

# Microbiological evaluation of the effects of hyperbaric oxygen on periodontal disease

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## SUMMARY

The term periodontitis indicates a variety of clinical manifestations of infectious disorders in which the supporting tissues of the teeth are attacked. The initiation and progression of periodontal disease are attributed to the presence of elevated levels of pathogenic bacteria within the gingival crevice. Approaches to periodontal treatment range from surgical to regenerative therapy and anti-infective chemotherapy. Anti-infective drug therapy should be rationally based on the composition of the pathogenic microbiota. It is also important to recognize that the periodontopathic plaque constitutes a bacterial biofilm infection that may render the resident microorganisms more resistant than the same organisms grown planktonically. Hyperbaric oxygen (HBO) has been successfully used in several medical applications. The therapeutic effect is related to elevated partial oxygen pressure in the tissues. The aim of this study was to evaluate the effects of HBO on a selected number of patients suffering from adult chronic periodontitis in comparison with surgical intervention (scaling and root planning, SRP), as well as the effects of a combination of both therapies on the evolution over time of the microflora of the periodontal pockets. Bacteria were detected either by culture or by a molecular method (PCR). Microbiological data indicate that the combination of HBO and SRP substantially reduced (by up to 99.9%) the gram-negative anaerobe loads of the subgingival microflora. The low values of pathogens persisted for at least two months after the therapy. HBO or SRP alone produced a temporarily more limited effect on periodontal anaerobes. Additional experimental confirmation of these results was provided by molecular detection of the main periodontopathic bacteria with a significant reduction in the number of dental sites which harboured them. It is also shown that HBO both alone and in combination with SRP reduced the Gingival Index value to zero and gingival health persisted for 3 months at least. Thus, in parallel with the loss of periodontopathic bacteria, a substantial improvement in oral health was observed. In conclusion, this study has shown that HBO may represent a useful aid, especially in combination with SRP, as far as non-surgical periodontal therapy is concerned.

**KEY WORDS:** Oral microbiology, Periodontitis, Hyperbaric oxygen

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## INTRODUCTION

The term periodontitis indicates a variety of clinical manifestations of infectious disorders in which the supporting tissues of the teeth are at-

tacked. In periodontal diseases the junctional epithelial tissue at the base of the gingival crevice migrates down the root of the tooth with the consequent formation of a periodontal pocket.

The initiation and progression of periodontal disease are attributed to the presence of elevated levels of pathogenic bacteria within the gingival crevice (Nishihara and Koseki, 2004, Socransky and Haffajee, 2005). Several hundred recognized species of microorganisms inhabit the gingival crevice; however, it has been shown that only a few of these play a significant role in the aetiology of periodontal disease. Indeed, it is generally

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accepted that a consortium of bacteria, and not just a single microorganism, is involved in such diseases. However, the mere presence of putative periodontopathogens in the gingival crevice is not sufficient to initiate the inflammation process. Elevation of the relative proportions of these bacteria is the crucial step, such that a sufficient critical mass is achieved to mount an effective tissue-damage response.

Susceptibility to advanced periodontal disease is not universal; there is a moderate prevalence of destructive periodontal disease (Hansen *et al.*, 1990; Sheiham, 1991; Brown and Löe, 1993; Gjermo, 1998; Sheiman and Netuveli, 2002), while the worldwide prevalence of severe periodontal disease is very low (Gjermo, 1998; Albandar *et al.*, 1999; Corbet, 2006; Meyle, 2006). On the contrary, mild gingivitis is common and most adults present some loss of bony support and loss of probing attachment (Sheiman and Netuveli, 2002). Considering the common occurrence of periodontitis and the vast amount of periodontal research performed during the last three decades, it is to be expected that the most appropriate management of the various types of periodontal disease should now be a matter of agreement among dentists. Approaches to periodontal treatment range from surgical to regenerative therapy and anti-infective chemotherapy. Controversy exists as to when to perform periodontal surgery or whether to employ nonsurgical antimicrobial therapy. It is generally accepted that anti-infective therapy is a fundamental issue in periodontal treatment. Anti-infective drug therapy should be rationally based on the composition of the pathogenic microbiota. It is also important to recognize that the periodontopathic plaque constitutes a bacterial biofilm infection that may render the resident microorganisms up to 1,000-fold more resistant than the same organisms grown planktonically (Gilbert *et al.*, 1997; Socransky and Haffajee, 2002). Therapy should be specific for the periodontal pathogens identified, and bactericidal agents are preferred. A combination of antimicrobial agents and approaches is often required to achieve broad-spectrum coverage against multiple pathogens, to provide drug synergy and to prevent the development of resistance (Roberts, 2002). In general, treatment of progressive periodontitis should be multifaceted and vigorous, including mechanical, antibiotic

and antiseptic therapy, and the various treatment approaches should be employed within a short time period (Slot and Jorgensen, 2002).

Because of the need, as indicated by the literature, to eliminate/reduce the periodontopathic bacteria in order to cure or prevent periodontal disease, novel approaches (both surgical and non-surgical) deserve attention, in that they may provide useful alternatives to the conventional approaches, or in combination with them. Hyperbaric oxygen (HBO) has been successfully used in several medical applications. The therapeutic effect is related to elevated partial oxygen pressure in the tissues. The pressure itself enhances oxygen solubility in the tissue fluids (Kindwall *et al.*, 1991). Well known are the effects of HBO on osteointegration (Granström, 1992). Principally, HBO has been shown to affect angiogenesis (Marks *et al.*, 1994), bone metabolism and bone turnover (Johnsson *et al.*, 1999). Finally, of by no means of secondary importance may be the role of HBO on the growth and survival of anaerobic bacteria, the commonest aetiological agents of periodontal diseases. The possibility, thus, of interfering with bacterial viability by means of a novel approach other than the prescription of antibiotics or oral disinfectants, could allow us to overcome the difficulties arising during antibiotic chemotherapy as a result of the fact that oral bacteria form a biofilm.

In this study we evaluated the bacterial load in the subgingival plaque in patients with adult chronic periodontitis before, immediately after and some time after a treatment cycle of HBO. Bacteria were detected either by culture or by a molecular method (PCR). Results show a dramatic reduction in the most common periodontopathogens as well as an improvement in clinical signs.

## MATERIALS AND METHODS

### *Patient selection*

Twenty patients were enrolled from among those attending the Dental Clinic of the University of Verona (12 males and 8 females, aged 30-45 years). Adult chronic periodontitis was diagnosed for all of them and their periodontal pockets were deeper than 6 mm. The patients enrolled had not received periodontal therapy in the previous 2

years at least and presented no chronic diseases such as allergies, diabetes, liver and kidney disease. None of the patients were either smokers or drinkers. All were aware of the experimental design and gave their written informed consent. Before proceeding with any therapy or sample collection, all the patients enrolled underwent supragingival oral hygiene by ultrasonic application.

#### *Experimental design*

The oral cavity of each patient was vertically divided into two portions to identify the right and left halves. One half did not receive any treatment, while the other was surgically treated by scaling and root planning (SRP). Ten patients were exposed to a cycle of HBO consisting in 10 daily sittings. At each sitting the patients breathed HBO at 2.5 ATA (0.25 pa) pressure for a total of 92 minutes. The hyperbaric therapy was carried out at the Istituto Iperbarico S.p.A located in Villafranca, Verona (Italy).

The following 4 types of dental sites were identified:

- 1) pockets treated with both SRP and HBO (SRP+/HBO+);
- 2) pockets treated with HBO alone (SRP-/HBO+);
- 3) pockets treated with SRP alone (SRP+/HBO-);
- 4) pockets receiving no treatment (SRP-/HBO-).

#### *Sample plaque collection*

Two plaque samples (ca. 1 mg) were collected from each periodontal pocket with a sterile curette. One sample was dispersed in 0.5 ml of thioglycollate medium (Difco) and immediately transferred to the microbiology laboratory for bacterial detection by culture. Sample processing was performed within one hour of collection. The second sample was dispersed in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and stored at -80°C before molecular analysis. Plaque samples were collected at time zero, then at 15 days corresponding to the end of the HBO therapy if performed, and 45 and 75 days later.

#### *Microbiological evaluation by culture*

Before plate seeding, samples were treated for 45 s in an ultrasonic bath in order to disgregate the bacterial mass. This was the minimum time period needed to obtain the highest bacterial count.

The following media were used: to enumerate total bacteria, plates of Schaedler Blood Agar (S-BA) supplemented with vitamin K (DID) were used, while to enumerate gram-negative anaerobes Schaedler Agar containing vitamin K, kanamycin and vancomycin (KKVA) was used. Plates were incubated at 37°C for 72 hours in an anaerobic atmosphere in an MK3 anaerobic work station and incubator (du Scientific). The colonies appearing were counted and numbers referred to one mg of plaque.

#### *Detection of bacteria by molecular method*

Plaque samples were thawed and the resulting DNA was purified with the aid of the Extragen kit (Amplimedical) according to the manufacturer's instructions. Two distinct Multiplex PCR amplifications were performed using the primers described by others (Majanagi *et al.*, 2004; Montebugnoli *et al.*, 2004). One Multiplex PCR (annealing temperature 58°C) allowed us to detect *Porphyromonas gingivalis* (404 bp) and *Prevotella intermedia* (259 bp) in the plaque sample, while with the other (annealing temperature 60°C) *Fusobacterium nucleatum* (705 bp), *Treponema denticola* (316 bp) and *Actinobacillus actinomycetemcomitans* (273 bp) were identified.

#### *Statistical analysis*

The data collected for each case and sample were typed onto a spreadsheet and statistically analysed using SPSS for Windows (SPSS Inc., Chicago, IL). The data for each dental site were analysed for normality of distribution with  $\pm 2$ . Differences in total microbial counts, counts of specific microorganisms and clinical features between subgroups were considered statistically significant at  $P < 0.05$ .

## **RESULTS**

#### *Enumeration of bacteria in the subgingival plaque by culture*

Total bacterial load (Table 1) and gram-negative anaerobes (Table 2) were evaluated on SBA and KKVA plates, respectively. Table 1 shows that there were no statistically significant variations ( $P > 0.05$ ) in the mean total bacterial load of the various groups, either in the case of the single (SRP-/HBO+ and SRP+/HBO-) or double treatment

TABLE 1 - Mean values of total culturable bacterial load per mg of subgingival plaque (growth on SBA plates) in the different groups. In each column, P values are calculated versus the SRP-/HBO- group.

Group	Time (days)			
	0	15	45	75
SRP+/HBO+	$5.83 \times 10^6 \pm 4.25 \times 10^6$ p=0.407	$1.72 \times 10^6 \pm 1.16 \times 10^6$ p=0.409	$5.05 \times 10^5 \pm 5.0 \times 10^5$ P=0.0542	$1.50 \times 10^6 \pm 3.04 \times 10^6$ p=0.981
SRP-/HBO+	$3.92 \times 10^6 \pm 4.02 \times 10^6$ p=0.962	$2.57 \times 10^6 \pm 1.67 \times 10^6$ p=0.0588	$1.40 \times 10^6 \pm 1.69 \times 10^6$ p=0.995	$1.49 \times 10^6 \pm 2.22 \times 10^6$ p=0.963
SRP+/HBO-	$1.90 \times 10^6 \pm 2.26 \times 10^6$ p=0.349	$1.22 \times 10^6 \pm 1.01 \times 10^6$ p=0.887	$1.02 \times 10^6 \pm 571 \times 10^5$ p=0.378	$7.693 \times 10^5 \pm 6.92 \times 10^5$ p=0.17 <sup>6</sup>
SRP-/HBO-	$3.81 \times 10^6 \pm 5.88 \times 10^6$	$1.29 \times 10^6 \pm 1.06 \times 10^6$	$1.39 \times 10^6 \pm 1.19 \times 10^6$	$1.53 \times 10^6 \pm 1.55 \times 10^6$

TABLE 2 - Mean values of anaerobe load per mg of subgingival plaque (growth on KKVA plates) in the different groups. In each column, P values are calculated versus the SRP-/HBO- group.

Group	Time (days)			
	0	15	45	75
SRP+/HBO+	$1.41 \times 10^5 \pm 2.44 \times 10^5$ p=0.342	$3.90 \times 10^2 \pm 3.65 \times 10^2$ p=0.00142	$3.32 \times 10^2 \pm 3.59 \times 10^2$ p=0.0248	$2.28 \times 10^2 \pm 3.10 \times 10^2$ p=0.0219
SRP-/HBO+	$6.86 \times 10^4 \pm 7.90 \times 10^4$ p=0.832	$5.35 \times 10^3 \pm 3.90 \times 10^3$ p=0.0440	$1.41 \times 10^4 \pm 3.09 \times 10^4$ p=0.2263	$8.87 \times 10^3 \pm 1.59 \times 10^4$ p=0.1583
SRP+/HBO-	$4.71 \times 10^4 \pm 7.58 \times 10^4$ p=0.730	$5.45 \times 10^3 \pm 4.27 \times 10^3$ p=0,040	$9.47 \times 10^3 \pm 8.26 \times 10^3$ p=0.07317	$1.19 \times 10^4 \pm 1.12 \times 10^4$ p=0.1975
SRP-/HBO-	$6.02 \times 10^4 \pm 9.08 \times 10^4$	$4.30 \times 10^4 \pm 9.9 \times 10^3$	$3.61 \times 10^4 \pm 4.34 \times 10^4$	$2.52 \times 10^5 \pm 2.96 \times 10^4$

(SRP+/HBO+) compared to the group receiving no treatment (SRP-/HBO-). In fact, the mean values remained roughly constant and of the order of  $10^6$  CFU per mg of subgingival plaque. As far as the CFU counts on KKVA were concerned (Table 2), treatment with SRP alone enabled us to detect a statistically significant decrease ( $P=0.040$ ) in the mean anaerobe load immediately after treatment.

Within a short time period (30 days), however, the anaerobe load reverted to values very close to the initial load ( $P>0.05$ ). A similar behaviour was detected when HBO therapy alone was evaluated. In this case, too, a statistically significant reduction ( $P=0.044$ ) was observed immediately after treatment, but the lower anaerobe load was not consistently maintained in subsequent plaque col-

lections one month later. Very different, on the contrary, was the behaviour of the dental sites that received the double treatment (SRP+/HBO+). In these cases, a statistically significant reduction in anaerobe load was recorded immediately after the double treatment, but the very low values (of the order of  $10^2$  per mg of subgingival plaque corresponding to a 99.9% loss of anaerobes) persisted after one month ( $P=0.025$ ) and three months ( $P=0.022$ ).

Figure 1 shows, by way of an example, one of the best results as far as the anaerobe count was concerned. In this case, the zero value was reached, but, what is more, this value persisted for one month at least. Worthy of note is the fact that this behaviour was detected in 2 of the 10 cases examined.

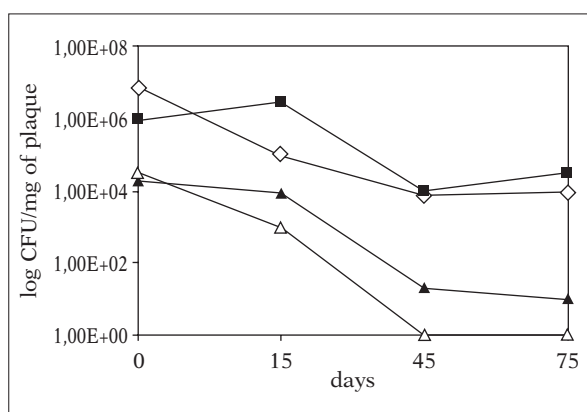


FIGURE 1 - Total (■, □) and anaerobe (▲, △) CFU counts per mg of subgingival plaque of HBO+ patient treated (open symbols) or not (closed symbols) with SRP.

Detection of the main anaerobes with PCR

To further support the culture results, detection of selected periodontopathogens (*P. gingivalis*, *P. intermedia*, *T. denticola*, *F. nucleatum* and *A. actinomycetemcomitans*) was performed by PCR. Samples of subgingival plaque collected from dental sites that received no treatment showed that *T. denticola* was present in 67% of them, while 63% were positive for *P. gingivalis*, 59% for *F. nucleatum*, 35% for *P. intermedia* and 18% for *A. actinomycetemcomitans* (Figure 2). SRP treatment alone did not induce significant variations in the percentage of positive sites for the specific pathogens.

Interesting results in terms of reduction of positive sites were obtained for *P. gingivalis*, *T. denti-*

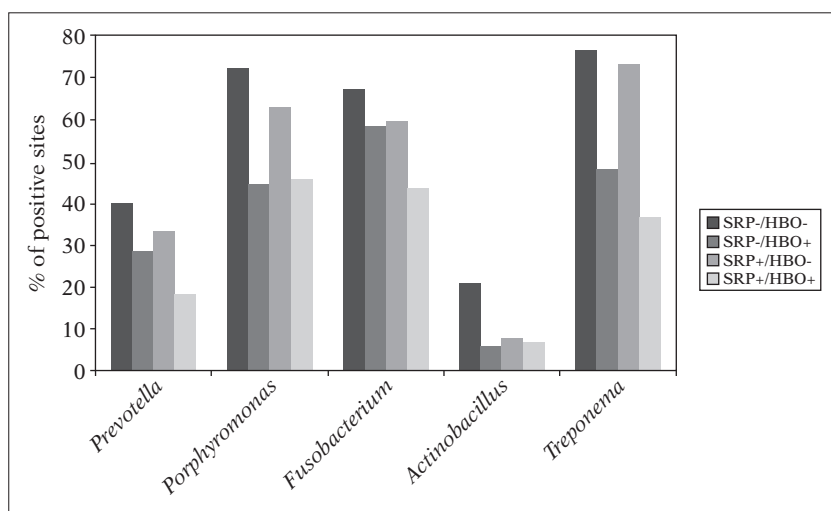


FIGURE 2 - Percentage of positive dental sites in the four treatment groups for specific periodontopathogens detected by PCR.

TABLE 3 - Evaluation of mean values of the Gingival Index in the different groups. In each column, P values are calculated versus the SRP-/HBO- group.

Group	Time (days)			
	0	15	45	75
SRP+/HBO+	2.44 ± 0.53 P=0.63	0 P=0.000	0 P=0.000	0 P=0.000
SRP-/HBO+	2.56 ± 0.54 P=0.75	0 P=0.000	0 P=0.000	0 P=0.000
SRP+/HBO-	2.12 ± 0.35 P=0.98	1.25 ± 0.46 P=0.094	0.75 ± 0.30 P=0.0029	0.71 ± 0.32 P=0.031
SRP-/HBO-	2.12 ± 0.35	2.00 ± 0.27	1.75 ± 0.36	1.71 ± 0.49



*cola* and *A. actinomycetemcomitans* as far as HBO treatment alone was concerned, but the best results were again obtained by combined therapy (SRP+/HBO+). In the latter case, only 32% of the sites harboured *T. denticola*, 40% *P. gingivalis*, 38% *F. nucleatum*, 16% *P. intermedia* and 6% *A. actinomycetemcomitans*.

#### *Evaluation of the gingival index (GI) of the dental sites*

The GI is a reliable parameter for evaluating the degree of gingival inflammation and, thus, one of the best parameters for predicting the recovery or worsening of the periodontal disease (Löe and Silness, 1963).

Table 3 shows that this parameter did not change with time in dental sites that received no treatment. Surgical treatment alone induced a significant reduction in GI. Interestingly, both HBO alone and the double treatment removed all signs of inflammation and this picture persisted for at two months at least after the therapeutic intervention.

## DISCUSSION

Several studies have described the beneficial role of HBO in the treatment of various human pathologies either alone or in combination with other therapies (Costantino *et al.*, 1995). Very few studies have been conducted to analyse the effects of HBO therapy on periodontal disease. Chen *et al.* (2002) showed that HBO increases local oxygen distribution, especially at the base of the periodontal pocket. On the one hand, this could inhibit the growth of anaerobe bacteria, and, on the other hand, would allow the ischaemic tissues to receive an adequate intake of oxygen sufficient for a rapid recovery of cell metabolism.

The aim of this study was to evaluate the effects of HBO on a selected number of patients suffering from adult chronic periodontitis in comparison with surgical intervention (SRP), as well as the effects of a combination of both therapies on the evolution over time of the microflora of the periodontal pockets. The microbiological data presented here clearly indicate that a combination of both HBO and SRP substantially reduced (by up to 99.9%) the gram-negative anaerobe

loads of the subgingival microflora. These very low values of pathogens persisted for at least two months after the therapy. HBO or SRP alone produced a temporarily more limited effect on the periodontal anaerobe load, which later reverted to the pre-treatment values.

That the effects of HBO were specific for periodontopathogenic bacteria is shown by the observation that none of the therapies, whether alone or in combination taken into consideration during this study, significantly affected the total microflora of the subgingival plaque. Thus, HBO exerts its killing action specifically against those microorganisms which, via the production of sophisticated virulence determinants, are responsible for direct tissue damage. The presence of high counts of periodontopathogenic bacteria enhances the inflammation response which, in turn, is mainly responsible for the clinical signs of periodontal diseases such as gingival oedema, bleeding and migration of the gingival crevice with eventual formation of a periodontal pocket. These pathological effects can be measured by means of specific parameters. One of these is the GI, which is a reliable parameter for evaluating the degree of inflammation at the gingival level. Here we show that HBO both alone and in combination with SRP reduces the GI value to zero and gingival health persists for at least two months. Thus, in parallel with the loss (or very great reduction) of periodontopathogenic bacteria, a substantial improvement in oral health was observed. Additional experimental confirmation of these results was provided by molecular detection of the main periodontopathogenic bacteria. Although, in this study, we evaluated the presence of selected bacteria by PCR which does not allow us to enumerate them, we detected a significant reduction in the number of dental sites which harboured them. We observed roughly a halving of the number of the dental sites for *T. denticola*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum*, while *A. actinomycetemcomitans* underwent a 3-fold decrease. Worthy of note is the fact that the extreme sensitivity of PCR allows us to detect very few bacteria, and therefore it would be reasonable to believe that our results may be overestimated.

In conclusion, this study has shown that HBO may be a useful aid, especially in combination with SRP, as far as non-surgical periodontal therapy is concerned. The excellent microbiological

results are supported by the clinical evidence with disappearance of the gingival inflammation. If these preliminary results are confirmed in further, more sizeable clinical and microbiological experiences, it may be possible to suggest an innovative, non-invasive protocol for the non-surgical therapy of periodontal disease.

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