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COMPARATIVE EVALUATION OF ANTIBACTERIAL EFFECTS OF HYDROGEN WATER AND CHLORHEXIDINE MOUTHWASH ON *PORPHYROMONAS GINGIVALIS* AND *FUSOBACTERIUM NUCLEATUM*: AN IN – VITRO MICROBIOLOGICAL STUDY

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ABSTRACT

Introduction: Periodontitis is an inflammatory disease of supporting tissues of teeth initiated by microorganisms resulting in progressive destruction of tissues and eventual loss of tooth. The use of chemical agents such as chlorhexidine to combat microbial load in oral cavity is proven to be gold standard but duration of action of such agents is short lived and cannot be used lifelong. Therefore, use of natural agents such as "hydrogen water" which can be consumed on daily basis and also have antibacterial effects on oral bacteria is desirable. **Objective:** To compare the antibacterial effect of hydrogen water and chlorhexidine mouthwash *on P. gingivalis* and *F. nucleatum*. **Methodology:** In this in – vitro study, antibacterial effect of hydrogen water will be tested on *P. gingivalis* and F. nucleatum cultures by using disc diffusion method and compared with chlorhexidine mouthwash and saline. After incubation colony forming units of respective pathogens will be measured and compared. **Result:** The antimicrobial activity of 100% hydrogen water against *P. gingivalis* showed statistically insignificant difference ($p > 0.05$) when compared to 0.2% Chlorhexidine mouthwash. While against *F. nucleatum* a statistically significant difference ($p < 0.05$) was seen. **Conclusion:** Though 0.2% Chlorhexidine remains the gold standard, 100% hydrogen water also has an almost equivalent antimicrobial activity against *P.gingivalis* and *F. nucleatum*. However, when 100% hydrogen water is diluted the antimicrobial activity decreases and becomes negligible beyond 50% hydrogen water.

KEYWORDS: Hydrogen water, 0.2% Chlorhexidine mouthwash, antimicrobial activity, P.*gingivalis, F. nucleatum.*

INTRODUCTION

Periodontitis is defined as "an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with increased probing depth formation, recession, or both."[1] Dental plaque biofilm is responsible for the initiation and progression of periodontitis, which may be tooth or tissue associated. Tooth surface-associated plaque is characterized by Gram-positive rods and cocci including *Streptococcus mitis, Streptococcus sanguis, Actinomyces viscosus, Actinomyces naeslundii,* and *Eubacterium* species. The tissue-associated plaque shows a predominance of *Streptococcus oralis, Streptococcus intermedius, Peptostreptococcus micros, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythus,* and *Fusobacterium nucleatum.*[2]

To prevent periodontal disease and limit its progression, an overall reduction of bacteria present in dental plaque is the prerequisite.[3] Till date, mechanical plaque control remains the primary and most widely accepted means of controlling plaque and maintaining good oral hygiene.[4] As an adjunct to this, various chemotherapeutic agents are used to combat the microbial load in oral cavity.[5]

The most frequently used antiplaque agent is chlorhexidine. Chlorhexidine mouthrinse is an effective agent in preventing and controlling plaque formation, breaking up existing plaque and inhibiting & reducing development of gingivitis.[6] Although clinical effectiveness of CHX has been well documented, it has

abounding adverse effects such as brownish discoloration or staining of teeth, restoration and tongue, irritation, soreness and desquamation of oral mucosa, altered taste sensation, unilateral or bilateral parotid swelling, enhanced supragingival calculus formation[7,8], hence restricting long-term utilization.

These enumerous limitations led to the development of safer and natural alternative oral hygiene products, which can be consumed on a daily basis and also have antibacterial effects on oral bacteria. One such undercover agent is "*Hydrogen water*".

Hydrogen is one of the nature's most simple elements. As a gas it is a colourless, tasteless, odourless, lightest, highly flammable, with smallest diatomic molecule and most abundant element in space.[9,10]

According to contemporary surveys, H_2 owns antioxidant, anti-inflammatory, antiapoptotic, anti-allergy and anti- cancer effects.[11,12] It is known to significantly reduce oxidative stress by selectively reducing free radicals such as hydroxyl (OH^-) and peroxynitrite ($ONOO^-$).[13] Hydrogen water (HW) has been known to show antibacterial activities on standard strains *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia* and *Treponema Denticola.*[14]

It has also been proven to be an antifungal agent by showing its activity against *C. albicans* biofilm.[15] Furthermore, Hydrogen water has also been beneficial in suppressing the progression of periodontitis by decreasing gingival oxidative stress.[16]

To forecast the above applications of hydrogen water, we conducted a study on periodontopathogens mainly *Porphyromonas gingivalis* and *Fusobacterium nucleatum* to evaluate and compare the antibacterial effect of various concentrations of hydrogen water with 0.2% Chlorhexidine digluconate mouthwash and 0.9% normal saline.

MATERIAL AND METHODS

1. **Generation of hydrogen water: -** Hydrogen water was generated using a Commercially available Hydrogen generator supplied by **Firstmark Hydrogen rich Alkaline water solution (Tyent, India)**

2. **PROCEDURE:-** The present study was an in- vitro microbiological study conducted in Maratha Mandal's Central Research Laboratory, Belgaum, to assess and compare the antibacterial effect of various concentrations of hydrogen water (Group1- 100% HW, Group 2 – 75% HW, Group 3 – 50% HW) with 0.2% Chlorhexidine digluconate mouthwash (Group 4- Chlohex Plus) and 0.9% normal saline (Group 5 – negative control) against *Porphyromonas gingivalis* and *Fusobacterium nucleatum.*

2.1 Preparation of various concentrations of hydrogen water – 1200 µL of 100% hydrogen water was taken without dilution, whereas for 75%. HW, 900 µL of HW was diluted with 300µl of distilled water and for 50% HW, 600µl of HW was diluted with 600µ distilled water.

2.2 Culturing of *Porphyromonas gingivalis* and *Fusobacterium nucleatum*

Porphyromonas gingivalis was cultured on Brucella blood agar which was incubated anaerobically for 4- 5 days at 37°C in an anaerobic jar. The same culture conditions as that of P. gingivalis were carried out for *Fusobacterium nucleatum*. After pertinent incubation period, the growth of bacteria was checked.

2.3 Preparation of bacterial suspension

Using a sterile loop, the colonies of *P. gingivalis* & *F. nucleatum* were transferred to separate test tubes both containing Thioglycollate broth as a growth media for preparation of *P. gingivalis* suspension and *F. nucleatum* suspension respectively.

Visually, the turbidity of suspension was adjusted to equal that of 0.5 McFarland turbidity standard that has been vortexed.

2.4 Preparation of broth-by Broth dilution method

For each 200 µl of *P. gingivalis* suspension and *F. nucleatum* suspension, 200 µL of each component i.e 100% HW (group 1), 75% HW (Group 2), 50% HW (group 3), 0.2% CHX mouthwash (group 4) and 0.9 % normal saline (group 5) were added and inoculums were formed.

2.5 Incubation of inoculum

The prepared inoculums were incubated anaerobically for 72 hours at 37°C.

2.6 Streak Culture Method

For surface plating, three agar plates were taken to assess the antibacterial effect by measuring the colony forming units.

The first agar plate was divided into 3 parts for Group 1, 2, 3 & tested against prepared *P. gingivalis* inoculum. The second agar plate was again divided into 3 parts for Group 1, 2, 3 & tested against prepared *F. nucleatum* inoculum. The third agar plate was divided into 4 parts for Group 4 and 5 & tested against prepared inoculum for *P. gingivalis* and *F. nucleatum* respectively.

The above surface plating was done using a sterile loop and the prepared inoculums were transferred by streaking zig-zag movements without digging into the agar plates.

2.7 Incubation of agar plates

The surface plated agar plates were incubated anaerobically for 48-72 hours at 37°C. If the bacterial growth was confluent or nearly confluent, only then the reading of the plates were carried out.

2.8 Checking of colony forming units:

The colony forming units of *P. gingivalis* and *F. nucleatum* were checked using colony counter. After measurements, intergroup comparison was done.

STATISTICAL ANALYSIS

To assess the reliability of data obtained, statistical analysis was performed using Statistical Package for Social science (SPSS) version 21 for Windows. Descriptive quantitative data was expressed in mean and standard deviation respectively. Data normality was checked by using Shapiro – Wilk test. Confidence interval was set at 95% and probability of alpha error (level of significance) set at 5%. Power of the study was set at 80%.

Overall intergroup comparison among five groups for each periodontal pathogen in relation to CFU counts was done using One-way Anova 'F' test followed by Tukey's post hoc test for pairwise intergroup comparison between each group.

RESULTS

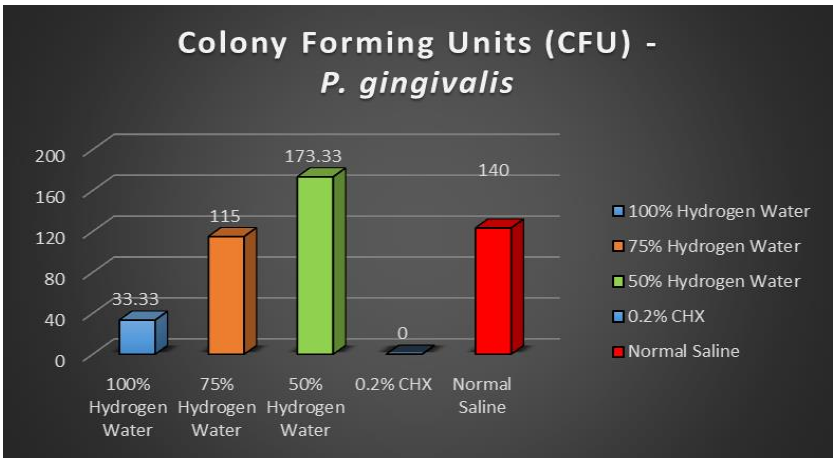
The antibacterial effect of hydrogen water and Chlorhexidine mouthwash on bacterial count of *P.gingivalis* and *F. nucleatum* were bestowed in the following results.

A. *P. gingivalis*

1. Colony Forming units of different components against *P. gingivalis*

In- vitro bacterial growth of *P. gingivalis* was observed in all groups, except group 4 (CHX) which did not show any colonies and accentuated growth in group 2,3,5 as compared to group 1.

It was found that *P. gingivalis* is highly sensitive to 0.2% CHX while with 100% HW the mean CFU is 33.33 (Graph 1)



Graph 1: Colony Forming Units of different groups.

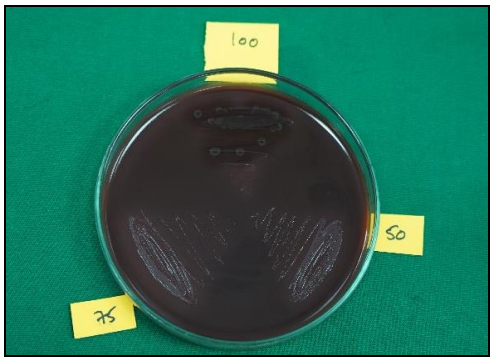


Figure 1: P. gingivalis growth in presence of different concentration of Hydrogen water on blood agar.

2. Intergroup Comparison of anti-microbial activity (CFU) of different components against *P. gingivalis*

Intergroup comparison was done using one way ANOVA 'F' test (Table 1, Graph 1) which shows p - value of < 0.001, suggestive of statistically highly significant difference.

Table 1: Comparison of anti - microbial activity (colony forming units) of different media against *P. gingivalis* using One-way Anova 'F' test.

CFU numbers (*P.gingivalis*)	Mean	SD	One-way Anova 'F' test	p value, Significance
100% Hydrogen Water	33.33	16.63	F = 30.161	p<0.001**
75% Hydrogen Water	115.0	17.88		
50% Hydrogen Water	173.33	35.02		
0.2% CHX	0.0	0.0		
Normal Saline	140	56.09		

****p<0.001 – highly statistically significant difference**

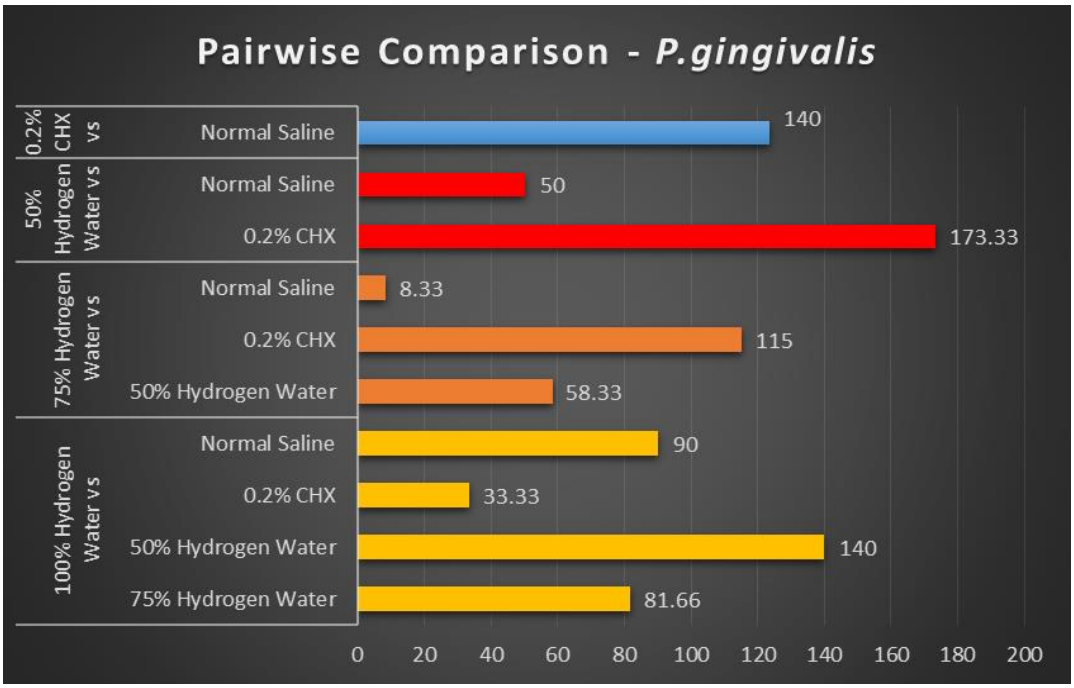
3. Pair wise comparison of antimicrobial activity (CFU) of different components against *P. gingivalis*

It was done using Tukey's post hoc test (Table 2, Graph 2). There was a statistically highly significant difference (p <0.001) when 100% HW was compared to 50 % HW and 0.9% normal saline, and when 0.2% CHX was compared to 75% HW, 50% HW and 0.9% NS. A statistically significant difference (p < 0.05) was seen when 100% HW was compared to 75% HW and when 75% HW compared with 50% HW. However, statistically insignificant difference (p > 0.05) was seen when100% HW was compared to 0.2% CHX and when 0.9%. NS was compared to 75% HW and 50% HW.

Table 2: Pairwise comparison of anti - microbial activity (colony forming units) of different media against *P. gingivalis* using Tukey's post hoc test.

Group	Comparison Group	Mean Difference	P value, Significance
100% Hydrogen Water vs	75% Hydrogen Water	81.66	P =0.001*
	50% Hydrogen Water	140.0	P<0.001**
	0.2% CHX	33.33	p =0.379
	Normal Saline	90.0	p<0.001**
75% Hydrogen Water vs	50% Hydrogen Water	58.33	p =0.028*
	0.2% CHX	115.0	P<0.001**
	Normal Saline	8.33	P =0.990
50% Hydrogen Water vs	0.2% CHX	173.33	P<0.001**
	Normal Saline	50.0	P =0.075
0.2% CHX vs	Normal Saline	140	P<0.001**

p>0.05 – no significant difference, *p<0.05 – significant, **p<0.001 – highly significant



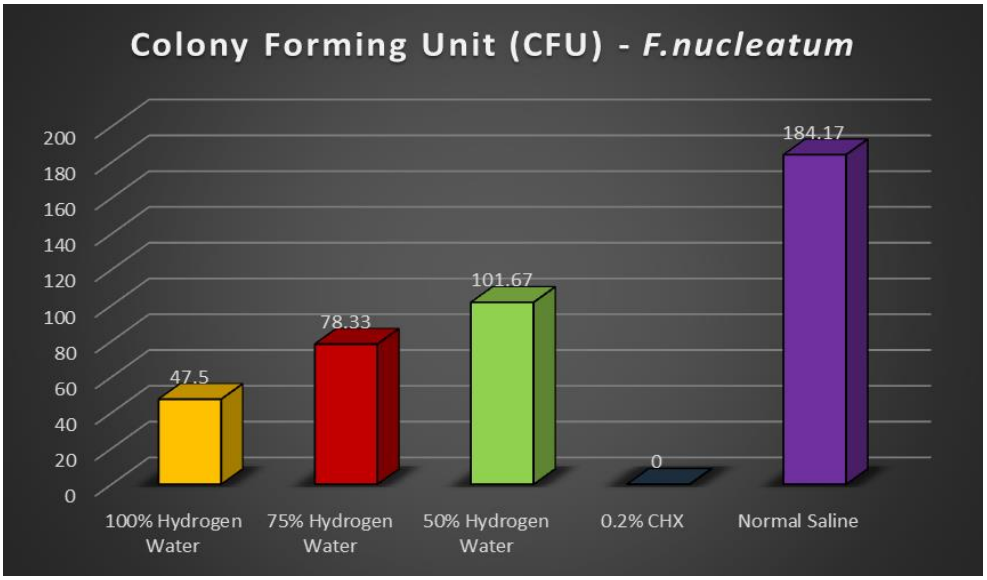
Graph 2: Pairwise comparison of anti - microbial activity (colony forming units) of different media against *P. gingivalis* using Tukey's post hoc test.

B. *F. nucleatum*

1. Colony forming units of different components against *F. nucleatum*

In- vitro bacterial growth of *F. nucleatum* was observed in all groups except group 4 (CHX) which did not show any colonies, followed by accentuated growth observed in Group 1, 2, 3and 5 respectively.

Thus, it was found that *F. nucleatum* is highly sensitive to 0.2% CHX, while with 100% HW the mean CFU were 47.5 (Graph 3).



Graph 3: Colony forming units of different groups against *F. nucleatum*.

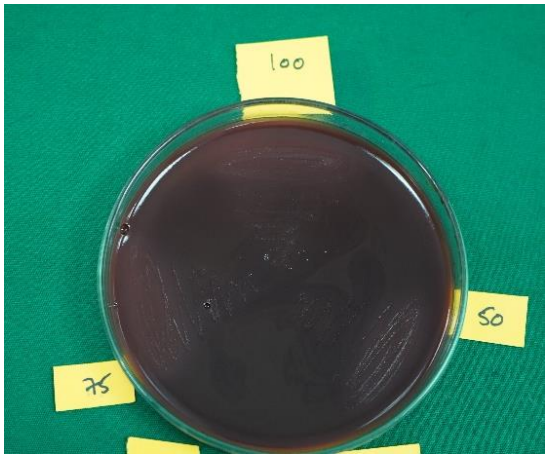


Figure 2: *F. nucleatum* growth in presence of different concentration of Hydrogen water on blood agar.

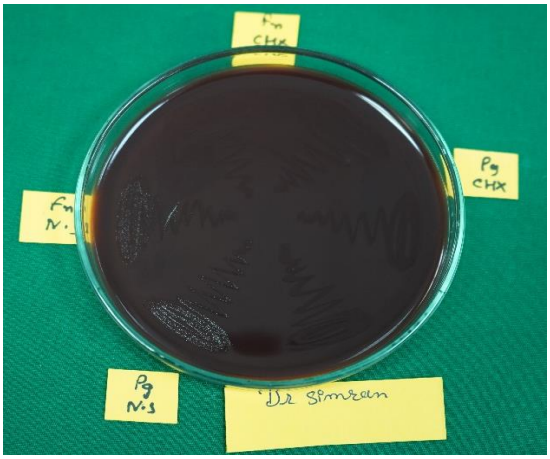


Figure 3: *P. gingivalis* and *F. nucleatum* growth in presence of Chlorhexidine and normal saline.

2. Intergroup comparison of antimicrobial activity of different components against *F.nucleatum*

It was done using one Anova F test (Table 3, Graph 3) which shows p- value of < 0.001 suggestive of statistically highly significant difference.

Table 3: Comparison of anti - microbial activity (colony forming units) of different media against *F.nucleatum* using One way Anova 'F' test.

CFU numbers *(F.nucleatum)*	Mean	SD	One way Anova 'F' test	p value, Significance
100% Hydrogen Water	47.5	19.42	F = 52.185	p<0.001**
75% Hydrogen Water	78.33	28.75		
50% Hydrogen Water	101.67	33.56		
0.2% CHX	0.0	0.0		
Normal Saline	184.17	19.08		

****p<0.001 – highly significant**

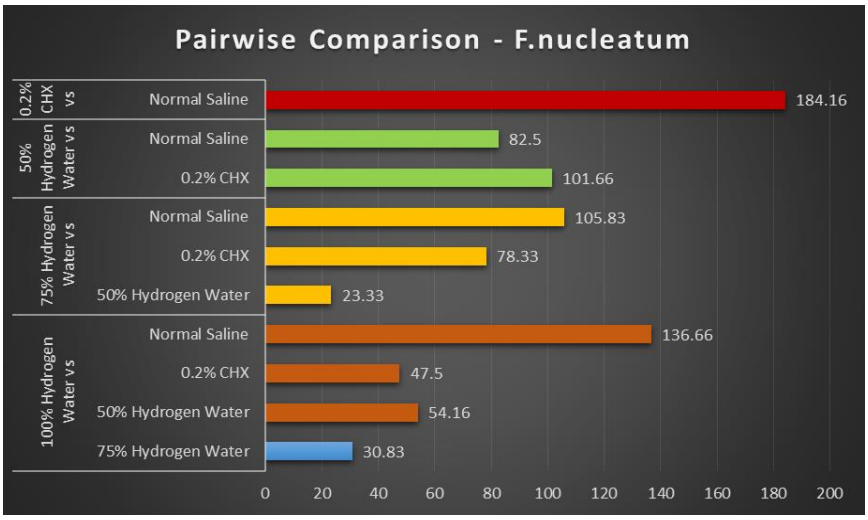
3. Pairwise comparison of antimicrobial activity of different component against *F. nucleatum*

It was done using Tukey's post hoc test (Table 4, Graph 4). There was a statistically highly significant difference (p <0.001) when 0.9% NS compared to all groups and when 0.2% CHX compared to 75% HW and 50% HW. A statistically significant difference (p <0.05) was seen when 100% HW was compared to 50% HW & 0.2% CHX. However, statistically insignificant difference (p > 0.05) when 100% HW was compared to 75% HW & when 75% HW compared to 50 % HW.

Table 4: Pairwise comparison of anti - microbial activity (colony forming units) of different media against *F.nucleatum* using Tukey's post hoc test.

Group	Comparison Group	Mean Difference	P value, Significance
100% Hydrogen Water vs	75% Hydrogen Water	30.83	P =0.178
	50% Hydrogen Water	54.16	P =0.004*
	0.2% CHX	47.5	p =0.013*
	Normal Saline	136.66	p<0.001**
75% Hydrogen Water vs	50% Hydrogen Water	23.33	p =0.429
	0.2% CHX	78.33	P<0.001**
	Normal Saline	105.83	P<0.001**
50% Hydrogen Water vs	0.2% CHX	101.66	P<0.001**
	Normal Saline	82.5	P<0.001**
0.2% CHX vs	Normal Saline	184.16	P<0.001**

p>0.05 – no significant difference, *p<0.05 – significant, **p<0.001 – highly significant



Graph 4: Pairwise comparison of anti - microbial activity (colony forming units) of different media against *F.nucleatum* using Tukey's post hoc test

The above results stipulate that 0.2% CHX mouthwash shows the best anti-bacterial effect against both *P. gingivalis* and *F. nucleatum* respectively, while amidst the remaining groups, group 1- 100% HW shows an almost equivalent anti-bacterial effect against *P. gingivalis* and *F. nucleatum* respectively.

DISCUSSION

Benjamin Franklin once quoted, **"An ounce of prevention, is worth a pound of cure".** This couldn't be better elucidated than through our understanding and knowledge of prevention of periodontal diseases. Prevention of periodontal diseases consists of patient performed control of dental biofilm and professional interventions which includes patient motivation, home care instructions & anti-infective therapy. Anti-infective therapy encompasses a combination of mechanical and chemical plaque control approaches which serves as an aid to eradicate the primary etiology of gingivitis and periodontitis. Chemotherapeutic agents play an adjunctive role to mechanical debridement therapy and not surprisingly, till date a large number of chemical agents have been tested for their ability to reduce plaque accumulation. A literature review, highlighted chlorhexidine as not only a plaque control agent but also as an effective antimicrobial agent.[17] Though, chlorhexidine is gold standard, it is well said by **Sir Thomas** that, "**Natural things are glorious, and to know them is Glorious**."

Hence, pioneering of masked natural agents depicting antibacterial effect on oral microorganism is prudent. One such natural agent proven to have antibacterial property is Hydrogen water.[4,14,18] The antibacterial activity of hydrogen water has been proven because of the combined action of hydrogen ion concentration, high oxidation- reduction potential and presence of extremely bactericidal hypochlorous acid (HOCl).[14]

The present study is an in-vitro microbiological study conducted to assess and compare the antibacterial effects of various concentration of hydrogen water and 0.2% CHX against *P. gingivalis* and *F. nucleatum*.

In this study, the antibacterial effect was evaluated by measuring the colony forming units by surface plating on agar plate. After speculated incubation period, it was found that the antibacterial activity of 100% HW is almost equivalent to that of CHX which showed the best antibacterial activity against *P. gingivalis* and *F. nucleatum* respectively ($p<0.001$). As 100% HW was diluted, its antibacterial activity also diminished.

Amidst the sparse evidences on antibacterial effect of hydrogen water, on the whole studies are in line with the result of the present study. A study by **Nayak et al (2021)**[5] also showed a similar result of antibacterial effect of HW. They found that HW has an antibacterial effect on microorganisms associated with chronic periodontitis ($p<0.001$). The results of our present study were similar to the above study where 100% HW and 0.2% CHX showed better efficacy in reduction of colony forming units of *P. gingivalis* and *F. nucleatum* respectively.

Another study by **Lee and Choi (2006)**[14], also demonstrated the antibacterial effect of electrolyzed water against periodontopathogens in-vitro. The electrolysed tap water (puri-water) significantly showed reduction in the growth of *P. gingivalis* and *F. nucleatum* in culture ($p<0.05$). The result of our study is analogous to the above study.

Yet another study by **Baek et al (2013)**[18] also showed results in accordance with our present study. They evaluated the antibacterial activity of hydrogen rich water against *F. nucleatum* and *P. gingivalis* & compared with tap water. They found a significant difference ($p<0.05$) in colony forming units of *P. gingivalis* and *F. nucleatum*. Also, they concluded that HW may have a positive impact on oral hygiene by aiding to remove cariogenic bacteria and periodontopathogens.

This might may be attributable to the similar methodology and the antibacterial mechanism of hydrogen water. The hydrogen water has the property of scavenging reactive oxygen species and induction of certain antioxidant enzymes thus suppressing the progression of disease. However, the exact mechanism by which the hydrogen water reacts with bacterial membrane is beyond the scope of this study.

Besides the antibacterial effects of HW, it possesses certain other properties such as anti-inflammatory, anti-apoptotic, anti-allergy, anti-cancer, antioxidant and cytoprotective roles.[13]

Azuma et al (2015)[19] conducted a pilot study to compare the effects of HW along with non-surgical periodontal treatment in periodontitis patients. They found greater improvements in probing pocket depth and clinical attachment level in HW group than in the control group. This is accountable to the antioxidative capacity of HW that suppressed the inflammation in periodontal tissues.

However, our present study is strictly confined to evaluate the antibacterial effect of hydrogen water and therefore further research and clinical trials on antioxidant potential and various other applications of Hydrogen water needs to be explored.

LIMITATIONS

The present study has certain deficiencies, firstly, it is an in-vitro study so does not simulate the oral cavity conditions, hence need of clinical trials is mandatory. Secondly, only two periodontopathogens mainly *P. gingivalis* and *F. nucleatum* were considered while periodontitis is polymicrobial disease. Also, with time

the efficacy of hydrogen water may be lost as its half-life is 0-8 hours and accordingly the antibacterial activity may be hampered.

CONCLUSION

Though 0.2% Chlorhexidine remains the gold standard, 100% hydrogen water also has an almost equivalent antimicrobial activity against *P.gingivalis* and *F. nucleatum*. However, when 100% hydrogen water is diluted the antimicrobial activity decreases and becomes negligible beyond 50% hydrogen water comparable to 0.9% normal saline. Thus, consumption of 100% hydrogen water without any dilution can serve as an alternative to limit the growth of potential putative periodontopathogens. This astonishing inference of something as legitimate as water having antibacterial property without any adverse effect has led to progressive research of hydrogen water and its application in dentistry.

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