DOI: 10.7860/JCDR/2026/78604.23185

Original Article

Dentistry Section

Comparative Evaluation of Antifungal Efficacy of Ozonated Water Against Candida albicans in Heat Cure Acrylic Resin: An In-vitro Study

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ABSTRACT

Introduction: Ozonated water has been therapeutically used in different fields of Dentistry. Ozone usage has become more extensive because of its antimicrobial action, especially against *Candida albicans*. The anticandidal efficacy of ozonated water generated by commercially available portable machines that are used commonly in household is not investigated. The purpose of the study was to evaluate the effectiveness of ozonated water as a denture cleanser generated by portable ozonator which is easily accessible and affordable to patient.

Aim: To evaluate the effect of ozonated water on *Candida albicans* adhered to heat cure acrylic resin at different time intervals.

Materials and Methods: The present in-vitro comparative study was conducted in Central Research Laboratory, Maratha Mandal's Nathajirao G Halgekar Institute of Dental Sciences and Research Centre, Belagavi, Karnataka, India from January to June 2017. A total of 120 (N=12 in each group) heat cure acrylic resin disc specimens were inoculated with *Candida albicans* American Type Culture Collection (ATCC) 2091 for 24 hours and were subjected to flowing (Group A), immersion (Group B), and ultrasonication (Group C), in 0.5 mg/L ozonated water and commercially available denture cleanser (Efferdent-Group D) for different time intervals. Distilled water and 50 mg of nystatin 1:20 dilution (Group E) was used as negative and positive control, respectively. The Colony Forming Units (CFU) of *Candida albicans* of each group were evaluated under Scanning Electron Microscopy (SEM). Data were analysed by One-way ANOVA and Post-hoc Bonferroni test and the p-value <0.05 was considered as statistically significant.

Results: One-way ANOVA test showed that there was a significant difference with a p-value <0.001 between the groups overall. The antifungal effect of ozonated water at a concentration of 0.5 mg/L for 15 minutes immersion showed the maximum reduction of microbial counts of *Candida albicans* with mean value of CFU 5.26±1.62 whereas flowing ozonated water for one minute revealed the least reduction of CFU showing the mean score of 29.65±4.16 when compared to all the other groups. Post-hoc Bonferroni test showed significant differences in mean CFU counts were found with respect to various groups (p<0.001).

Conclusion: Ozone is found to be a potent denture cleanser. Ozonated water shows better antifungal activity against *Candida albicans* in heat cure acrylic resin than commercially available denture cleanser. Ozonated water could be used in denture cleaning protocol effectively in candidiasis patients at lower concentrations.

Keywords: Denture base, Denture cleanser, Oral candidiasis, Ozone

INTRODUCTION

Ozone therapy has been gaining popularity in dentistry. Ozone has been successfully used as an antimicrobial agent against various intraoral infections [1-4]. The word 'ozone', originated from the Greek word 'ozein' which means to smell. Ozone is a highly reactive form of oxygen and it is generated by passing oxygen through high voltage. Ozone came into use as a disinfectant of drinking water as early as 1906 at the Eon Voyage plant in Nice, France; since then, more than l000 amenities throughout Europe have adopted the practice of ozone water treatment [5].

Ozonated water has been therapeutically used in various fields of dentistry which includes disinfection of dental unit waterlines [6], biofilm purging [1], periodontal and osseous infections [7], irrigation in endodontics [8], treatment of dental caries [9], peri-implantitis [10], oral candidiasis [11], oral ulcers [12], and Temporomandibular Joint (TMJ) pain [13]. Ozone is also used following tooth extraction and surgical interventions to enhance haemostasis [14]. Ozone has also been used in denture cleansing [15,16]. Ozone results in local damage of cytoplasmic membrane of the microbes. The secondary oxidant effects are ozonolysis of double bonds and ozone induced modification of intracellular contents [1].

Studies have shown the ozone effectiveness in inhibiting oral microbes especially in cases of oral candidiasis [17,18]. The most common form of oral candidiasis seen in patients is denture stomatitis, which mainly affects the palatal mucosa covered by a denture. This condition is frequently symptomless, but when symptoms occur, they appear as mucosal bleeding, burning or painful sensations and dryness of the mouth. The onset and severity of denture stomatitis is multifactorial in origin. The predisposing factors which influence this disease are decreased salivary flow, poor denture hygiene, surface defects and porosities on the intaglio surface of the denture base, denture trauma, denture wearing at night without adequate tissue rest, smoking and nutritional insufficiencies. *Candida albicans* is the predominant oral pathogen associated with denture stomatitis [19]. The Candidal proliferation and its survival in the oral cavity depend on its capability to get adhered to the mucosal tissues and to abiotic surfaces [10]. Candidiasis occurs when the host provides environmental conditions which are essential for attachment, growth and reproduction [11]. Several studies have

been performed to investigate the adhesion of *Candida albicans* on acrylic resin surfaces [13-15]. The factors which determine the adhesion of *Candida albicans* to acrylic resin are surface roughness produced by machining, the relative surface energies of Candida species and the effect of different types of saliva. Le PH et al., noticed that Candida attachment may be more greatly influenced on denture materials possessing higher roughness values (>0.5 µm) [20]. The roughness and microporosities on the surface of the denture harbour microorganisms that resist removal by mechanical and chemical methods of denture cleansing.

Denture hygiene is essential for the maintenance of healthy supporting tissues. Effective plaque removal by mechanical method is a difficult task in case of elderly individuals, due to their poor manual dexterity [21]. However, brushing alone is insufficient for plaque removal from dentures. To facilitate an effective removal of plaque, various chemical solutions are used for denture cleansing. Many investigations have been conducted to assess the antimicrobial effect of denture cleansers which have shown that there was incomplete eradication of the microorganisms from the denture [21-24]. A study conducted by Peracini A et al., and Paranhos HFO et al., has also shown that the immersion type denture cleansers will have effect on physical and mechanical properties of the heat cure resin [25,26].

Antifungal efficiency of different forms of ozone generated by industrial machines are well studied but there is a dearth of the investigations carried out on the ozonated water generated by indigenous portable machine that are used commonly in household for various purposes. Hence, a need was felt to investigate antifungal efficacy of ozonated water which is more accessible for the patients and could be used by them for the effective denture cleansing on daily basis. The aim of the study was to evaluate and compare the antifungal efficacy of ozonated water with commercially available denture cleanser on heat cure acrylic resin at different time intervals. The alternate hypothesis of the study was that the ozonated water was superior in inhibiting *Candida albicans* when compared to commercially available denture cleanser in heat cure acrylic resin. The null hypothesis was that the ozonated water was inferior in inhibiting *Candida albicans* when compared to commercially available denture cleanser in heat cure acrylic resin.

MATERIALS AND METHODS

The present in-vitro comparative study was conducted in Central Research Laboratory, Maratha Mandal's Nathajirao G Halgekar Institute of Dental Sciences and Research Centre in Belagavi, Karnataka for a period of six months from January to June 2017. The study was registered and conducted with the approval of Institutional Review Board. (2015-16/1122).

Sample size calculation: Sample size calculation was based on the CFU of the study by Arita M et al., [3]. The mean CFU at the ozone concentration of 0mg/1 was 17X103 with standard deviation of 6. Mean CFU for study group was 4X103 with standard deviation of 1. Sample size was calculated based on the following formula: N=2 Sp 2 (Z1- a/2 + Z1 - B)/D2 α error assumed at 5%, β error assumed at 20% (Power of the study - 80%), Sp= Pooled standard deviation, D is the mean difference between ozone groups. The sample size was calculated to be 3 per group. To apply the statistical tests with an accuracy, a sample size of 12 per each group was determined to be adequate. A total of 120 disks were prepared for the study.

Study Procedure

Preparation of the specimens: The test samples were prepared from heat cure acrylic resin (DPI, Mumbai, India) with a diameter of 20 mm and thickness of 2 mm and were divided into groups: A1, A2, B1, B2, B3, B4, C1, C2, D and E. The resin disks were ground with # 180 emery sand paper, sonicated in water for 60 minutes, and immersed in water for 24 hours to remove the residual monomer. All samples were checked for surface roughness under the surface roughness tester (Tally surf model no.201, Mitutoyo America Corporation, USA). The average Ra value was maintained to 0.05 µm. Then the samples were subjected to autoclaving at 120°C for 15 minutes to maintain a sterile environment before the microbiological procedure. The disks with porosities, surface irregularities and defects were discarded.

Stock solution preparation and inoculation: *Candida albicans* ATCC 2091 was cultured in Sabourad's dextrose broth at 37°C for 48 hours. After the growth of *Candida*, it was used to prepare the stock solution of 2×107 CFU/ mL which was immediately used as a fungal solution. The resin disks were placed in a glass test tube containing brain heart infusion broth. Each of these test tubes were inoculated with 0.1 mL of *Candida albicans* stock solution and incubated at 370C for 24 hours without agitation. After 24 hours of incubation the disks were removed from the test tubes and washed three times with sterile distilled water to remove loosely attached microorganisms. Samples were stored in sterile petridish at room temperature for 20 minutes [3].

Treatment with ozonated water: Commercially available portable ozone generator (Multipurpose ozone sterilizer, Ozone Engineers Pvt Ltd, Chinnavedampatti, Coimbatore, Tamilnadu, India) [Table/Fig-1] was used for the study. Ozonated water was generated at a concentration of 0.5 mg/L. Sterile distilled water was used as negative control (0 mg/L). Group A1 samples were subjected to flowing distilled water for one minute. Group A2 samples were subjected to flowing ozonated water at a concentration of 0.5 mg/L for one minute. Test samples of Group B1 and B3 were immersed in distilled water for five and 15 minutes, respectively. B2 and B4 samples were immersed in 150 mL of ozonated water at a concentration of 0.5 mg/L for five and 15 minutes, respectively. Group C1 samples were subjected to ultrasonification with sterile distilled water and Group C2 were subjected to ultrasonification with 0.5 mg/L of ozonated water for one minute, respectively.

[Table/Fig-1]: Portable ozone generator.

Treatment with denture cleaner: Group D samples were immersed in Efferdent denture cleanser solution according to manufacturer's instruction for 15 minutes. Efferdent contains sodium bicarbonate as active ingredient.

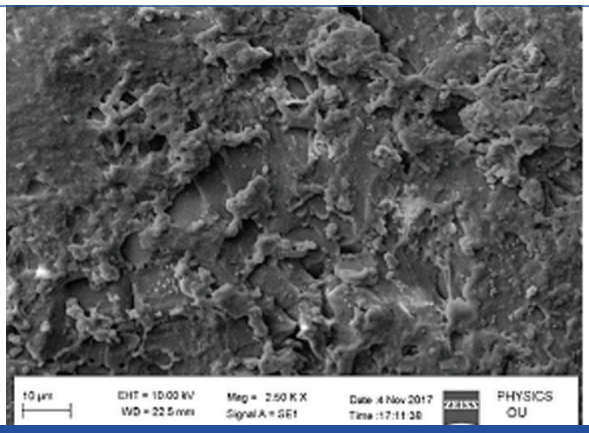
Treatment with Nystatin: Group E samples were placed in a freshly prepared 50 mg Nystatin (Himedia, India) suspension (1:20 dilution) for 10 minutes, Nystatin was taken as positive control.

Microscopic examination: The test samples were examined under the scanning electron microscope. Each sample was observed under the magnification of 2.50 KX, 5 KX, and 10 KX for microbial counts in 10 different fields prior to ozone treatment [Table/Fig-2] and after treating with ozonated water at a concentration of 0.5 mg/L [Table/Fig-3].

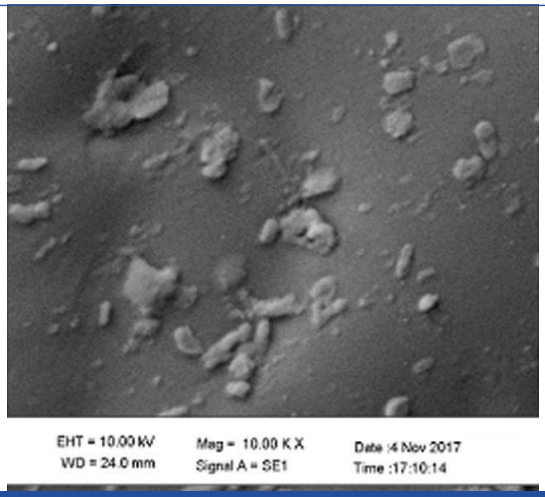
A1: Flowing distilled water for one minute; A2: Flowing ozonated water 0.5 mg/L for one minute;

B1: Immersion in distilled water (0 mg/L) for five minutes; B2: Immersion in 0.5 mg/L of ozonated water for 5 minutes, B3: Immersion in distilled water for 15 minutes; B4: Immersion in 0.5 mg/L of ozonated water for 15 minutes; C1: Ultrasonification with distilled water for one minute; C2: Ultrasonification with 0.5 mg/L

of ozonated water for one minute; D: Efferdent denture cleanser; E: Nystatin.



[Table/Fig-2]: Pretreatment SEM image showing the quantity of Candidal colonies adhered to the surface of heat cure acrylic resin disk specimen.



[Table/Fig-3]: Posttreatment SEM image with ozonated water at 0.5 mg/L showing the reduced quantity and size of the colonies.

STATISTICAL ANALYSIS

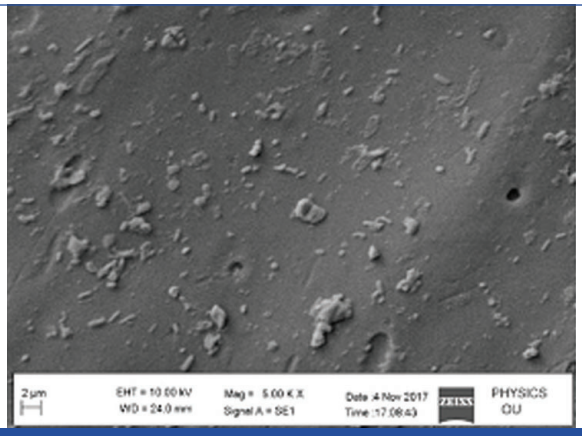
The data were arranged using Microsoft Excel software. Statistical analyses were conducted using SPSS software (version 29.0, IBM SPSS Statistics Inc., Chicago, IL, USA), Level of significance was predefined to α=0.05 for all tests. Descriptive statistics, consisting of mean and standard deviations, were computed for each group. The data was subjected to one way ANOVA followed by post-hoc Bonferroni test groups' comparison.

RESULTS

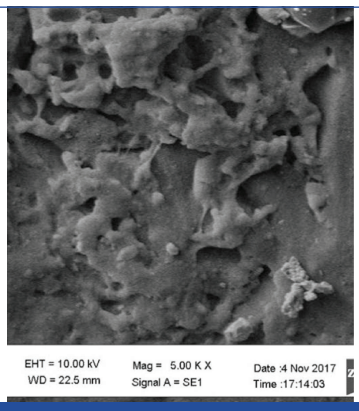
The results of the study showed significant differences ($p<0.001$) in mean microbial counts with respect to various groups [Table/Fig-4]. The least mean microbial counts were found for Group E Nystatin (2.37±1.11) [Table/Fig-5] followed by Group B4 immersion in 0.5 mg/L of ozonated water for 15 minutes (5.26±1.62), Group C2 ultrasonification with 0.5 mg/L of ozonated water for one minute (10.18±1.68), Group D Efferdent denture cleaner (10.23±6.18), Group C1 ultrasonification with sterile distilled water for one minute (12.25±2.79) [Table/Fig-6] and Group B2 immersion in 0.5 mg/L of ozonated water for five minutes (16.02±1.87). Other groups had higher mean microbial counts. Post-hoc analysis showed no significant differences in mean microbial counts between- Group E gold standard Nystatin versus Group B4 immersion in 0.5 mg/L of ozonated water for 15 minutes (p=0.624); Group D Efferdent denture cleanser versus Group B4 immersion in 0.5 mg/L of ozonated water for 15 minutes (p=0.265); and Group D Efferdent denture cleanser versus Group C2 ultrasonification with 0.5 mg/L of ozonated water for one minute (p=0.120) [Table/Fig-7].

Groups	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Group A1	12	85.79	7.87	80.79	90.79	73.90	98.30
Group A2	12	29.65	4.16	27.01	32.29	23.20	34.90
Group B1	12	43.11	5.35	39.71	46.51	36.20	51.40
Group B2	12	16.02	1.87	14.83	17.20	12.80	18.80
Group B3	12	36.40	4.87	33.31	39.49	30.80	45.20
Group B4	12	5.26	1.62	4.23	6.29	3.30	8.40
Group C1	12	12.25	2.79	10.48	14.02	7.60	18.30
Group C2	12	10.18	1.68	9.11	11.24	8.10	13.00
Group D	12	10.23	6.18	6.30	14.15	3.90	25.00
Group E	12	2.37	1.11	1.66	3.07	1.30	4.60

[Table/Fig-4]: ANOVA test showing mean scores in various groups.
Note: Analysis of variance (ANOVA) test applied shows: F-ratio 409.112, p-value <0.001, Significant differences between the groups overall.A1: Flowing distilled water for one minute; A2: Flowing ozonated water 0.5 mg/l for one minute; B1: Immersion in distilled water (0mg/l) for five minutes; B2: Immersion in 0.5 mg/l of ozonated water for 5 minutes, B3: Immersion in distilled water for 15 minutes; B4: Immersion in 0.5 mg/l of ozonated water for 15 minutes; C1: Ultrasonification with distilled water for one minute; C2: Ultrasonification with 0.5 mg/l of ozonated water for one minute; D: Efferdent denture cleanser; E: Nystatin



[Table/Fig-5]: SEM images of the Candidal colonies treated with 50mg Nystatin suspension. (1:20 dilution).



[Table/Fig-6]: SEM images of the Candidal colonies treated with distilled water.

DISCUSSION

The study design followed the conventional denture cleansing protocol. The resin disk specimens were first subjected to running ozonated water followed by immersion in ozonated water and then the specimens were subjected to ultrasonification in ozonated water.

The study results showed that 0.5 mg/L concentration of ozonated water was the most effective in reducing microbial counts of *Candida albicans* when the samples were immersed in it for 15 minutes. The differences in Candidal elimination were not significant between immersion in 0.5 mg/L of ozonated water for 15 minutes and Nystatin. Ultrasonification with 0.5 mg/L of ozonated water also showed a significant reduction in Candidal colonies. Both immersion

Comparison ↓ vs →	A-0	A-0.5	B-0-5min	B-0.5-5min	B-0-15min	B-0.5-15min	C-0	C-0.5	D	E
A - 0 mg/L	—	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
A - 0.5 mg/L		—	<0.001	<0.001	0.010	<0.001	<0.001	<0.001	<0.001	<0.001
B - 0 mg/L - 5 min			—	<0.001	0.011	<0.001	<0.001	<0.001	<0.001	<0.001
B - 0.5 mg/L - 5 min				—	<0.001	<0.001	>0.05	0.058	0.064	<0.001
B - 0 mg/L - 15 min					—	<0.001	<0.001	<0.001	<0.001	<0.001
B - 0.5 mg/L - 15 min						—	0.006	0.288	0.265	>0.05
C - 0 mg/L							—	>0.05	>0.05	<0.001
C - 0.5 mg/L								—	>0.05	0.001
D									—	0.001

[Table/Fig-7]: Post-hoc Bonferroni tests for inter group comparison.
Mean values from lowest to highest: Group E < Group B - 0.5 mg/L - 15 min < Group C - 0.5 mg/L < Group D < Group C - 0 mg/L < Group B - 0.5 mg/L - 5 min < Group A - 0.5 mg/L < Group B - 0 mg/L - 15 min < Group B - 0 mg/L - 5 min < Group A - 0 mg/L

and ultrasonification methods revealed remarkable reduction in microbial counts. Flowing ozonated water showed the least reduction in microbial counts amongst all the groups. The Efferdent denture cleanser solution showed lesser antifungal effect when compared to immersion in 0.5 mg/L ozonated water for 15 minutes. Studies have shown that the commercially available denture cleansers were capable of reducing *Candida albicans* biofilm. However, none of the denture cleansers were effective in completely eliminating established *Candida albicans* biofilms [21-24]. A study carried out by Arita M et al., quantified the antimicrobial effect of ozonated water at various concentrations 0, 0.5, 2, 4 mg/L for 1, 5, 10, 30 and 60 minutes and a combined technique of ozonated water and ultrasonification for one minute [3]. They found that the combination of ozonated water and ultrasonification eradicated almost all the viable *Candida albicans* cells adhering to the acrylic resin plates when compared to commercially available denture cleansers. Murakami H et al., carried out a study to assess the effectiveness of ozoniser generating ozone at a concentration of 10ppm and concluded that Candida [15]. albicans decreased to about 1/10 after 30-min and to 1/103 after 60-min exposure to ozone. In the present study, ozonated water of concentration 0.5 mg/L was used as the generation of higher concentration of ozone requires expensive equipments which could not be affordable for the patients to use it in denture cleansing.

Nagayoshi M et al., conducted a study to examine the effect of ozonated water at a concentration of 4 mg/L against microorganisms present in dental plaque [1]. Dental plaque samples were treated with 4ml of ozonated water for 10 seconds. They evidenced that ozonated water was effective in killing gram positive and gram negative oral microorganisms and oral *Candida albicans* in pure culture as well as bacteria in plaque biofilm, and concluded that ozonated water could be useful to control oral infectious microorganisms in dental plaque. A study conducted by Huth KC et al., has shown that higher concentrations of aqueous ozone 20 µg ml-1 could be considered as useful in the reduction of periodontal pathogens [7]. Oizumi M et al., conducted a study was to compare the microbicidal effect of gaseous ozone with that of ozonated water in disinfecting dentures on three standard strains of oral microorganisms [16]: Streptococcus mutans (strain IID 973), Staphylococcus aureus (strain 209-P), and *Candida albicans* (strain LAM 14322). The numbers of cells of all three strains decreased below the detection limit by 3-min when gaseous ozone was. The study concluded that direct exposure to gaseous ozone to be a more effective microbicide compared with ozonated water, and that gaseous ozone could be clinically useful for disinfection of dentures. Ozaki M et al., reported the antimicrobial effect of ozone in the microbubbled state [17]. More recently a study was conducted by Mirmortazavi A et al., has shown that the home-generated ozone water could inhibit Candida growth and colonization on the acrylic denture base material [27]. The null hypothesis of the study was accepted.

Contraindications of ozone therapy are pregnancy, hyperthyroidism, severe anaemia, severe myasthenia, acute alcohol intoxication, recent myocardial infarction, haemorrhage from any organ and ozone allergy [4]. The concentration of ozone is a significant factor when it is used for treating patients, as data suggests that short-term exposure to ozone can increase bronchial allergen responsiveness in subjects with mild allergic asthma or rhinitis [28]. Aqueous form of ozone was found to be less cytotoxic than gaseous form on the biologic system [29].

Limitation(s)

Limitations of the study were that lesser concentration of ozonated water was used to test the antifungal effect as the study utilised the multipurpose ozone sterilizer which is a portable machine which produces ozone only at a concentration of 0.5 mg/L. Even though the equipment which produces higher ozone concentrations has shown more efficient reduction in *Candidal* counts, it is impractical to prescribe that to a patient for denture cleansing. The equipments which produce higher concentration of ozonated water are mainly used for industrial purposes. Ozone exposure time was lesser in the present study due to the dissolving nature of ozone as it would be difficult to maintain same concentration of ozonated water for a longer time. So the time interval was kept for a shorter duration of 1, 5 and 15 minutes. The study conducted on only one strain of *Candida albicans*, further more investigations should be carried out on other oral microbes present in edentulous patients. More in-vivo research should be conducted to evaluate ozone antimicrobial potential in patients with denture stomatitis.

CONCLUSION(S)

Ozone is a relatively new therapeutic agent which promises to be an effective denture cleanser due to its antimicrobial effect. The antifungal activity of ozonated water at a concentration of 0.5 mg/L was efficient in reducing Candidal colonisation in heat cure resin when disk specimens were immersed for 15 minutes as compared with commercially available denture cleanser. Immersion and ultrasonication in ozonated water has shown to more effective against. Ozone could be used beneficially in denture cleaning protocol on regular basis for candidiasis patients by 15 minutes immersion of dentures in ozonated water generated by portable ozone generator.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Feb 17, 2025
- Manual Googling: Jul 05, 2025
- iThenticate Software: Jul 11, 2025 (15%)

ETYMOLOGY: Author Origin

EMENDATIONS: 6

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Feb 13, 2025**
Date of Peer Review: **May 03, 2025**
Date of Acceptance: **Jul 16, 2025**
Date of Publishing: **May 01, 2026**