diagonal measurement errors. A high load produces a large impression, and it is easier to measure the indentation diagonal [24,31].

Enamel and dentin have specific microstructures, thus their microhardness may depend upon indentation loads or times. There is no standard condition for enamel and dentin microhardness testing, therefore, selection of testing conditions depended on the researcher's decision [24,31].

In tooth microhardness studies the Vickers indenter is more useful than the Knoop because a square shape must always be conserved, and because the indentation produced on a non-flat surface, or by the difference in microhardness of enamel and dentin, is easily detected. The tooth enamel microhardness has been reported in the range of 314 to 361 for KHN or 322 to 353 for VHN [24,31].

Test loads of 0.98 N, 1.96 N and 2.94 N are the most used in previous studies. A load of 2.94 N for enamel produced a Knoop indentation diagonal of approximately 100 μm, while the 400× magnification of the attached optical microscope can measure a maximum length of about 300 μm. A higher load may be impractical for a softer surface in the pre-post experiment because, after treatment, it produces a larger impression than the optical microscope can measure. The lowest loads, 0.98 N for enamel create Vickers diagonals longer than 20 μm, which is recommended to prevent errors in optical measurement [24,31].

The difference of loading times (10, 20 and 30 seconds) is not significant for either enamel or dentin. This suggests that an indentation time of 10 seconds is sufficient for a permanent indentation on the tooth surface to take place [24,31].

Decrease in tooth microhardness reflects loss of mineral in the enamel but may have been not enough to induce visible changes in surface morphology [24,30].

The saliva used for in vitro studies should has a known composition to standardize the procedure of a test. It is important to understand that it is impossible to obtain an artificial saliva that reproduces exactly the same characteristics of human saliva, which is very inconsistent and unstable.

Natural saliva is a mixture of fluids secreted by the parotid, submaxillary and sublingual glands that have been shown to differ from each other in composition and in volume, with numerous constituents and variable according to the time of day. However, artificial saliva may have a similar composition to natural saliva, that's why has been used in numerous studies in odontology. The SAGF (Artificial Saliva Gal Fovet-SAGF) is a reference, a solution without protein and organic compounds (except for urea and/or organic acids for pH adjustment) which can be used as storage medium [32-35].

1.2.2. Sports Drinks

In order to maintain body temperature within narrow physiological limits, the metabolic heat generated by exercise must be dissipated. When environmental temperature exceeds skin temperature, heat loss can occur only by evaporation of sweat from skin surface. Although, significant rates of sweat production also occur in a cool environment if the work rate is high. Trained acclimated individuals exercising in warm, humid conditions can maintain sweat rates exceeding 2 $l.h^{-1}$ for many hours [36].

The water content of sweat is derived from all compartments, the free exchange of water among body fluid compartments ensures that, with the distribution being influenced by sweat rate, sweat composition, total water and electrolyte loss [36].

The primary cation lost in sweat is sodium, with typical concentrations of about 40-60 $mmol.l^{-1}$, compared with about 4-8 $mmol.l^{-1}$ for potassium. Given the higher sodium loss and the distribution of these cations between body water compartments, the primary water loss is likely to be from extracellular space [36].

Exercise capacity is influenced by the decrease of plasma volume that accompanies dehydration. Blood flow to the muscles must be maintained at a high level during exercise to supply oxygen and substrates, however, a high blood flow to the skin is also necessary to transfer heat to the body surface where it can be dissipated [36].

Hipohydration is associated with higher cardiovascular strain, impaired thermoregulation and with loss of protection conferred by acclimation. Loss of intracellular volume may be particularly important during recovery, however, a reduced intracellular volume can reduce the rates of glycogen and protein synthesis and a high cell volume can stimulate these processes [36].

The salivary flow rate presented a significant reduction during and after training and a number of undesirable consequences occur, such as swallowing difficulty, great discomfort, speech problems, loss of taste, pH reduction, buffering capacity decrease, and oral micro flora changes, which increase the risk for caries and periodontal diseases. Indeed, the saliva quality and quantity are important as a defence against orally transmitted microorganisms related to respiratory tract infections, common in athletes. This occurrence is a consequence of the reduction in immunoglobulins, responsible for infection resistance [37].

The normal values reported for stimulated salivary flow in healthy adults vary from 1.0 to 3.0 mL/min, being defined as 2.0 ± 0.8 mL/min for men and 1.7 ± 0.7 mL/min for women, while other authors have demonstrated values of 0,9 mL/min, considered to be reduced [37].

Dehydration will lead to loss of performance, and suggests that a 68 kg adult undertaking prolonged exertion will require 1250 ml/h fluid replacement (2% body weight loss of a typical exercising 70 kg male equates to 1.4 l of fluid requiring replacement) [4].

Therefore, noncariogenic drink ingestion at regular intervals and maintenance of hydration levels during training, are recommended [37]

Sports scientists and nutrition specialists are stressing the importance of adding carbohydrate and salt to water and encouraging competitors to drink more during exercise. Sports Drinks is a rapidly growing market, worth $US 1.2 billion in the year 2000 in the USA alone. Indeed, a Euromonitor report revealed that the off-trade volume of isotonic drinks in the global market has increased from 7563.0 million litres in 2004 to 9678.8 million litres in 2009, which translates to an average annual increase of 5% [7,8].

An athlete using a widely researched carbohydrate-electrolyte sport drink as a nutritional strategy is likely to consume the fluid both during and after exercise. Ingestion of sports drinks during and after moderate-intensity exercise replaces fluids and energy lost more effectively than water and a placebo, leading to an improvement in subsequent endurance capacity [36].

Commercial carbohydrate-electrolyte sports drinks are formulated to include carbohydrate as an energy source to supplement liver and muscle glycogen stores [4,38], fluids to counteract the dehydration and hyperthermia [39], and electrolytes, mainly sodium, to replace losses via sweating and to promote intestinal glucose and water uptake [40]. Sodium is included in most sports drinks, mainly because it is the primary cation lost in sweat [41].

Protein supplements, as stated before, are collectively marketed as a supplemental nurture for athletes. WP, which have high concentrations of branched-chain amino acids, are known for having a good indispensable amino acid balance, high nutritional quality [9,10] and digestibility [11].

Protein or amino acid supply is closely associated with muscle mass gain or loss and oral solutions are a practical way of delivering supplements designed to promote muscle protein anabolism in exercising subjects [12]. Supplements of 1g per kg of body mass consumed orally immediately after the training sessions by young male elite athletes appeared to be a safe strategy commonly used [13].

Several properties related to sports drinks such as pH, titratable acidity, buffer capacity, calcium and phosphate content have an impact on the erosive potential of the drink [23].

1.2.3. Tooth Bleaching

The first dental bleaching technique was described by Chapple in 1877, but it was in 1989 that Haywood and Haymann introduced the at-home vital tooth bleaching technique [42,43].

Tooth bleaching to remove tooth discolorations has been considered to be a safe, effective, minimally invasive, and non-destructive. Traditionally, vital teeth have been whitened while supervised by dentists using two different main treatment methods [44].

One method is the “at-home vital tooth bleaching treatment”, while the other one has become known as “power tooth bleaching” an in-office procedure. Of course, the application of tooth bleaching

procedures to whitening multiple teeth has become increasingly popular. Tooth bleaching is one of the most rapidly growing oral care sectors, fuelled by patient demands for both healthy teeth and cosmetically attractive smiles. Indeed, any discoloration that may form within enamel or dentin will affect aesthetic qualities [44].

CP ($CH_6N_2O_3$) gel at a concentration of 10% to 16% is frequently used in at-home vital tooth bleaching treatments [45,46]. The CP-based gels have the advantage of promoting a slow and gradual release of hydrogen peroxide, which prevents its quick diffusion through enamel and dentin at high concentrations [44,47,49].

CP breaks down to hydrogen peroxide and urea (CH_4N_2O) [42]. Subsequently, the hydrogen peroxide breaks down into water and oxygen molecules. The latter penetrates the tooth and liberates or chemically changes the pigment molecules leading to a more or less pronounced whitening of the tooth. In contrast, sodium chlorite liberates a small amount of chlorine dioxide (ClO_2) in the presence of acid, thus also serving as a bleaching agent [44,48].

Bleaching products contain some form of hydrogen peroxide as the whitening agent. The chemistry of this agent is based in the ability to generate free radicals in most solvents. Hydrogen peroxide decomposes in aqueous solutions to yield hydroxyl radicals which are highly reactive because the radicals lack one electron, they are extremely electrophilic and unstable, and will attack most other organic molecules to achieve stability, generating other radicals. The basis of their decolorizing ability lies in the fact that when these agents react with highly conjugated organic molecules, they will disrupt the electron conjugation and change the absorption energy of the molecule. This can result in a shift of the visible absorption. The ability of hydrogen peroxide to generate free radicals which interact with adsorbed coloured organic molecules allows for its successful whitening action on enamel. Free radical reactions, however, are not specific and can potentially react with other organic structures [43,44,46]

To fulfil patient's requirement for faster tooth bleaching outcomes, CP-based gels with concentrations varying from 15 to 22% have been currently recommended. However, according to Soares et al. in 2013 [49], was concluded that 10 and 16% CP bleaching gels reduced the mineral content and increased the surface roughness of dental enamel, producing a more irregular and porous surface. However, the 16% CP gel promoted the most intense alterations on enamel, even after a single 8-h application.

As general known, enamel has been shown to behave as a membrane permeable to small ions. The small size of the molecule allows it to diffuse into the interprismatic spaces of the semi-permeable enamel structure. However, the possible alteration in the organic substances caused by the uncontrolled reaction of the peroxide radical could lead to some changes in the mechanical properties of the enamel and dentin [43,44]. Peroxides can increase the permeability of enamel and remove surface enamel matrix, causing morphological changes on the tooth surface such as pores, erosion, surface roughness, mineral alterations, and reduction in tooth microhardness. Soft tissue of the oral cavity can also be affected [44,46,49].

These changes may be correlated to the composition, concentration, instructions for use of the product, exposure time, and pH values [50]. About the composition, products containing sodium chlorite reduce the enamel microhardness due to its combination with an acid as activator agent, as citric acid [51], which have a low pH-value, and solutions with a low pH value are assumed to soften enamel, and even to produce erosion [52]. About concentration of the agent, CP at a concentration of 10% is less toxic to pulp cells when compared with CP at 16% [53] the last one also causes major alterations in the enamel surface, such as loss of mineral structure and increased roughness [49,54].

The literature is not consensual. Some studies have shown that there are no significant changes in enamel surface post-bleaching [55-57]. Others studies have reported structural changes that occur with CP even with a 10% concentration [58,59].

Some detrimental effects have to be taken into account such as:

(i) Sensitivity due to the increased enamel porosity (which allows the diffusion of the bleaching gel into the dentin through the dentinal tubules and further into the dental pulp [42,52];

(ii) Gingivitis [42,60];

(iii) Throat and gastric irritation [42];

(iv) The potential reduction of the adhesive strength capacity in the presence of bleaching-generated oxygen [60];

(v) Changes in enamel microhardness and surface roughness. Although at-home vital tooth bleaching does not produce macroscopic effects, structural changes and superficial roughness may occur at a microscopic level, leading to plaque accumulation and, subsequently, tooth decay and periodontal disease [61].

It is important to minimize risks to ensure that the integrity and longevity of the dental structure are maintained. Preserving enamel microhardness is essential to maintain dental health and the ability to resist masticatory, mechanical and chemical forces. [62,63].

Both sport drinks consumption and tooth bleaching treatment have dramatically increased among population, however the implication of these habits, alone and combined, to tooth enamel microhardness are not well documented in the literature.

2. OBJECTIVES

The aim of the study was to evaluate the potential effect of the ingestion of isotonic drinks or protein supplements and tooth bleaching in tooth enamel microhardness:

H0 – Enamel microhardness does not change between T0, T1 and T2.

H0 - Sports drinks do not influence enamel microhardness.

H0 – Tooth bleaching does not influence enamel microhardness.

3. MATERIALS AND METHODS

3.1. ENAMEL SAMPLES

The study comprised 25 caries-free human molars were used after storage in 1% chloramine-T trihydrate solution for a week, followed by a maximum period of 6 months in distilled water at 4ºC (ISO/TS 11405/2015).

Enamel specimens were prepared by sectioning the crowns from the roots using a diamond bur mounted in a high-speed hand-piece. The tooth crowns were sectioned longitudinally in a mesial-distal direction. The enamel samples were obtained from the vestibular and lingual or palatine side of the tooth crown and the other side was coated with clear nail varnish. The middle region of each sample was ground using 600 to 2000 grit abrasive paper and polished with diamond paste to provide a flat surface with approximately 9 mm^2.

3.2. SPORTS DRINKS

Four popular and commercially available SD were selected: Isostar powder formulations (IS), IsoCarb powder formulations (IC), 100% Whey Protein powder formulations (WP), UltraRecovery powder formulations (UR) (Table 1) (Figure 2).

The dilution protocol was: IS 40 g/ 500 ml of Penacova water; IC 60 g/ 375 ml of Penacova water; WP 50 g/ 400 ml of Penacova water; UR 80 g/ 400 ml of Penacova water.

Figure 2. Sports Drinks used: Isostar (IS), IsoCarb (IC), 100% Whey Protein (WP), Ultra Recovery (UR).

Table 1. Isotonic drinks selected, their manufacturer, ingredients and instructions for dilution. Food Colour: 102 = Tartrazine / Food Acid: 330 = Citric acid; 331 = Sodium citrate.

	Brand/ Lot/ Expire Date	**Manufacturer**	**Flavour**	**Ingredient (as listed by manufacturer)**	**Manufacturer recommended dilution (g/mL)**
Isotonic Drinks	Isostar/ L8059/ 08.2019	Isostar	Lemon	Sucrose, glucose syrup, acidity regulator (citric acid), sodium citrate, maltodextrins, calcium phosphate, natural lemon flavour, magnesium carbonate, sodium chloride, potassium chloride, antioxidant (ascorbic acid), dye (beta-carotene), vitamin B1.	40g in 500 ml of water.
	IsoCarb/ LI1714747/ 10.2019	Prozis	Lemon	Maltodextrin, fructose, vitamins and minerals (potassium citrate, sodium citrate, magnesium citrate, L-ascorbic acid, pteroylmonoglutamic acid, zinc sulfate, nicotinamide, calcium D-pantothenate, D-Biotin, cyanocobalamin, pyridoxine hydrochloride) , aroma, multienzymatic complex DigeZyme (alpha-amylase, neutral protease, cellulase, lactase, lipase), acidifier (citric acid, sweetener (sucralose), dye (beta-carotene).	80 g to 500 ml of moderately cold water.

Table 2. Protein Supplements selected, their manufacturer, ingredients and instructions for dilution. Food Colour: 102 = Tartrazine / Food Acid: 330 = Citric acid; 331 = Sodium citrate.

	Brand/ Lot/ Expire Date	**Manufacturer**	**Flavour**	**Ingredient (as listed by manufacturer)**	**Manufacturer recommended dilution (g/L)**
Protein Supplements	100% Whey/ LI1718343/ 12.2019	Prozis	Chocolate	Whey Protein Concentrate (soy lecithin emulsifier) (91%), cocoa, aroma, thickeners (guar gum, xanthan gum, sweeteners (acesulfame K, sucralose).	25 g with 200-250 ml of water.
	Ultra Recovery/ LI1717362/ 11.2019	Prozis	Lima-Lemon	Maltodextrin, whey protein concentrate, fructose, vitamin and mineral blend (sodium citrate, magnesium citrate, potassium citrate, L-ascorbic acid, DL-alpha-tocopheryl acetate, zinc gluconate, nicotinamide, acetate (L-leucine, L-isoleucine, L-valine), L-leucine, L-leucine, L-leucine, L-leucine, L-isoleucine, L-valine -glutamine, acidifying (citric acid), aroma, HMB (beta-hydroxy-beta-methylbutyrate), atomized pomegranate juice (maltodextrin, Punica granatum juice extract), CherryPure (Montmorency cherry powder), Peptopro casein hydrolyzate, curcumin C3 complex, sweetener (sucralose), multienzyme complex DigeZyme (alpha-amylase, neutral protestase, cellulase, actase, lipase), dyes (beet red), LactoWise (symbiotic combination of hay seed extract greek (trigonella foenum gracum) and Lactobacillus sporogenes).	80 g with 400 ml of water.

3.3. BLEACHING AGENT

The bleaching agent used was Opalescence PF, a 16% Carbamide Peroxide with potassium nitrate and fluoride.

Figure 3. 16% Carbamide Peroxide.

Table 3. 16% Carbamide Peroxide selected, their manufacturer, ingredients, pH and instructions.

	Brand/ Lot/ Expire Date	**Manufacturer**	**Ingredient (as listed by manufacturer)**	**pH**	**Instructions**
16% Carbamide Peroxide	Opalescence/ LBFMCZ/ 31.03.2020	Ultradent Products. INC	Glycerin, Water, Urea (Carbamide) Peroxide, Xylitol, Carbomer, PEG-300, Sodium Hydroxide, Potassium Nitrate, EDTA, Sodium Fluoride.	6.5	Application time: 4-6 hours

3.4. EXPERIMENTAL DESIGN

The 50 enamel samples (25 lingual/palatine, 25 vestibular side) were randomly allocated into 10 groups (n=5) (Figure 4).

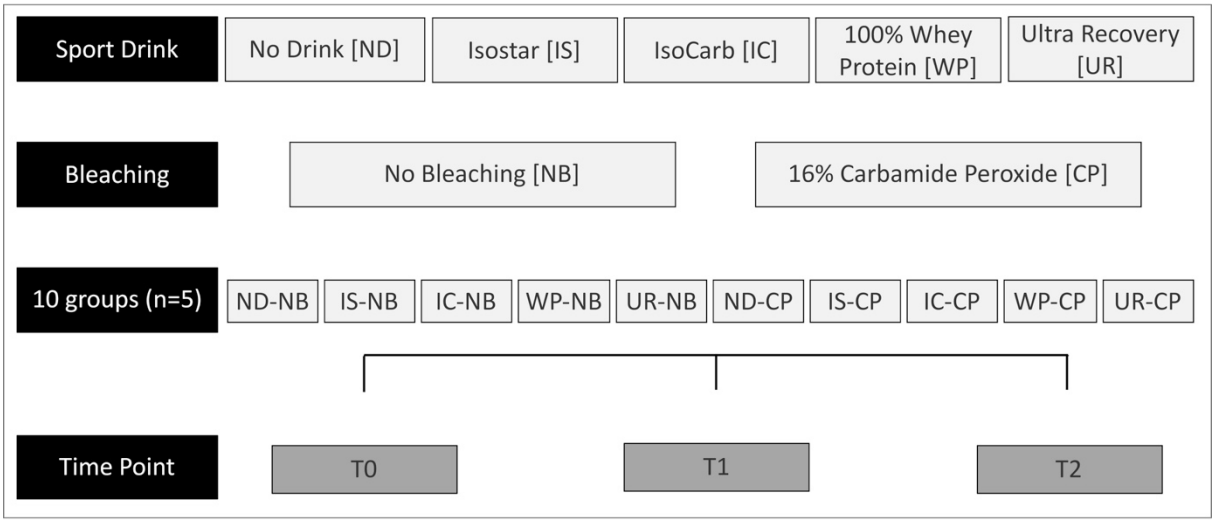

Figure 4. Experimental Design.

3.5. EXPERIMENTAL PROTOCOL

3.5.1. T0 - Baseline

In time point T0, all samples stored in distilled water were removed from respective storage container (10 containers, one for each experimental group), washed in distilled water and dried.

Three Vickers microhardness measurements were made across the enamel surface of each sample equidistant at 0.5 millimetres, using a microhardness tester, at 0.98 N load and 10 s dwell time (24,76). The mean Vickers microhardness numbers (VHN) was used as the microhardness value of the respective sample.

After the measurements all the samples were stored in the respective container with distilled water for 24 hours at 37ºC incubator temperature.

3.5.2. Immersion/ Exposure Cycles

All samples underwent to 14 immersion/exposure cycles, one cycle per day.

Between immersion/ exposure cycles the samples were stored in a container filled with 10 ml of artificial saliva, Artificial Saliva Gal Fovet-SAGF, which was changed every day and maintained at 37ºC incubator temperature.

The samples of the group ND-NB (No Drink-No Bleaching) (Figure 4) were removed from the storage container, washed and immerged in distilled water for a 5 hours period. After the immersion cycle, the samples were washed with distilled water and stored in the respective storage container with artificial saliva at 37ºC incubator temperature.

The samples of de groups IS-NB (Isostar-No Bleaching), IC-NB (IsoCarb-No Bleaching), WP-NB (100% Whey Protein-No Bleaching), UR-NB (Ultra-Recovery-No Bleaching), IS-CP (Isostar – 16% Carbamide Peroxide), IC-CP (IsoCarb-16% Carbamide Peroxide), WP-CP (100% Whey Protein-16% Carbamide Peroxide) and UR-CP (Ultra-Recovery-16% Carbamide Peroxide) (Figure 4) were removed from the storage container and suspended in 167mL of respective sport drink for 1 hour period in a platform shaker at room temperature. The pH of each sport drink was measured with a EC-25 pH/ Conductivity Portable Meter, Phoenix Instrument GmbH, according to the producer's specification.

After the immersion cycle, the samples of the groups IS-NB; IC-NB; WP-NB; UR-NB were washed and immerged in a respective distilled water container for a 4 hours period. At the same time, the samples of the groups ND-CP; IS-CP; IC-CP; WP-CP; UR-CP underwent to 4 hours exposure to 16% Carbamide Peroxide gel.

When a cycle ended, all samples were washed with distilled water and stored in the respective storage container with artificial saliva at 37ºC incubator temperature.

3.5.3. T1 – immediately after 14 cycles

In time point T1, immediately after the14th immersion/ exposure ended, all samples were washed in distilled water and dried. After Vickers microhardness measurements all samples were washed with distilled water and stored in the respective storage container with 10 ml of artificial saliva at 37ºC incubator temperature.

3.5.4. T2 – 24 hours after 14 cycles

In time point T2, 24 hours after the 14 cycles immersion/ exposure ended, all samples were removed from artificial saliva containers, washed in distilled water and dried. The standardized Vickers microhardness measurements were performed again.

All samples were washed with distilled water and stored in the respective storage container with 10 ml of artificial saliva at 37ºC incubator temperature.

3.6. STATISTICAL ANALYSIS

Data were statistically analysed with IBM SPSS Statistics v20 (IBM Corp).

As normality (Shapiro-Wilk; $p<0.05$) and homogeneity of variance (Levene test; $p<0.05$) have been not verified, non-parametric tests were used for inferential statistical analysis.

Friedman's test (2-way ANOVA to Friedman's classes for repeated measurements) was used to study microhardness changes over time (α=0.05).

Kruskal-Wallis tests followed by multiple comparisons were used to analyse the influence of the sport drink on the enamel microhardness one for each time point (α=0.05).

The influence of tooth bleaching on each time point was analysed with Mann-Whitney tests (α=0.05).

4. RESULTS

4.1. PH OF SPORTS DRINKS

The pH for each sports drinks are shown in table 4. Isostar had the lowest pH (4.03) and 100% Whey Protein had the highest pH (6.63). Of the isotonic drinks, Isostar had the lowest pH (Table 4). Of the protein supplements, Ultra Recovery had the lowest pH (Table 4).

Table 4. pH of the sports drinks used.

	Brand	Flavour	pH
Isotonic Drinks	Isostar	Lemon	4.03
	IsoCarb	Lemon	4.75
Protein Supplements	100% Whey	Chocolate	6.63
	Ultra Recovery	Lima-lemon	4,60

4.2. MICROHARDNESS MESUREMENTS

4.2.1. Descriptive Statistics

The mean and median values of tooth enamel microhardness according to time point (T0-Baseline; T1-Immediately after 14 cycles; T2-24 hours after 14 cycles) along with the respective standard deviation (SD) and interquartile range (IR) are shown in Table 5.

Table 5. Descriptive statistics. T0-Baseline; T1-Immediately after 14 cycles; T2-24 hours after 14 cycles; ND-No drink; IS-Isostar; IC-IsoCarb; WP-100% Whey Protein; UR-Ultra Recovery; NB-No bleaching; CP-16% Carbamide Peroxide; Mean; SD-Standard Deviation; Median; IR-Interquartile Range.

		T0		T1		T2	
		Mean (SD)	Median (IR)	Mean (SD)	Median (IR)	Mean (SD)	Median (IR)
ND	NB	283.9 (27.94)	290.7 (47.17)	284.3 (22.48)	289.3 (38.83)	301.3 (33.49)	308.0 (62.33)
IS		314.1 (28.78)	316.0 (48.50)	30.7 (18.39)	25.7 (29.50)	29.9 (12.36)	30.0 (22.83)
IC		326.7 (31.83)	307.7 (59.50)	150.5 (45.71)	156.7 (88.17)	125.4 (28.68)	122.7 (50.50)
WP		293.7 (27.46)	284.7 (37.83)	258.5 (25.94)	251.3 (46.33)	234.5 (49.05)	241.7 (96.33)
UR		321.8 (25.17)	326.3 (43.33)	215.8 (35.95)	224.7 (52.17)	229.3 (28.72)	237.0 (41.83)
ND	CP	291.5 (51.43)	304.0 (94.67)	220.7 (91.74)	263.7 (137.50)	232.0 (89.25)	254.7 (130.00)
IS		307.6 (24.20)	322.7 (45.33)	109.5 (36.49)	103.3 (63.50)	143.4 (53.83)	148.0 (104.17)
IC		320.1 (32.73)	323.3 (56.17)	105.6 (22.13)	105.0 (37.50)	107.1 (28.50)	97.0 (49.83)
WP		319.7 (39.56)	310.0 (74.00)	265.8 (32.81)	266.3 (57.33)	237.7 (15.57)	231.3 (30.00)
UR		325.9 (23.53)	340.3 (40.50)	198.3 (45.93)	199.7 (89.83)	242.8 (27.45)	249.7 (51.17)

4.2.2. Influence of Time Point

In T0 there were no statistically significant differences (p=0.128). A statistically significant decrease (p<0.001) of the VHN was observed from T0 to T1 and T2. No significant differences were found between T1 to T2 (p=1.000) (Figure 5).

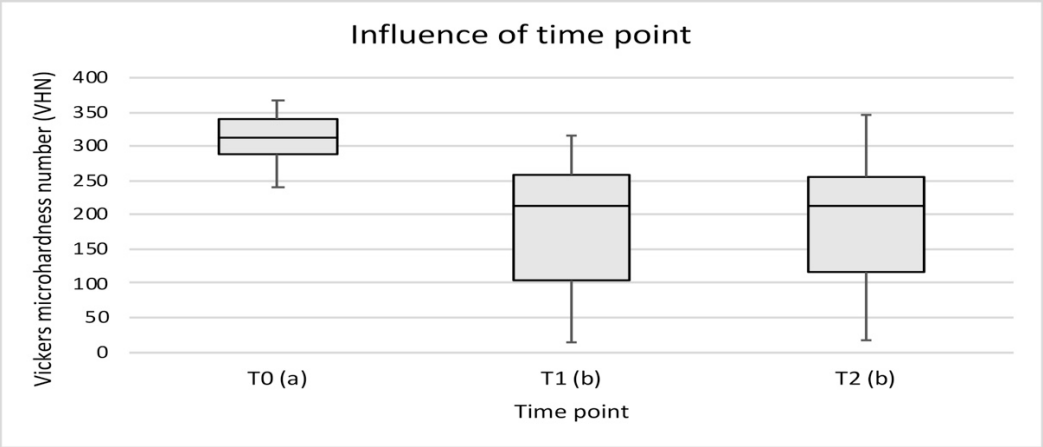

Figure 5. Influence of microhardness determination moment. There were no statistically significant differences (p≥0.05) between time points marked with the same letter in parentheses (a or b) (T0 – Baseline; T1 – immediately after 14 cycles; T2 – 24 hours after 14 cycles).

4.2.3. Influence of Sport Drink

The VHN was statistically significantly influenced (p<0.001) by the sport drink immersion, both in T1 and T2. At both time points, immerging enamel in the isotonic drinks, IS and IC, resulted in a significant (p<0.05) decrease of the VHN, when compared with the NB-ND group. The mean HVN obtained after immersion in each protein supplements, WP and UR, was statistically (p>0.05) similar to the HVN observed in the ND-NB group (Figure 6).

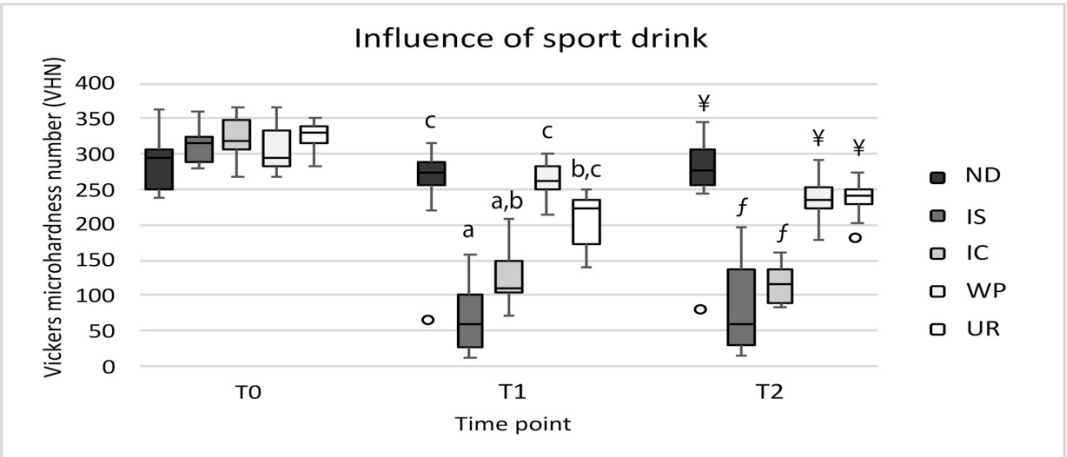

Figure 6. Influence of the sports drinks according to the moment of microhardness determination. No diferences were found between sports drinks at T0 (p=0.128). For T1 and T2 drinks with equal letters do not present statistically significant differences (p≥0.05) (ND-No Drink; IS-Isostar; IC-IsoCarb; WP-100%Whey Protein; UR-Ultra Recovery; T0-Baseline; T1-immediately after 14 cycles, T2-24 hours after 14 cycles).

4.2.4. Influence of Tooth Bleaching

None statistical influence was observed by exposing enamel to 16% carbamide peroxide, neither at T0 (p=0.485), nor at T1 (p=0.635), or at T2 (p = 0.915) (Figure 7).

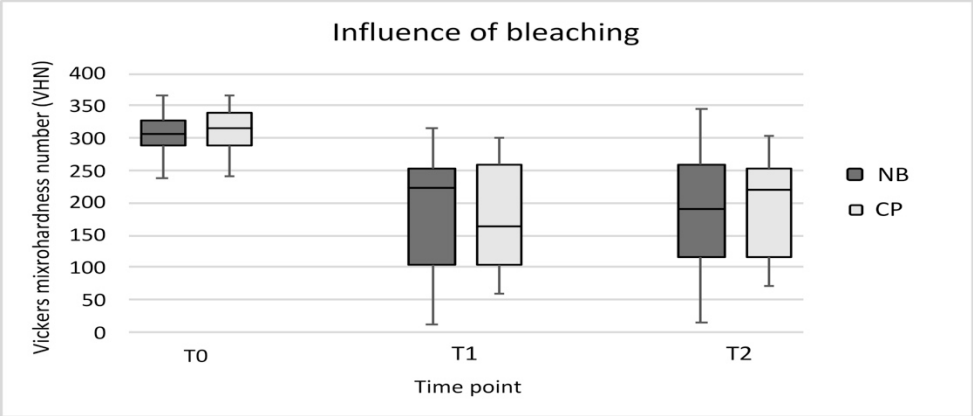

Figure 7. Influence of tooth bleaching according to the moment of microhardness determination (NB-No Bleaching; CP – 16% Carbamide Peroxide; T0-Baseline; T1-imideatelly after 14 cycles, T2-24 hours after 14 cycles).

5. DISCUSSION

The sports drinks evaluated are commonly used by athletes, a fitter body requires exercise, which needs hydration and supplementation. Isostar and IsoCarb are an important component of the diet of athletes because provides a proper hydration, supplements and minerals which are lost through sweating during physical activity [6]. Isostar and IsoCarb are specially formulated carbohydrate–electrolyte products designed to provide fast rehydration [7]. 100% Whey Protein and Ultra recovery are also a frequent choice to athlete's diets for muscle mass gain or loss and muscle recovery [9,12].

The increasing demand for a better appearance and whiter smiles, has made tooth bleaching, an increasingly popular dental procedure [1,2]. The widely used and approved 16% carbamide peroxide makes possible to have results after only a 4 hours diary application for 14 days (4-6 hours according manufacturer specification) [49].

The study protocol attempted to simulate an athlete experience of sports drinks consumption by immersing the samples for a significant period of time. One-hour immersion period for 14 days is equivalent to a 7 months of exposure, 4 minutes each day hypothetically, which simulate a prolonged consumption of the respective sport drink [5,64-66].

Saliva is an important discussion topic because, in vivo, it bathes natural teeth in the mouth and very efficiently counteracts the lowering of mouth pH values caused by sports drinks. Saliva is of special importance in reducing demineralization, enhancing remineralisation, and acting as lubricant. In laboratory research, usually, the artificial saliva is used as a potential substitute for human saliva. It is important to understand that it is impossible to obtain an artificial saliva that reproduces exactly the same characteristics of human saliva, which is very inconsistent and unstable [25,26]. In the present study, the SAGF (Artificial Saliva Gal Fovet-SAGF), a solution without protein and organic compounds (except for urea and/or organic acids for pH adjustment) was the artificial saliva chosen for storage mediumecause it is a reference in dentistry in vitro studies [25-28].

Microhardness tests are widely used to measure enamel microhardness, usually correlated with its mineral content [24]. This method is easy, accurate and requires only a small area of specimen surface for testing [24]. Vickers indenter was chosen for this study, because according to Gutiérrez-Salazar and Reyes-Gasga [35], Vicker indenter is more useful than the Knoop's because a square shape has to be always conserved and a small elongation of the diagonals of the indentations, that produce errors in hardness measurements, is easily detected. A 0.98N load was used because, besides being commonly used in other studies, it creates Vickers diagonals longer than 20 μm, which was recommended to prevent errors in measurement. An indentation time of 10 seconds is sufficient for a permanent indentation on the tooth surface to take place. The difference of indentation times was not influential VHN of enamel for the same indentation loads [24].

Since, microhardness has changed between time points, the first null hypothesis was rejected. The reduction of VHN from T0 to T1 and T2 shows that 14 cycles, with 1 hour diary immersion period in sports drinks results in a decrease of tooth enamel microhardness, which may reflect lost of mineral

content in enamel [5,64-66]. At T2, microhardness did not recover the initial T0 VHN, so 24 hours in saliva was not time enough to adequate tooth remineralisation.

The second null hypothesis was also rejected, as sports drinks have influenced enamel microhardness. In T0 there were no statistically significant differences among groups, which validate the randomness of samples distribution, all samples were at the same initial condition and well distribute among the experimental groups. VHN was influenced by immersion in sports drinks both in T1 and T2. The immersion in Isostar (pH 4.03) and IsoCarb (pH 4.75) (Table 4), resulted in a significant decrease in VHN. However, protein supplements immersion, 100% Whey Protein (pH 6.63) and Ultra Recovery (pH 4.60) (Table 4), did not result in a significant decrease in VHN. According to previous studies, the highest decrease induced by Isostar may be explained by pH, the presence of citric acid, calcium ion activity, inorganic phosphate ion activity and buffering capacity when compared to the lowest decrease in VHN of 100% Whey Protein [7]. The greater the buffering, the longer time it will take for saliva to restore the pH value (salivary clearance) [66].

Previous studies emphasized the consumption detrimental effect in enamel of some sports drinks. Sirimaharaj et al. [15] in 2002, stated that athletes who drinks isotonic drinks are in a high-risk group regarding enamel lesions. Meurman et al. [17] attribute the strong decalcifying properties of isotonic drinks to the citric acid (pH 1.8) within them. Citric acid is particularly damaging to the teeth as the citrate anion is able to chelate calcium in addition to the erosive effect of the protons released. Owens et al. [65] in 2007, with similar isotonic drink than the ones used in this study, states that the samples immersed in Gatorade revealed extensive enamel dissolution with an exposed dentin substrate. Additionally, the evaluation of the enamel microhardness test protocol to sports drinks and the explanation for the results were supported by Cochrane et al. [7] in 2012, who measured the erosive potential of isotonic drinks by changes in KHN, also with similar isotonic drinks than the ones used in this study. The majority of the sports drinks (Powerade, Gatorade and Staminade) caused the enamel microhardness to decrease by approximately 30% to 50% [7]. The products with erosive potential have pH values ranging from 2.81 to 3.55, titratable acidity (to pH 7.0) from 35.81 to 59.22 mmol OH^-/L and low levels of calcium (< 0.19 mM). The high titratable acidity can be attributed to the high content of citric acid and sodium citrate contained in these isotonic drinks. All this results are consistent to the results obtained in this study [7].

The third null hypothesis could not be rejected. Exposing enamel to 16% CP had not an impact in VHN neither in T1 nor in T2. The pH (6.5) of 16% CP (according to manufacturer), above the critical pH (5.5) for erosion of enamel to occur, may help to explain these results once demineralization can be affected by the degree of acidity of the gel. The higher the pH of the gel, the lower the solubility of calcium and phosphate present in the enamel will be [48].

The exposure time to the bleaching agent, 4 hours period is the minimum according to manufacturer specification [48,50], and the presence of the ingredient fluoride in 16% CP may also give us an explanation for this result [67]. Fluoride ions would increase the bleaching gel ions saturation and decrease mineral loss during bleaching action and exchange of ions. It is generally accepted that the major effects of fluoride ions in enhancing crystal growth and retarding dissolution of dental enamel

minerals, increasing supersaturation or decreasing undersaturation [48].

The study limitations were related to the collection of samples, alteration in the morphology and physical properties of the enamel can occur due to the use of enamel samples at different age groups (teeth erupted or not). Also, the sports drinks should be analysed not only by pH measurements, but also by titratable acidity, the content of calcium and inorganic phosphate and degree of saturation with respect to hydroxyapatite. Microindentation hardness tests do not provide specific information about the qualitative changes within a substance, so only an elemental or histochemical analysis is able to identify the specific alteration in enamel.

In future is important to standardize a methodology to study this matter, use microscope or profilometer evaluation of enamel surface changes, an elemental or histochemical analysis in order to identify the specific alteration in enamel, increase the number of of time points microhardness measurements (8 days after, a month after the cycles ended), use combination of other variables, as erosive diet regular components or toothpastes and tooth brushing.

6. CONCLUSIONS

Enamel microhardness decreased after 14 cycles of demineralization and did not increased after 24 hours in saliva.

The enamel microhardness was affected by the sport drink. Exposure to isotonic drinks, Isostar and IsoCarb, had a negative impact in enamel VHN. Enamel VHN was not significant influenced by exposure to protein supplements, 100% Whey Protein and Ultra Recovery.

Tooth bleaching with 16% Carbamide Peroxide did not influence enamel microhardness.

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9. APPENDIX I

Table 1. Vickers Microhardness Number (VHN) in T0 – Baseline.

				T0			
Sample	Sports Drinks	Tooth Bleaching	Groups	VHN1	VHN2	VHN3	VHN - Mean
1	1	1	No Drink - No Bleaching	304	275	356	311,6666667
2	1	1		221	248	248	239
3	1	1		267	277	356	300
4	1	1		289	323	260	290,6666667
5	1	1		282	255	298	278,3333333
1	2	1	Isostar - No Bleaching	306	288	277	290,3333333
2	2	1		348	289	311	316
3	2	1		347	360	372	359,6666667
4	2	1		290	299	276	288,3333333
5	2	1		356	302	290	316
1	3	1	IsoCarb - No Bleaching	311	326	286	307,6666667
2	3	1		338	292	264	298
3	3	1		376	357	363	365,3333333
4	3	1		297	326	293	305,3333333
5	3	1		294	387	390	357
1	4	1	100% Whey Protein - No Bleaching	285	258	302	281,6666667
2	4	1		361	368	296	341,6666667
3	4	1		291	293	270	284,6666667
4	4	1		274	270	273	272,3333333
5	4	1		343	238	283	288
1	5	1	Ultra Recovery - No Bleaching	331	334	387	350,6666667
2	5	1		336	357	309	334
3	5	1		272	270	308	283,3333333
4	5	1		323	326	330	326,3333333
5	5	1		292	306	346	314,6666667
1	1	2	No Drink - 16% Carbamide Peroxide	238	244	240	240,6666667
2	1	2		235	256	233	241,3333333
3	1	2		353	347	387	362,3333333
4	1	2		285	291	351	309
5	1	2		298	295	319	304
1	2	2	Isostar - 16% Carbamide Peroxide	294	321	361	325,3333333
2	2	2		336	327	305	322,6666667
3	2	2		293	383	307	327,6666667
4	2	2		302	259	280	280,3333333
5	2	2		288	266	292	282
1	3	2	IsoCarb - 16% Carbamide Peroxide	320	340	368	342,6666667
2	3	2		314	392	350	352
3	3	2		350	314	306	323,3333333

4	3	2		303	333	307	314,3333333
5	3	2		274	250	280	268
1	4	2	100% Whey Protein - 16% Carbamide Peroxide	256	324	350	310
2	4	2		252	254	298	268
3	4	2		327	408	361	365,3333333
4	4	2		312	303	292	302,3333333
5	4	2		364	336	359	353
1	5	2	Ultra Recovery - 16% Carbamide Peroxido	424	282	328	344,6666667
2	5	2		343	307	371	340,3333333
3	5	2		337	299	385	340,3333333
4	5	2		271	248	350	289,6666667
5	5	2		373	299	271	314,3333333

Table 2. Vickers Microhardness Number (VHN) in T1 – Immediately after 14 cycles.

				T1			
Sample	Sports Drinks	Tooth Bleaching	Groups	VHN1	VHN2	VHN3	VHN - Mean
1	1	1	No Drink - No Bleaching	232	244	286	254
2	1	1		284	270	266	273,3333333
3	1	1		248	295	325	289,3333333
4	1	1		286	290	295	290,3333333
5	1	1		241	362	341	314,6666667
1	2	1	Isostar - No Bleaching	19	26	20	21,66666667
2	2	1		12	12	14	12,66666667
3	2	1		35	52	96	61
4	2	1		40	30	27	32,33333333
5	2	1		23	24	30	25,66666667
1	3	1	IsoCarb - No Bleaching	171	203	156	176,6666667
2	3	1		195	144	131	156,6666667
3	3	1		222	180	226	209,3333333
4	3	1		95	115	102	104
5	3	1		93	159	65	105,6666667
1	4	1	100% Whey Protein - No Bleaching	264	273	214	250,3333333
2	4	1		266	293	267	275,3333333
3	4	1		238	185	249	224
4	4	1		265	221	268	251,3333333
5	4	1		299	308	268	291,6666667
1	5	1	Ultra Recovery - No Bleaching	266	241	186	231
2	5	1		190	202	282	224,6666667
3	5	1		200	248	214	220,6666667
4	5	1		202	260	283	248,3333333
5	5	1		145	168	150	154,3333333

1	1	2	No Drink - 16% Carbamide Peroxide	260	301	284	281,6666667
2	1	2		192	301	298	263,6666667
3	1	2		65	58	64	62,33333333
4	1	2		215	266	179	220
5	1	2		296	244	287	275,6666667
1	2	2	Isostar - 16% Carbamide Peroxide	102	104	89	98,33333333
2	2	2		138	103	139	126,6666667
3	2	2		105	195	177	159
4	2	2		65	69	47	60,33333333
5	2	2		79	102	129	103,3333333
1	3	2	IsoCarb - 16% Carbamide Peroxide	163	113	76	117,3333333
2	3	2		101	107	107	105
3	3	2		103	106	97	102
4	3	2		63	62	91	72
5	3	2		149	127	119	131,6666667
1	4	2	100% Whey Protein - 16% Carbamide Peroxide	199	280	296	258,3333333
2	4	2		288	266	307	287
3	4	2		349	273	283	301,6666667
4	4	2		227	161	259	215,6666667
5	4	2		270	267	262	266,3333333
1	5	2	Ultra Recovery - 16% Carbamide Peroxido	248	248	251	249
2	5	2		180	287	243	236,6666667
3	5	2		97	147	179	141
4	5	2		169	134	192	165
5	5	2		224	229	146	199,6666667

Table 3. Vickers Microhardness Number (VHN) in T2 – 24 hours after 14 cycles.

				T2			
Sample	Sports Drinks	Tooth Bleaching	Groups	VHN1	VHN2	VHN3	VHN - Mean
1	1	1	No Drink - No Bleaching	318	325	307	316,6666667
2	1	1		280	267	377	308
3	1	1		264	282	285	277
4	1	1		358	318	359	345
5	1	1		260	215	305	260
1	2	1	Isostar - No Bleaching	15	16	31	20,66666667
2	2	1		13	14	22	16,33333333
3	2	1		28	28	34	30
4	2	1		34	42	67	47,66666667
5	2	1		29	45	31	35
1	3	1	IsoCarb - No Bleaching	102	132	134	122,6666667

2	3	1		143	180	160	161
3	3	1		127	123	102	117,3333333
4	3	1		86	72	95	84,33333333
5	3	1		174	183	68	141,6666667
1	4	1	100% Whey Protein - No Bleaching	194	203	176	191
2	4	1		249	237	239	241,6666667
3	4	1		160	197	177	178
4	4	1		276	275	262	271
5	4	1		320	274	278	290,6666667
1	5	1	Ultra Recovery - No Bleaching	214	230	267	237
2	5	1		225	165	148	179,3333333
3	5	1		215	246	240	233,6666667
4	5	1		173	295	277	248,3333333
5	5	1		177	290	278	248,3333333
1	1	2	No Drink - 16% Carbamide Peroxide	301	337	274	304
2	1	2		190	272	302	254,6666667
3	1	2		74	54	105	77,66666667
4	1	2		234	337	265	278,6666667
5	1	2		233	251	251	245
1	2	2	Isostar - 16% Carbamide Peroxide	116	114	97	109
2	2	2		209	193	184	195,3333333
3	2	2		156	161	127	148
4	2	2		74	76	64	71,33333333
5	2	2		182	184	214	193,3333333
1	3	2	IsoCarb - 16% Carbamide Peroxide	78	61	119	86
2	3	2		102	99	90	97
3	3	2		120	139	91	116,6666667
4	3	2		94	89	67	83,33333333
5	3	2		173	126	158	152,3333333
1	4	2	100% Whey Protein - 16% Carbamide Peroxide	250	258	254	254
2	4	2		256	261	247	254,6666667
3	4	2		241	202	239	227,3333333
4	4	2		202	249	243	231,3333333
5	4	2		196	217	251	221,3333333
1	5	2	Ultra Recovery - 16% Carbamide Peroxido	191	230	191	204
2	5	2		266	266	250	260,6666667
3	5	2		258	253	238	249,6666667
4	5	2		241	237	203	227
5	5	2		291	273	254	272,6666667