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Antimicrobial effect of mushroom and ozone gas individually or combined

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ABSTRACT
Purpose: The purpose of this in-vitro study was to evaluate the effect of mushroom extract and ozone gas on streptococcus mutans.
Materials and methods: Suspensions of streptococcusmutans (NCTC 10449) in salt buffer were used to check the antimicrobial activity of aqueous extracts of mushroom and ozone. The test samples were divided as follow:Group I: Aqueous extract of mushroom, Group II: ozone gas, Group III: Aqueous extract of mushroom+ ozone gas. After exposure to different treatment, aliquots of the samples were spread on agar plate which was incubated at 37 C for 2 days. The number of bacterial colonies (CFU) on the plates was counted.
Results: There was a significantdifference between different groups ($f=31.69$, $p<0.001$). The highest value of bacterial count was recordedfor ozone therapy (8.97±0.18), followed by mushroom extract (8.69±0.45), while the lowest value of bacterial count was recorded for mushroom extract + ozone therapy (3.36±0.12).
Conclusions: mushroom extract + ozone gas show synergistic effect on streptococcus mutans.

Keywords: mushroom, ozone, streptococcus mutans.

Introduction
The minimal intervention dentistry nowadays is important for conservation of enamel and dentin in addition to elimination of cariogenic bacteria to maintain the process of remineralization through the caries removal procedure(1). Thus, cavity preparation should include removal of infected carious dentin and further maintain a sound and sterile cavity surface(2).The residual bacteria left after the preparation of the lesion can induce secondary caries, postoperative sensitivity, and even lead to pulp damage(3). To overcome the bacterial consequences, cavity disinfecting plays an effective role to eliminate the residual bacteria left in the cavity. Disinfection of remaining dental tissue is numerously recommended to be complemented by chemical agents with antibacterial properties (4).
Streptococcus mutans are considered to be the main etiological agents of dental caries due to effective colonization on the dental surface, carbohydrate metabolism, and lactic acid generation(5). Although cariogenic streptococci are present among the normal oral flora of humans, both diet and inefficient oral hygiene is the trigger for disease initiation and progression(6).
Mushroom extracts have a wide range of biomolecules withnutritional and medicinal substances with anti-inflammatory and antimicrobial properties. Studies have shown that the mushroom extracts have antimicrobial activity against gram-positive organism(7).Medicinal mushrooms, including *Lentinulaedodes*or shiitake, have been used in Asia for centuries

knowing to have numerous health benefits, shiitake mushroom contain many chemical compounds which protect DNA from oxidative damage[8].

Ozone is an energy-rich, and a highly unstable form of oxygen. It is a strong and fast cell wall and cytoplasmatic membranes oxidizingagent of bacteria and is considered to be one of the best bactericidal, antiviral and antifungal agents[9]. Despite that it has shown highlyantibacterial capacity, ozone probably lacks the ability to discriminate between various targets in an environment containing other substances than bacteria which affect the outcome of the antibacterial treatment[10].

The aim of the present in vitro study is to evaluate andcompare the antibacterial effect of mushroom extract, ozone gas and their combination against Streptococcus mutans.The null hypothesis for the present study was that the combination has a synergistic antibacterial effect on streptococcus mutans.

MATERIALS AND METHODS:

Sample size calculation:

A power analysis was designed to have adequate power to apply a statistical test of the null hypothesis that there is no difference would be found between tested groups. By adopting an alpha (α) level of (0.05), a beta (β) of (0.2) (i.e. power=80%), and an effect size (f) of (0.560) calculated based on the results of a previous study [4]; the predicted sample size (n) was found to be (30) samples (i.e. 10 samples per group). Sample size calculation was performed using G*Power version 3.1.9.7[11].

Preparation of mushroom extract:

About 50 g of dried shiitak mushroom was boiled in 500 mL of deionized distilled water to a final volume of 10–20 mL. The concentrated mixturewas filtered and stored in a refrigerator for 24h[12].

Determining Minimal Inhibitory Concentration (MIC) End Pointsof mushroom extract:

Pure strains of streptococcus mutans (ATCC 25175) were obtained from a standard microbiology lab. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes or microdilution wells as detected by the unaided eye or measured OD at 600 nm. Viewing devices intended to facilitate reading microdilution tests and recording of results may be used as long as there is no sample compromise in the ability to discern growth in the wells.

The amount of growth in the wells or tubes containing the antimicrobial agent compared with the amount of growth in the growth-control wells or tubes (no antimicrobial agent) used in each set oftests when determining the growth end points. For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) must occur in the growth-control well.Results are represented as ul/ml in table (1).

Table (1): Minimal Inhibitory Concentration End Points of mushroom extract:

sample / Pathogenic microorganism	Mushroom extract
Strept. Mutans ATCC25175	31.25ul/ml

Ozone generation:
The ozone was generated using coaxial dielectric barrier discharge (DBD) technique at the Center of Plasma Technology, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt as described by Khaled H. Metwaly et al. [13]. The DBD cell was fed by oxygen gas. The concentration of the generated ozone was controlled by the discharge current and the gasflow rate was adjusted to 615 ml/min. the ozone concentration of the gas was 2100 ppm, ±10%. The concentration of ozone inside each tube was measured using ozone analyzer (Model H1-AFX-Instrumentation, USA)

Sample grouping:
A total of 30 tubes were divided into three groups:
Group I: Aqueous extract of mushroom
Group II: ozone gas
Group III: Aqueous extract of mushroom+ ozone gas
Aliquots of 100 ul of the suspensions (10^10/ml) of S. mutanswere added to 9.9 mlsalt buffer in tubes. Ten falcon tubes were treated with mushroom extract at pre minimal inhibitory concentration (31.25 ul/ml). Other ten falcon tubes were exposed to ozone for 30 sec.The delivery of ozone wasstarted when bubbles were detected in the buffer[10]. The last ten were treated with mushroom extract at the pervious concentration then exposed to ozone for 30sec. After serially diluted in a salt buffer solution, 100 ul aliquots of the samples were spread on Mitissalivarius agar (Himedia, india) supplemented with bacitracin (10 mg/l) and potassium tellurite (3.3 mg/l) which were incubated in 5% CO2 and 95% air at 37 C for 2 days. The number of bacterial colonies (CFU) on the plates were counted.

Statistical analysis:
Numerical data was represented as mean and standard deviation (SD) values. Normality was checked using Shapiro-Wilk's test and by viewing data distribution. Bacterial count data were non-parametric and were positively skewed. They were log transformed, rechecked for normality and were found to be normally distributed. They were analyzed using one-way ANOVA followed by Tukey's post hoc test. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.1.3 for Windows.

Results
Results showed that there was a significantdifference between different groups ($f=31.69$, $p<0.001$). The highest value of bacterial count was recorded for ozone therapy (8.97±0.18), followed by mushroom extract (8.69±0.45), while the lowest value of bacterial count was recorded for mushroom extract + ozone therapy (3.36±0.12). Post hoc pairwise comparisons showed mushroom extract + ozone therapy to have a significantly lower value than other groups ($p<0.001$). Mean and standard deviation values for log bacterial count in different groups were presented in in table (2) and figure (1).

Table (2): Intergroup comparison for log bacterial count

Log bacterial count (Mean±SD)			*f-value*	*p-value*
Mushroom extract	*Ozone therapy*	*Mushroom extract + Ozone therapy*		

8.69±0.45^{A}	8.97±0.18^{A}	3.36±0.12^{B}	**31.69**	**<0.001***

Means with different superscript letters within the same horizontal row are significantly different*significant ($p<0.05$)

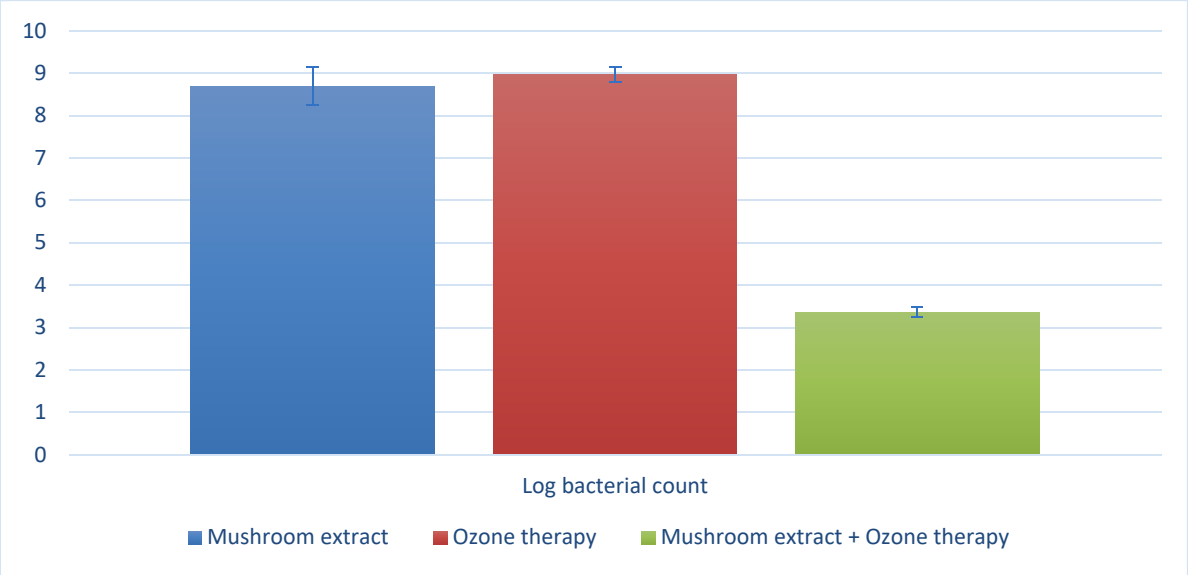

Figure (1): Bar chart showing mean and standard deviation valuesof log bacterial count in different groups

DISCUSSION

The removal of the cariogenic biomass appears to be essential for control of caries progression. A significant reduction of causative bacteria such as streptococcus mutanssuggested that there was absence of caries lesion activity, the restored microenvironment has less activity, and is less acidic as compared to carious tissue environment[14].

This study was designed to evaluate the antibacterial effect of mushroom extract, Ozone gas and combination of both on cariogenic bacteria.

Streptmutans was chosen as the test organism because they are one of the predominant inhabitants of dental plaque and have been implicated in the formation of dental caries because of their acidogenicand aciduric properties[12].

Mushroom contains erythritol which is 1,2 and 3,4-butanetetrol which has 70–80% sweetness more than that of sucrose. It is classified as a noncariogenic sweetener based on a study [15].

Ozone gas is among the pharmaceutically least invasive techniques used for caries preventionand treatment. It can be used alone or in combination with other techniques to treat carious lesions[16]. Caries management using ozone is conservative, and requires shorterperiods of mouth opening. In addition, ozone is efficient in killing and reducing different cariogenic micro organisms such as streptococcus mutans, streptococcus sobrinus and lactobacilli[17].

Colony forming unit (CFU) is a measure of viable bacterial cellsnumbers in CFU/mL. These are an indication of the number of remaining viable cells that able to proliferate and form small colonies[18].The two advantages of CFU are the capacity for counts of any number of

bacteria using dilutions, if too many, or concentrations if too few and viable bacteria are the only bacteria counted with this method as the CFU method excludes dead bacteria and debris[19].

In the present study, the highest value of bacterial count was recorded for ozone therapy, followed by mushroom extract, while the lowest value of bacterial count was recorded for mushroom extract + ozone therapy. The difference in mean *S. mutans*among the groups was found to be statistically significant ($p < 0.001$).

The mushroom extract showed antibacterial activityagainst *S. mutans*. Mushroom extracts have some active, low-molecular-weight compounds (plectasin, confuentin, grifolin, neogrfolin) that show antibacterial action[20]. The low molecular mass (LMM) fraction of extracts from an edible mushroom called Shiitake mushroom (Lentinusedodes) are mainly secondary metabolites such as sesquiterpenes and other terpenes, steroids, and benzoic acid derivatives[21]. High molecular mass compounds are mostly peptides and proteins.

This proves bacteriostatic action of Shiitake mushrooms by inhibiting thesynthesis of DNA. This bacteriostatic effect is also confirmed by morphological effects by the LMM fractions which show elongation of the bacteria with interrupted septa. The morphogenetic effects which induced by the mushroom similar to those observed in streptococcal thermosensitive temperature or exposed to inhibitory doses of B-lactam antibiotics [22].

Moreover, the*S. mutans*showed no adherence to glass in the presence of erythritol which suggests that erythritol is a sugar not used by bacteria for synthesis of glucans. Since it is not used by these bacteria, the byproduct lactic acid is not produced[15].

The present study showed S. mutans sensitivity to the ozoneexposurefor 30s which is lower than mushroom extract. Ozone gas is reported to have a strongoxidizing power with a reliable bactericidal effect by mediating oxidation that destroys the cell walls and cytoplasmic membranes of bacteria beside its low cytotoxicity with a rapid degrading just after contact with organic compounds[4]. It showed significant effectiveness in reducing the numbers of *S.mutans*in dental samples via a mechanisminvolving the rupture of their membranes[23].

Previous studies stated that not only the application time of ozone but also the dose used plays an important role on the antibacterial effect of ozone. An application of a higher ozone dose would probably need a lower application time in order to get higher disinfecting effect[24].

Furthermore, another study stated that 80sozone application time seems to be insufficient to exert an optimal antibacterial effect on the infected dentin surface and vacuum applicatorof the ozone device might have been inadequate forreaching the cylindrical cavity surfaces[4].

The combination between mushroom extract and ozone gas in the present studyhas a significant lower value of bacterial count than other groups. The bacteriostatic action of Shiitake mushrooms by inhibiting the synthesis of DNA of bacteria was added to a strongoxidizing power of ozone which destroys the cell walls and cytoplasmic membranes of bacteria leading tosynergisticantibacterial effect.

The null hypothesis that the mushroom extract and ozone have synergistic antibacterial effect on streptococcus mutans was accepted as the results demonstrated that there was a statistically significant difference between the combination and each material alone.

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