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Impact of Daily Using Alcohol-containing Mouthwashes on Tongue, Cementum, and Enamel Surface; *In vitro* and *In vivo* Studies

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Abstract

Mouthwashes (MWs) are liquid preparation, commonly used for oral care and applied on teeth and mucosa of the oral cavity and pharynx. MWs exert antiseptic, astringent, and sedative effects. The aim of the current study is to investigate the effect of daily use of alcohol-containing mouthwashes on the tongue, surface of cementum and enamel in rats. Four groups (10 rats/group) of male Sprague–Dawley rats were treated by local application of MWs by pledgets for 45 min for 41 days as follows: The control group treated with artificial saliva; the group treated with Lacalut® (MW1); the group treated with Listerine® (MW2); the group treated with Tricare® (MW3). At the beginning, mandibular of first molar was extracted from each animal and soaked into artificial saliva or in MW for an *in vitro* study. At the end of the treatment, tongue, and extracted mandibular first molar from each group were collected for histological examinations and Transmission Electron Microscopes (TEM). Alcohol-containing mouthwashes showed sever histological changes and keratosis of tongue. Scanning electron microscope revealed that MW3 induced sever distortion of dorsal surface of the tongue and a distinguished harmful effect on enamel and cementum surface. It could be concluded that daily use of alcohol-containing mouthwashes induced harmful effect on dorsal surface of tongue, dental erosion on enamel and cementum surfaces.

Keywords: Mouthwashes; Oral lesions; Tongue; Cementum; Enamel surface; Alcohol

Introduction

Mouthwashes (MWs) are liquid preparations, used in treatment of oral disorders such as gingivitis, periodontitis, halitosis, and ulcerative conditions [1]. In addition, MWs are commonly used in the protection against dental caries and treatment of xerostomia [2]. They are widely used among other products for oral hygiene, were accounted with 12.7% of global market from 2009 to 2013 [3].

Over the few decades, mouthwashes are classified into different types according to its ingredients and their purpose of use. Chlorhexidine mouthwashes used for temporal eradication of bacteria at the oral cavity, inhibition of plaque formation and gingivitis, while cetylpyridinium chloride, sodium benzoate and triclosan used in protection against plaque formation [4]. In addition, fluoride-containing mouth rinses are commonly used in dental caries, and sodium bicarbonate used for patients with dry mouth and dental erosion [5]. Therefore, mouthwashes are being produced in different composition according to the therapeutic indications.

Alcohol (ethanol) is a main constituent of proprietary mouthwashes. Alcohol used in mouthwashes preparation as preservative, antiseptic and for dissolving the other ingredients [6]. In some mouthwashes, the ethanol concentration is between 5-26%. Recent research shows that alcohol-containing mouthwashes might increase the risk of oral cancer [7]. In addition, long-term use of alcohol-containing mouthwashes induced severs changes including distortion of taste buds, degeneration of nerve endings in oral mucosa and modulation of VR-1 variant amiloride-insensitive salt taste receptor [8]. Furthermore, ethanol of the mouthwashes might induce genotoxicity due to metabolic conversion from ethanol to acetaldehyde by bacterial flora of the oral cavity [9]. The results of the previous research are conflicting, because some research shows no sufficient evidence to accept the link between the alcohol-containing mouthwashes and oral cancer or other lesions in oral cavity. On the other hand, cumulative data from a large number of researches reveals that use of alcohol-containing mouthwash might induce development of oropharyngeal cancer. The first retrospective analysis of patients was conducted by Wynder et al. and reported that daily use of mouthwash increase the risk of oral cancer in females only [10]. Therefore, there are needs to further studies for better understanding of the role of alcohol-containing mouthwashes in development of oral lesions and other toxic manifestation such as oropharyngeal cancer [11].

Materials and Methods

Chemicals

Different mouthwashes were selected with different alcohol concentration, which are commonly used on the local market. Original Listerine® (Jonshon & Jonshon), Tricare® (Luna company), and Lacalut Active® (Arcam GmH) were purchased from the Egyptian local markets and the composition of mouthwashes were summarized in Table 1, according to attached insert of the manufacturer. All other chemicals or solvents were of the analytical or high performance liquid chromatography (HPLC) grade available.

Mouthwashes	Composition	Manufacturer
Lacalut Active	Aqua Castor oil Aroma Zinc sulphate Chlorhexidine Benzydamine hydrochloride Flouride	Arcam GmH
Original Listerine	Aqua Sorbitol Ethanol Benzoic acid Sodium Benzoate Thymol, Menthol Methyl Salicylate	Jonshon & Jonshon
Tricare	Sod. Lauryl Sulphate Glycerol Aqua Ethanol	Luna company

Table 1: Composition of different mouthwashes.

Animals

Four-month old male Sprague Dawley rats (200-250 g) were purchased from Animal House Colony, Pharmacology and Chemistry Research Centre (PCRC), Misr University for Science & Technology, 6th October City, Egypt. Animals were maintained on standard lab diet and housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12 h dark/ light cycle) and thermally controlled (25°C ± 1°C) at the Animal House Lab., Pharmacology & Chemistry Research Centre, Misr University for Science & Technology, 6th October City, Egypt. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the Pharmacology and Chemistry Research Centre, Misr University for Science and Technology. All the procedures described below were approved and carried out in accordance with guidelines of the ethics committee of faculty of dentistry, Ain Shams University.

In vitro study

The first molar of each rat within different groups was extracted at the first day of the present study, and then stored at the artificial saliva according Kuroshima et al. [12]. The extracted molar had been soaked daily in artificial saliva or in an assigned mouthwash for 45 min for 41 days based on the previous literature [13]. At the end of the treatment period, the extracted mandibular first molars were collected and prepared for scanning by Transmission Electron Microscope (TEM).

In vivo study

Experimental design: Forty male rats were randomly distributed into four groups (10 rats/ group) and the sample size was calculated according to previously published equation [14]. Animals were treated daily for 41 days at the following: Group 1 control treated with distilled water; Group 2 treated with Lacalut Active® mouthwash (MW1); Group 3 treated with Original Listerine® mouthwash (MW2); Group 4 treated with Tricare® mouthwash (MW3).

At the end of the treatment period (i.e., day 41), all animals were fasted for 12 h with free access to water ad libitum, then sacrificed by an over dose of ketamine (12.5 mg/kg, i.p.) and xylazine (1.5 mg/kg, i.p.) [15]. Sample of tongue from each animal was removed for histological examinations and scanning via Transmission Electron Microscope (TEM).

Determination of alcohol content of mouthwashes: The quantity of ethanol content in mouthwashes was determined by Gas chromatography (GC) according to a method of Teki and Bhat.

Application of mouthwashes: Pledgets (2.5 cm × 2.0 cm × 2.0 cm) were soaked daily into either distilled water (in case of control) or undiluted mouthwashes (MWs) for 5 min. Animals were sedated with ketamine (60 mg/kg, i.p.) and pledget applied into the dorsal surface of the tongue of each animal within different groups for 45 min for 41 days according to previously described procedure by Bernstein and Carlish [13].

Sample preparation for histological examinations: Anterior part of tongue was removed, and fixed in 10% neutral formalin solution for histological examinations [16]. The distorted filiform papillae of the tongue counted from three different areas in each specimen by image analysis program (Origin 7.0 software, Origin Lab, Northampton, MA).

Sample preparation for scanning electron microscopy: Another sample of tongue and cervical part of enamel and cementum from the previously extracted molars were fixed in a 2.5% glutaraldehyde in 0.1% phosphate buffer solution (pH 7.4). The samples were prepared for scanning by electron microscope (Philips XL30, Netherlands) [17].

Statistical analysis: All data were statistically analysed by analysis of variance (ANOVA) using the General Linear Model Procedure of the Statistical Analysis System [18]. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio [19]. All statements of significance were based on probability of $P<0.05$.

Results

Alcohol content of mouthwashes

The alcohol concentration of mouthwash (MW) was depicted in Table 2 and showed that alcohol content was highest concentration (29% w/v) in MW3, followed by MW2 with moderate concentration 17.8% (w/v) and MW1 with lower alcohol content <1% (w/v).

Mouthwashes	Alcohol percentage (%)
Lacalut (MW1)	-
Listerine (MW2)	17.80%
Tricare (MW3)	29.80%

Table 2: Alcohol content at different mouthwashes.

Effect of different mouthwashes on filiform papillae

The effects of different mouthwashes (MWs) on filiform papillae were graphically illustrated in Figure 1; Original Listerine® mouthwash

(MW2) and Tricare® mouthwash (MW3) induced significant distortion of filiform papillae, while Lacalut Active® mouthwash (MW1) showed non-significance difference in comparison to control group. The percentage of distortion of filiform papillae was graphically illustrated in regarding to alcohol contents of mouthwashes (MWs).

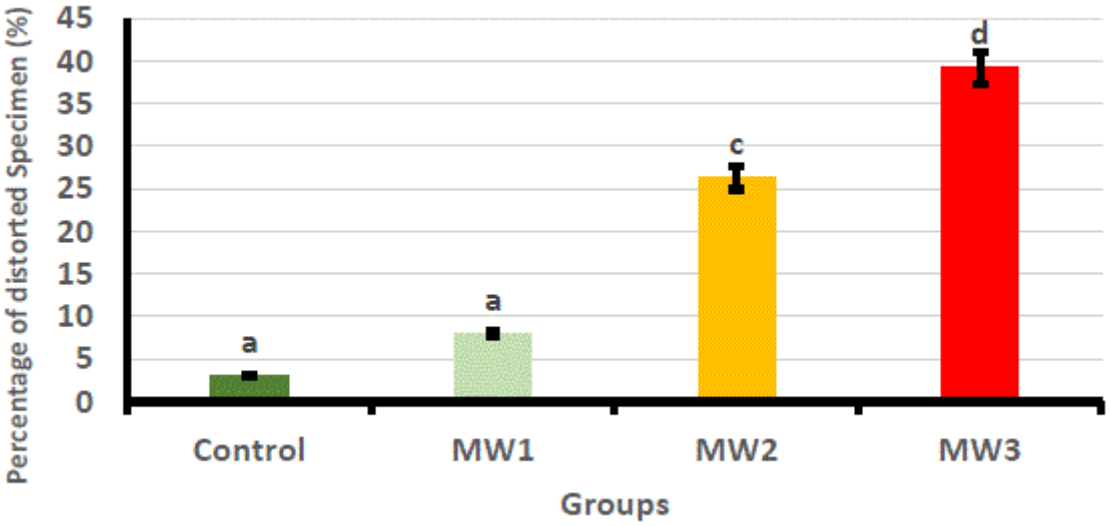

Figure 1: Effect of different mouthwashes on filiform papillae. Values represent mean ± SE for each group. Column superscripts with different letter are significantly different (P ≤ 0.05).

Microscopical examinations

The histological examinations of the anterior part of a tongue section of the control group showed normal morphological features of the filiform papillae that accompanied by regular slender distribution (Figure 2A). Photomicrograph of anterior part of a tongue section of animals treated with MW1; Lacalut Active® mouthwash showed the regular arrangement of the conical filiform papillae with underlying dense of connective tissue, slight loss of height in some regions and distortion of the epithelial covering in others (Figure 2B). The anterior part of tongue sections of animals treated with MW2; Original Listerine® mouthwash exhibit a slight decrease of height of the papillae associated with keratin formation at the giant conical papillae in lamellated pattern beside of an obvious scare of the connective tissue with some distortion of filiform papilla (Figure 2C). The anterior part of tongue section of rats treated with and Tricare® mouthwash (MW3) showed the most obvious distortion of filiform papillae at all types associated with torn keratin in many regions at the tip of the conical filiform papillae (Figure 2D).

Furthermore, histological examination of fungiform papillae of control group showed characteristic mushroom shaped morphology and stratified squamous epithelium (Figure 3A). Photomicrograph of fungiform papillae of MW1; Lactulate® mouthwash treated group showed mushroom shaped morphology more or less like control (Figure 3B). The anterior part of a tongue section of animals treated with MW2; Original Listerine® mouthwash showed general features similar to the control group, but mild changes in the taste bud were notified (Figure 3C). Photomicrograph of fungiform papillae of MW3; Tricare® mouthwash treated group showed dome shaped fungiform papillae with dense fibrotic connective tissue core and covering epithelium looked with foamy appearance (Figure 3D).

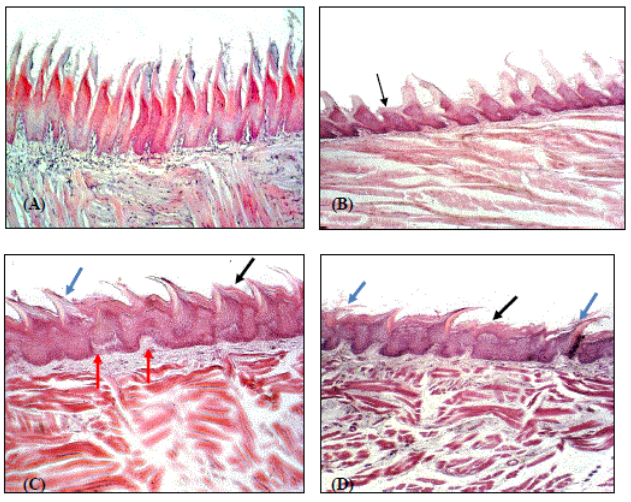

Figure 2: A photomicrograph of tongue section of (A) control group showing normal and regular slender type of filiform papillae; (B) Lacalut-treated group showing properly oriented slender type of filiform papillae of almost similar size except for some of less height (arrow); (C) Listerine-treated rats showing fifliform papillae with reduced height (black arrow) and karatin in lamellated pattern (blue arrow) and connective tissue papillae were scarce (red arrow); (D) Tricare-treated group showing ill-defined fifliform papillae (black arrow), while few observed papillae were of torn keratin (blue arrow) (H&E X100).

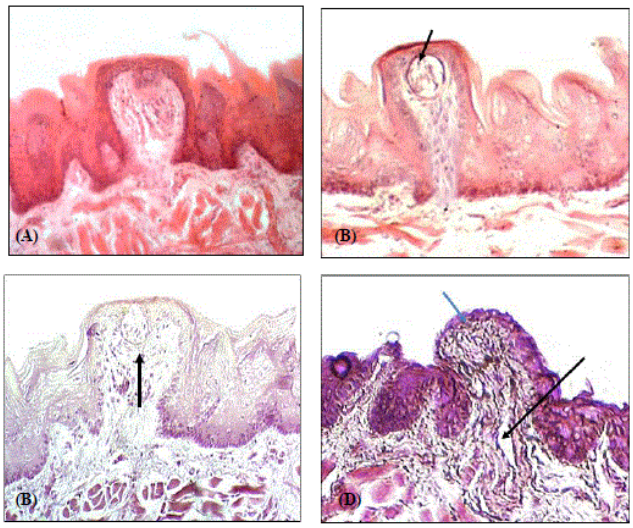

Figure 3: A photomicrograph of tongue section of (A) showing mushroom shape fungiform papillae with dorsal taste bud (arrow); (B) Lacalut-treated group showing mushroom shape fungiform papillae with dorsal taste bud (arrow); (C) Listerine-treated rats showing well defined mushroom shape fungiform papillae with taste bud showing slightly clear cellular appearance (arrow); (D) Tricare-treated group showing dome shape fungiform papilla with densely fibrotic connective tissue with areas of empty spaces (black arrow). The epithelium is relatively thick non-keratinized with foamy appearance (blue arrow) (H&E X200).

The histological examination of the anterior part of a tongue section of control group demonstrated that inverted cone shaped circumvallate papilla surrounded by an epithelial trough with large number of taste buds on the sides of the trough (Figure 4A). Photomicrograph of circumvallate papilla of MW1-treated group showed inverted cone shaped more or less like control group (Figure 4B). Photomicrograph of circumvallate papilla of MW2 treated rats showed significant widening in trough appeared with taste buds appeared empty in many regions and the connective tissue core showed some empty spaces (Figure 4C). Regarding the circumvallate papillae of the MW3 treated group showed obvious distortion in the papillae that accompanied with elongation and widening in the trough and taste buds of hollowed core (Figure 4D).

Scanning electron microscopic examinations

Electron microscopic examination of the anterior part of a tongue section of filiform papillae of the control group showed normal and regular arrangement of the three subtypes of the papillae, regularly arranged slender papillae, giant subtypes and true conical papillae were densely packed arrangement (Figures 5A-5C). MW1 treated group showed normal arrangement of filiform papillae, giant subtypes, and true filiform like control group (Figures 5D-5F). Electron microscopic examination of tongue of animals treated with MW2 showed a slight change in orientation representing bending of the tips and loss of vertical orientation in addition to obvious packing in true filiform papillae (Figures 5G-5I). Microscopic examination of three subtypes of filiform papillae of rats treated with MW3 showed obvious distortion of adjacent fungiform papillae besides splitting in the tips of papillae and adhesion of the giant filiform papilla (Figures 5J-5L).

On the other hand, electron microscopic examination of the enamel surface of cervical part of crown control group showed smooth and intact enamel surface with almost neither cracking nor erosive areas (Figure 6A). Scanning of enamel of animals treated with MW1 revealed few areas of mild and narrow cracks (Figure 6B). Microscopic examination of specimen of cervical part of enamel surface of MW2 treated animals showed smooth enamel surface, numerous depressed areas and well-defined enamel cracks (Figure 6C). Surface scanning of enamel specimen from cervical part of the crown of MW3 treated rats showed an increase in cracking with localized erosive areas on the surface of teeth (Figure 6D).

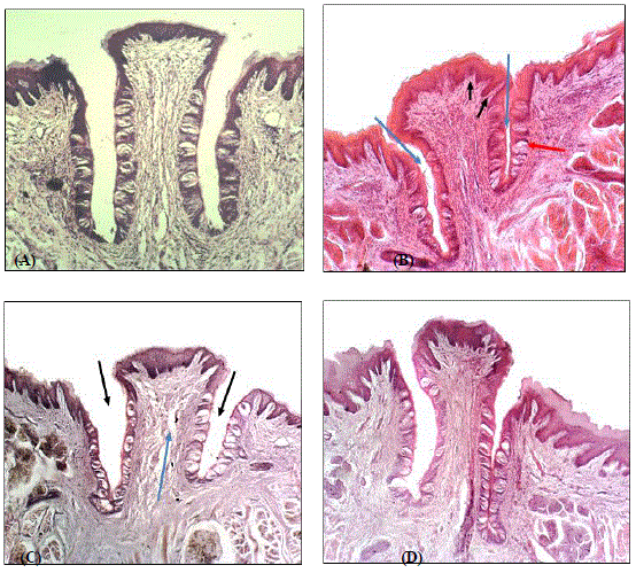

Figure 4: A photomicrograph of tongue section of (A) showing showing circumvallate papilla with connective tissue core with well-defined trough (blue arrows) and taste buds on sides of the trough (red arrows); (B) animals treated with Lacalut showing circumvallate papilla with dense connective tissue core and secondary papillae (black arrow) with well-defined trough (blue arrow), while taste buds on sides of the trough (red arrow); (C) Listerine-treated rats showing circumvallate papillae with wide trough (black arrows). Almost all taste buds are of clear content. Connective tissue core showed wide spaces (blue arrow); (D) Tricare-treated group showing distorted circumvallate papillae with wide and elongated trough and numerous taste buds (H&E X100).

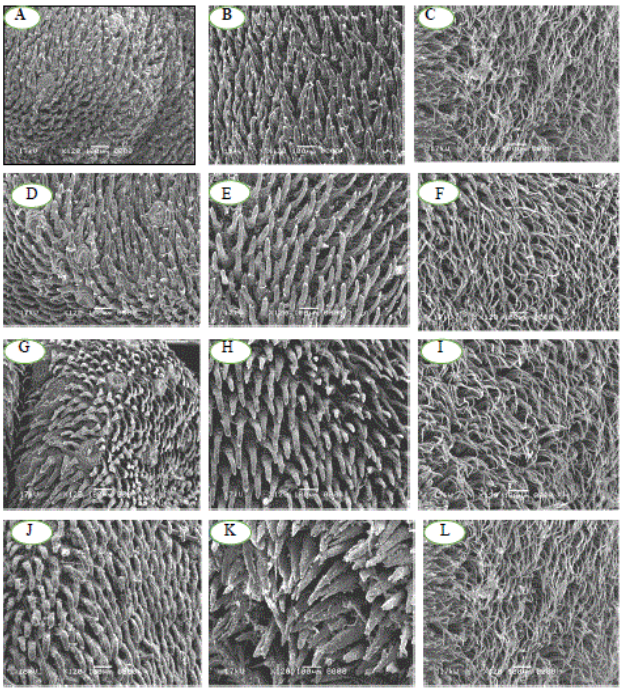

Figure 5: SE micrograph of tongue section of control group showing (A) regularly arranged simple conical filiform papillae; (B) regularly arranged giant conical filiform papillae with almost no distorted papillae; (C) densely packed small pointed true conical filiform papillae, Lacalut treated group showing (D) normal arrangement of simple conical filiform papillae with adjacent fungiform papillae (arrows); (E) regular arrangement of widely spaced giant conical filiform papilla; (F) true filiform papillae seemed to be like control group, SE micrograph of Listerine treated rats showing (G) simple conical filiform papillae with slight bending of their tips; (H) giant filiform papillae with slight loss of vertical orientation; (I) true filiform papillae with areas of adhesion between groups of papillae (arrow), electron microscopic examinations of tongue section of Tricare-treated rats showing (J) simple filiform papillae with almost horizontal orientation and adhesion between the neighbouring papillae (arrow); (K) giant conical filiform papillae with splitting in their tips and loss of normal arrangement; (L) true filiform papillae with more apparent areas of adhesions than other groups (arrows) (X120).

Examination of the root of the mandibular part of the control group showed no signs of resorption of the cervical part of cementum and cemento-enamel junction (CEJ) with intimately attached enamel, and regular cementum surface without surface defects (Figure 7A). The cervical part of cementum of animals treated with MW1 showed rough cementum with no resorption areas more or less like control group (Figure 7B). Scanning of cervical part of cementum surface of MW2 treated rats showed normal CEJ with a slight separation between enamel and cementum that accompanied with few depressions at sharpey's fibres and wide scattered concave areas (Figure 7C). Examination of MW3 treated group showed separation between enamel and cementum at CEJ beside cementum erosions, while sharpay's fibres were not absent (Figure 7D).

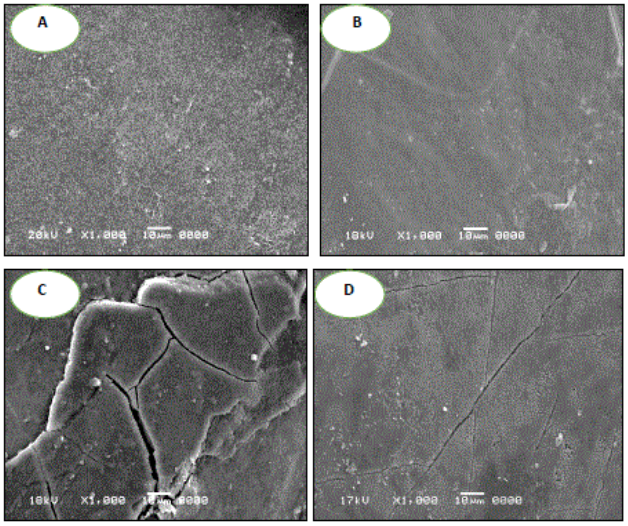

Figure 6: A photomicrograph of enamel surface (A) showing control group showing intact enamel surface; (B) animals treated with Lacalut group showing enamel surface with narrow cracks (arrows); (C) Listerine-treated rats showing Listerine group showing enamel cracks with variable width; (D) Tricare-treated group showing enamel cracks (black arrows) and erosive areas (white arrows) (X1000).

Discussion

Mouthwashes (MWs) are liquid preparations used by holding passively, or swallowing around the mouth by contraction or movement of the head for gargling purpose. MWs exert antiseptic, astringent, anti-bacterial, anti-inflammatory, and analgesic actions. Therefore, they are commonly used in treatment of oral disorders.

Mouthwashes (MWs) are an adjunctive procedure in treatment of oral diseases, but the extensive consumption of alcohol-containing mouthwashes might be associated with oral pain, mucosal dryness, and oral lesions [20]. Todkar et al. argue that daily use of mouthwashes leads to dental erosion including demineralization and impairment of the enamel surface [21]. Therefore, the current study aimed to investigate the effect of daily use of different mouthwashes for 41 days on the dorsal surface of the tongue, enamel, and cementum of Sprague-Dawly rats.

The results of gas chromatography analysis of mouthwashes revealed that alcohol content of MW1 i.e., Lactulate® mouthwash was small in comparison to other mouthwashes, while MW2; Original Listerine® mouthwash composed of moderate alcohol content, and MW3 i.e., Tricare® mouthwash showed the highest content of ethanol.

Tongue is a very sensitive, highly innervated, and well-coordinated muscular organ. Therefore, tongue is called as organ with a distinguished morphology, to deal with various diets and habitats [22]. The results of the present work showed that daily use of alcohol-containing mouthwashes induced sever changes in the dorsal surface of the tongue indicated by disruption of epithelium in some regions, distortion, and loss of integrity in papillae.

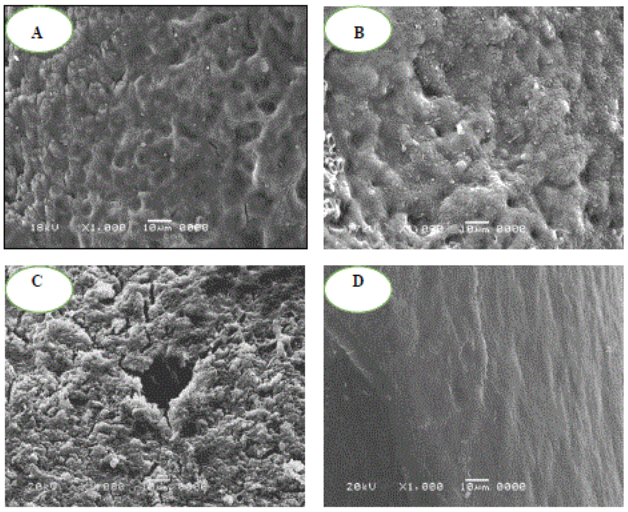

Figure 7: A photomicrograph of enamel surface (A) showing control group showing intact showing intact cementum surface with sites of fibre attachment; (B) animals treated with Lacalut showing no resorption sites and well defined sites of fibre attachment (arrows); (C) Listerine-treated rats showing cementum surface with slight wide concave area; (D) Tricare treated group showing many abrasions areas and notches in cementum surface with absence of sites of attachment of sharpeys fibers (X1000).

The histological examinations showed severe changes of dorsal surface of tongue in animals treated with MW3 more than MW2, while slightly changes were observed in MW1. These results were in agreement with previous work that demonstrated high concentrations of alcohol in mouthwashes resulted in deterioration of epithelial detachment, keratosis, and mucosal ulceration, while the alcohol deficient mouthwashes showed comparable efficacy [23]. According to Lachenmeier et al., evidence show that daily use of ethanol-containing-mouthwashes increase salivary acetaldehyde reaching concentration with DNA adducts and genotoxicity *in vitro* study [24].

Filiform papillae are widely distributed on the dorsal surface of the tongue. Therefore, it could be used papillae as an indicator for a good health status. At present, the current study show the degree of distortion of filiform papillae is in direct proportional to alcohol content of mouthwashes (MWs) that indicated by sever distortion of filiform papillae of animals treated with MW3 followed by MW2, but MW1 without any significant changes of filiform papillae. The results of the present work are in agreement with earlier study by McCullough and Farah, 2008, revealed that high alcohol concentration in mouthwashes preparations induced harmful effects including epithelial detachment, keratosis and mucosal ulceration [23]. Recent research shows that long-term use of alcohol-containing mouthwashes may lead to rapid atrophy of different types of papillae, disturbance of metabolic enzymes in oral cavity, and vascular insufficiency [25]. In addition, evidence indicates that regular alcohol consumption induces sever changes of oral mucosal cells due to substantial alteration of epidermal growth factor receptors (EGFR) and loss of keratin 13 proteins [26-28]. This clearly implies that alcohol showed a higher capacity to eliminate the lipid components of the cell barriers in the oral cavity, which surround the cellular granules at the epithelial spinous layer [6]. The results are in agreement with an earlier study was conducted by Johnson et al., reveal that long-term consumption of alcohol-containing mouthwashes (MWs) resulted in disruption of the epithelial lipid molecules, and decreased basal cell size of the mucosa in the oesophagus [29]. Based on these results, the degree of oral lesions correlated with the time of exposure to mouthwashes and the extent of alcohol content [30]. Thus, sever histological changes were pronounced on animals treated with MW3 i.e., Tricare® mouthwashes that indicated by hyperkeratotic white lesions [31]. Paradoxically, histological examinations of the current study did not show any changes in the oral mucosa that indicated to malignant or premalignant lesions. A general reason for this is that difference in experimental design and animal model beside the limitation for time of exposures mouthwashes. These results reinforced by previous work revealed that no sufficient evidence to argue the correlation between mouthwashes and oral cancer [30,32]. In contrast, some authors reported that the risk of oral cancer might increase with patients experienced with both alcohol and smoking in respective to the intensity and duration of exposure [33]. TEM of enamel surface showed irregular surface, concave areas, some cracks. The defect of enamel surface was pronounced in animals treated with MW3 followed by MW2, while treatment with MW1 showed slight changes in comparison to control group. According to earlier research was conducted by Tantbirojn et al., revealed that alcohol-containing mouthwashes may induce demineralization and dying changes of hard tissues like enamel [34]. The results of the current study showed that degree of enamel erosion and cementum hardness is in direct proportion with percentage of alcohol content in mouthwashes that indicated by potent stimulation on alcohol dehydrogenase enzyme (ADH III); which is an active modulator of matrix protease and important factor for enamel processing [35]. This clearly implies that progressive loss of hard tissues and roughness may be due to chemical dissolution of alcohol and fluoride in the mouthwashes [34]. In addition, daily use of alcohol-containing mouthwashes might induce dissolving of the enamel because of significant reduction of PH of saliva larger than buffering capacity that protect enamel pellicle in animal checks [36]. Mantonanaki et al. argues that alcohol-containing mouthwashes are an extrinsic etiologic factor for dental erosion. The above studies provides evidence for the current results of the present work that showed sever changes of enamel surface in animals treated with MW3 followed by MW2 and animals treated with MW1 showed no changes [37].

The results of the current study showed that MW3 induced surface defects on cementum associated with loss of Periodontal Ligament (PDL). Hornecker et al. argues that excessive alcohol consumption associated with periodontitis and damaging of supporting structures of teeth [38]. This evidence obviously explains that alcohol consumption increase the severity of loss of clinical attachment in periodontal diseases [39,40].

Based on the evidence shown above, the results of the current study support the previous findings of the earlier research studies that affirm alcohol-containing mouthwashes induce sever changes on the tongue, cementum, and enamel surface. The experimental design of the study was not able to detect such an effect of oral cancer. The current work is classified as a short-term study, so lifetime or at least 2-year studies are highly required to address the possibility of cancerous effects.

Conclusion

It can be concluded daily use of alcohol-containing mouth rinses induced distortion of dorsal surface of the tongue; sever changes of

cementum, and demineralization of enamel. Thus, using of alcohol-containing mouthwashes (MWs) should be restricted and controlled in a particular situation for a limited time; especially other forms of mouthwashes free from alcohol are equally effective. The harmful effects based on the degree of the alcohol content and time of exposure to alcohol-containing mouthwashes (MWs).

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