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Selective Effects of a Mouthwash Based on Pure Chlorine Dioxide. I. Effect on the Oral Mucosa and the Plaque Bacteria. Focused Review and Model Calculations

Kristóf Kály-Kullai¹, Ákos Zsembery², Arvin Shahbazi³, László Rosivall⁵, Marianna Megyesi⁴, Thuy B Lawson¹, Mária Wittmann¹, Zoltan Noszticzius¹

1. Department of Physics, Budapest University of Technology and Economics

2. Department of Oral Biology, Semmelweis University

3. Department of Periodontology, Semmelweis University

4. Solumium Ltd.

5. Institute of Translational Medicine and International Nephrology Research and Training Center, Semmelweis University

Abstract

The health of the oral cavity is largely determined by the composition of microbial community, the oral microbiom, living there. An antimicrobial mouthwash must therefore serve two opposing purposes. On the one hand, it must prevent the overgrowth of microbes, especially pathogenic bacteria, that is, it must kill these microbes. On the other hand, it has to maintain a renewable oral flora of useful bacteria, and for this you have to save at least some of these bacteria. According to published experimental data, which are cited in this article, mouthwash based on pure chlorine dioxide has proven to be able to do this. In our series of articles, we examine what models/mechanisms can explain that mouthwash containing pure chlorine dioxide is able to meet these seemingly contradictory requirements. In addition to reviewing the literature data, we also raise rational hypotheses, which will have to be verified with further scientific and clinical studies in the future. In the first part of our series, we give a general overview of the experimental results published so far, as well as the possible mechanisms of the selectivity of chlorine dioxide. Based on experimental data and theoretical considerations, we point out that pure chlorine dioxide mouthwash has practically no effect on the mucous membrane, but it does affect the bacterial flora of the oral cavity. According to published data, a significant proportion of bacteria survive oral rinses, and rinsing kills pathogenic microbes more effectively than others. The latter can be explained by colonization resistance. Then, with the help of a model calculation based on the diffusion of ClO₂ in plaque, we show that the majority

of dental plaque bacteria cannot survive a 15-second mouth rinse with mouthwash containing 15 ppm ClO₂. In other words, although the survival of a significant proportion of bacteria is obviously enabled by an oral biofilm, dental plaque cannot do so.

Keywords

chlorine dioxide, mouthwash, oral microbiom, selectivity

SELECTIVE EFFECTS OF A MOUTHWASH BASED ON PURE CHLORINE DIOXIDE

I. EFFECT ON THE ORAL MUCOSA AND THE PLAQUE BACTERIA. FOCUSED REVIEW AND MODEL CALCULATIONS

*Kristóf Kály-Kullai¹, Ákos Zsembéry², Arvin Shahbazi^{3,4,5}, László Rosivall⁶,
Marianna Megyesi⁷, Thuy B. Lawson¹, Mária Wittmann¹, Zoltán Noszticzius^{1,7}*

1 Department of Physics, Budapest University of Technology and Economics, Budapest

2 Department of Oral Biology, Semmelweis University, Budapest

3 Department of Anatomy Histology and Embryology, Semmelweis University, Budapest

4 Department of Restorative Dentistry and Endodontics, Semmelweis University,
Budapest

5 Department of Periodontology Semmelweis University, Budapest

6 Institute of Translational Medicine and International Nephrology Research and Training
Center, Semmelweis University, Budapest, Hungary

7 Solumium Ltd., Budapest

ABSTRACT

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In the first part of our series, we give a general overview of the experimental results published so far, as well as the possible mechanisms of the selectivity of chlorine dioxide. Based on experimental data and theoretical considerations, we point out that pure chlorine dioxide mouthwash has practically no effect on the mucous membrane, but it does affect the bacterial flora of the oral cavity. According to published data, a significant proportion of bacteria survive oral rinses, and rinsing kills pathogenic microbes more effectively than others. The latter can be explained by colonization resistance. Then, with the help of a model calculation based on the diffusion of ClO_2 in plaque, we show that the majority of dental plaque bacteria cannot survive a 15-second mouth rinse with mouthwash containing 15 ppm ClO_2 . In other words, although the survival of a significant proportion of bacteria is obviously enabled by an oral biofilm, dental plaque cannot do so.

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1. INTRODUCTION

The oral microbiome ranks second behind the intestinal flora in terms of flora diversity among the microbial communities that colonize the human body. More than 700 types of bacteria can occur in the oral cavity [Kilian et al., 2016, Pedersen and Belstrom 2019], which can coexist harmoniously with the human body under physiological conditions. (Of the 700 types of bacteria, only 250-300 types are present in healthy people at the same time.) This condition is usually called eubiosis. However, this healthy state can be overturned by the disproportionate proliferation of various pathogenic bacteria. The resulting dysbiosis [Lin et al., 2021] can lead to various local and systemic diseases [Tuganbaev et al., 2022; Peng et al., 2022].

To achieve adequate oral hygiene, in addition to mechanical methods (toothbrush, dental floss, etc.), the regular use of some kind of mouthwash is also recommended [Boyle et al., 2014]. The use of mouthwashes has a direct effect on the composition of the oral microbiome by killing bacteria. We expect good mouthwash to help restore eubiosis in case of dysbiosis; and in the case of eubiosis, it prevents the development of dysbiosis. The right mouthwash can replace dental floss [Bosma et al., 2022], helps prevent tooth decay, gingivitis, and the resulting periodontal disease by inhibiting the formation of dental biofilm (so-called "dental plaque") [Marsh, et al., 1995; Socransky and Haffajee, 2002; Gera 2005; Marsh 2006; Gurenlian 2007; Shinada et al., 2010], i.e. it is a significant factor in the prevention of oral diseases. In addition to dental biofilms, mouthwash also affects biofilms on the mucous membrane-covered surfaces of the oral cavity [Gurenlian 2007], and thus can help prevent e.g. bad breath.

In recent years, mouthwashes containing chlorine dioxide (ClO_2) have also become available. In addition to chlorine dioxide, these mouthwashes usually also contain sodium chlorite (NaClO_2). In fact, there are some that do not contain ClO_2 at all, but only chlorite ion (ClO_2^-), in a concentration of around 1000 ppm (0.1 %). These are counted among chlorine dioxide mouthwashes because sodium chlorite is also commonly referred to as "stabilized chlorine dioxide", which is of course not a very lucky name, since the two compounds are not the same. NaClO_2 is also bactericidal, but less effective than ClO_2 . In this series of articles, we only deal with the mechanism of action of mouthwashes in which ClO_2 is the only active ingredient. The chlorine dioxide concentration in these mouthwashes is very low, e.g. it is recommended to use hyperpure mouthwash concentrates¹ with an active ingredient concentration of only 15–30 ppm (0.0015–0.0030%) after dilution. This is a much lower active ingredient concentration than the 0.2%, i.e. 2,000 ppm concentration that is usual for chlorhexidine-based antibacterial mouthwashes referred to as the "gold standard". This makes it likely that the mechanism of action of chlorine dioxide-based mouthwash is different from that of previous "classic" mouthwashes.

An antimicrobial agent is considered to be more effective if its required concentration is lower and its action is faster compared to other antimicrobials. You can talk about selectivity in both chemical and biological terms. Chemical selectivity means that a reagent selects among possible reaction partners. Ideally, a reagent will react only with certain reaction partners and not at all with others. In practice, it is enough if the reagent reacts much faster with some reaction partners, but much slower with others.

Chlorine dioxide is highly effective both in terms of effective concentration and the speed of action. Tanner [Tanner 1989] found chlorine dioxide to be the most active biocidal substance among the surface disinfectants he examined. Benarde et al. [Benarde et al., 1967] had earlier shown that this disinfecting effect is extremely fast. The speed can be attributed to the fact that ClO_2 is well soluble both in water and in lipid phase, so it can easily penetrate any point of the lipid bilayer membrane of the bacterial cell, i.e. its entire outer surface, not just some special points. Such distinguished points are e.g. the ion channels of the membrane in the case of ionic components.

ClO_2 is a highly selective reagent from a chemical point of view [Noss et al., 1986], and this, as we shall see, is advantageous. Chlorine dioxide, for example, reacts very slowly with nucleic acids and

with the majority of the amino acids, but it reacts extremely quickly with three amino acids: cysteine, tyrosine and tryptophan [Hauchman et al. 1986; Napolitano et al. 2006; Ogata 2007].

By biological selectivity we mean that chlorine dioxide acts differently on different bacteria. From the point of view of mouthwash use, it is beneficial if it has a stronger effect on the pathogenic bacteria that cause dysbiosis than on the beneficial ones that maintain eubiosis.

Hyperpure ClO_2 is also suitable for treating wounds infected with antibiotic-resistant bacteria, since ClO_2 has the same efficiency against antibiotic-resistant microbes [Noszticzius, et al., 2013b]. Furthermore, it should be mentioned that fungi, viruses or planktonic bacteria can also be inactivated by chlorine dioxide, but in the present paper the focus is specifically on the effect on bacterial biofilms. Planktonic microbes are considered for comparison purposes only.

The purpose of this series of articles is to describe more precisely the mechanism of action of mouthwashes containing only chlorine dioxide, and to examine the effectiveness and selectivity of chlorine dioxide. In the series of articles, we examine several mechanisms that can cause biological selectivity.

In this first part, we provide an overview of the most important information regarding the use of chlorine dioxide as a mouthwash. We review the data and the present experimental results found in the literature, with particular attention to the mechanism of action and the possible causes of selectivity.

In the following second part, we will first examine whether ClO_2 can select among bacteria based on their metabolic processes. It is conceivable that aerobic bacteria performing oxidative phosphorylation with intracellular electron transport can defend themselves against chlorine dioxide more successfully than their anaerobic counterparts performing fermentation. According to the latest research, many bacterial species are capable of extracellular electron transfer (EET), which can provide an opportunity for even more pronounced selectivity. Furthermore, it may result in selectivity if, as they believe, Gram-positive bacteria are more resistant to ClO_2 than Gram-negative ones.

Our further aim is to build on the collected experimental data to develop reaction-diffusion models instead of the simple diffusion model discussed here. With these, we want to investigate how chlorine dioxide penetrates biofilms in the oral cavity that cover not hard, but soft surfaces. In this paper, as well as in later ones, the results of the model calculations are primarily evaluated from the point of view of whether a single oral rinse can kill the bulk of the bacteria in the biofilm in question, or, on the contrary, the model predicts the survival of a significant part of the bacteria.

2. SELECTIVITY OF ClO_2 IN THE ORAL CAVITY

2.1. Effect of chlorine dioxide on solitary cells in a glass reactor

The possibility of biological selectivity is first examined in a case where a mixture of solitary (isolated) human cells and solitary (planktonic) bacteria is reacted with chlorine dioxide in a glass reactor, with constant stirring. In the long term, we cannot expect selectivity here, because sooner or later chlorine dioxide will stop the vital activity of both bacteria and human cells in the same way. If, on the other hand, the process is interrupted after a sufficiently short time, the result can be quite different. In this case, there is a period of time (which also depends on the used ClO_2 concentration) during which bacteria, which are much smaller than human cells, already die, as their antioxidant reserves are used up, but human cells - thanks to their much larger antioxidant reserves - are not yet significantly damaged. For this reason, it is customary to call ClO_2 a size-selective antimicrobial agent [Noszticzius et al., 2013; Kály-Kullai et al., 2020] However, ClO_2 cannot differentiate between beneficial and pathogenic bacteria based solely on their size, as the difference between their sizes is neither significant nor definite. However, the situation may be different if two bacteria of almost the same size have different metabolic processes and therefore cannot protect themselves against ClO_2 in the same way. For example, among the pathogenic bacteria found in the mouth, anaerobes are

common, and their metabolism can be much more sensitive to chlorine dioxide than that of their aerobic counterparts [Lu, Imlay, 2021].

If we want to examine the question of biological selectivity in the human mouth, rather than in a glass reactor, we encounter a different situation, because here the role of planktonic bacteria is no longer decisive. In the mouth, the effect of bacterial biofilms, which are found on various surfaces and habitats, dominates. Habitat can affect the effect of chlorine dioxide on bacteria in two ways. On the one hand, if a part of the habitat consists of soft human tissues, it can "suck" some of the chlorine dioxide away from the biofilm, depending on the geometry of the habitat, which cannot happen in a glass reactor. On the other hand, it cannot be ruled out that the structure of the biofilm itself may be different in different habitats. So the effect of chlorine dioxide in the mouth may also depend on the habitat.

2.2. Experimental results supporting the selectivity of mouthwashes containing chlorine dioxide

As mentioned in the introduction, the purpose of mouthwash is not to kill all bacteria, because then the beneficial bacteria would also be killed. According to the experiments below, it is clear that surviving bacteria remain after the chlorine dioxide mouth rinse, and it is also possible that this mouth rinse selectively kills pathogenic bacteria.

First of all, we can refer to the recently published paper of Hercegh et al. [Hercegh et al., 2023]. The test subjects participating in the study rinsed with 5 ml of hyperpure chlorine dioxide solution with a concentration of 30 ppm for 1 minute. According to the data in Figure 4 of their paper, 5 minutes after the chlorine dioxide mouth rinse 94.1% of the *Streptococcus mutans* bacteria in the saliva were killed, while this figure for the total number of bacteria was only 66.8%, which is a significant difference in terms of specified error limits. These numbers mean that a *S. mutans* bacterium has approx. 5 times less chance of surviving the chlorine dioxide mouth rinse than the average bacteria. The number of surviving bacteria did not change significantly even after 90 minutes.

There is currently no reliable information on how the number of bacteria changes after a longer period of time (e.g. after half a day or even a full day), since this was the first such published study with a hyperpure chlorine dioxide solution, and it has not yet been measured what happens after 90 minutes. Such experiments have already been carried out with less pure (and unfortunately with only approximately known composition) chlorine dioxide containing solutions. In these so-called ASC (Acidified Sodium Chlorite) solutions sodium chlorite (NaClO_2) at acidic pH gradually decomposes into chloride, chlorate and chlorine dioxide, however, the authors did not provide the exact ClO_2 concentration of the solutions.

Shinada et al. [Shinada et al., 2010] precisely specified the ingredients of the ASC recipe they used: the solution contained 0.16% sodium chlorite and 1.13% citric acid, as well as some glycerin and peppermint oil. These latter components, however, do not play a role in the development of chlorine dioxide. In such a solution, ClO_2 is formed at a slow rate for days, but the concentration of ClO_2 at the moment of use is usually unknown. According to their experiments, as a result of using the ASC mouthwash by the test subjects twice a day for 7 days, the total number of bacteria that could be measured in the saliva of the test subjects decreased to 51% of the baseline, compared to the 65% result of the control group using the placebo mouthwash. The difference is not statistically significant, but it should be noted that the sampling took place 8.8 ± 1.53 hours after the last rinse, which may be enough time for the normal oral flora to be approximately restored.

According to the Discussion on page 7 of the article:

"The results of this study demonstrate that rinsing with a mouthwash containing ClO_2 , used over a 7-day period, was effective in reducing morning oral malodor, plaque, tongue coating accumulation and the counts of *Fusobacterium nucleatum* in saliva in healthy subjects."

What deserves special attention here is that the mouthwash had a selective effect on a pathogenic bacterium, as it reduced their number relatively more than other bacteria. According to their measurements, the chlorine dioxide mouthwash reduced the number of *F. nucleatum* to 20% of the

original number, i.e. by 80%, while in the control group the reduction was only half of this, i.e. 40%, which is a statistically significant effect.

ASC solutions were also used by Yates et al. [Yates, et al., 1997], but unfortunately they do not provide the exact composition of any ASC solution, only that the recipe used malic or mandelic acid as an acid in addition to NaClO_2 . They also do not mention the concentration of the resulting ClO_2 in the mixture. In the case of all three solutions, they found that after rinsing, the number of bacteria that could be measured in the saliva dropped to 10-20% of the original, and this remained so until the end of the 5th hour. By the end of the 7th hour, however, there was a significant increase. A previous publication [Goultschin, et al., 1989], also using an ASC recipe, reported that the plaque index decreased significantly with the use of mouthwash, but this was not accompanied by a significant decrease in the number of bacteria in the saliva.

Based on the above reports, it can be concluded that a significant proportion of bacteria (about 10-30%, or even more) survive the chlorine dioxide mouth rinse. At the same time, according to Benarde [Benarde et al., 1967], only 0.1% of *E. coli* planktonic bacteria survive in a test tube, in a solution containing 0.25 ppm chlorine dioxide at 30 °C after an exposure time of 21 s. This means that e.g. in a 5 ppm (i.e. 20 times more concentrated) chlorine dioxide solution, only $(21/20) = 1.05$, so approx. 1 second is enough to reduce the number of bacteria by 3 orders of magnitude, and in 2 seconds this reduction already reaches 6 orders of magnitude. In comparison, as mentioned, rinsing with 30 ppm mouthwash for 60 seconds reduced the number of bacteria measured in saliva by only 0.5 to 1 order of magnitude.

These experimental results mean that there is definitely at least one habitat in the mouth in which a significant proportion of the bacteria in the biofilm survive a chlorine dioxide mouth rinse. Since this is evidenced by the bacteria found in saliva, and since according to the research of Socransky and Haffajee [Socransky and Haffajee, 2005], most of the bacteria in saliva originate from the surface of the tongue, it can be assumed that the surface of the tongue is most likely such a habitat.

However, the antibacterial effect of chlorine dioxide has not only been investigated in connection with mouth rinses, data can be found in other areas as well, which indicate the selective antimicrobial effect of ClO_2 . In connection with the surface disinfection of food, for example, the extent to which different species of bacteria respond to a specific, low-dose chlorine dioxide treatment was examined [Vandekinderen, et al., 2009]. According to the measurements, in the case of different bacterial species, the number of germs that survive the treatment can differ by several orders of magnitude. For example, while the germ count of *Salmonella typhimurium* (a Gram-negative bacterium) decreased by more than 3 orders of magnitude, the germ count of *Pseudomonas fluorescens* (also a Gram-negative bacterium) practically did not change within the measurement error. Comparing Gram-negative and Gram-positive bacteria, they found that Gram-negatives were slightly more sensitive to chlorine dioxide treatment than Gram-positives. This was explained by the fact that Gram-positives have much thicker cell walls. *Pseudomonas fluorescens*, which is Gram-negative, is an obvious exception to this rule, but the reason for this has not been explained.

2.3. Possible causes of bioselective action in the oral cavity

Summarizing the above, we list the selection aspects according to which chlorine dioxide can select among biological organisms. Here we will deal exclusively with human cells and human tissues, as well as planktonic bacteria and bacterial biofilms.

A) Selectivity by size

This effect occurs when we examine the survival chances of two cells of different sizes. If both are exposed to the same chlorine dioxide concentration (c) with the same exposure time (T), then the parameters c and T can be chosen so that the larger cell survives and the smaller cell dies [Noszticzius et al., 2013; Kály-Kullai et al., 2020]. One of the reasons for this is that the larger the cell, the longer it takes for chlorine dioxide to reach all parts of the cell by diffusion. Another reason is that the passive defense of cells against oxidative stress (and thus chlorine dioxide) is based on the accumulated antioxidant reserves in the cell (primarily compounds containing SH groups, such as

glutathione or thioredoxin). Larger cells have larger stores of these and this helps them survive. The details can be found in publications [Noszticzius et al., 2013; Kály-Kullai et al., 2020].

B) Selectivity between unicellular microbe and multicellular organism

When unicellular organisms and multicellular organisms consisting of cells built into tissues encounter chlorine dioxide, their situation differs from one another in two respects. On the one hand, a single cell is usually surrounded on all sides by the chlorine dioxide medium. However, in the case of a multicellular organism, only the outer cells of the organism, or a part of them, come into contact with chlorine dioxide. The multicellular organism is thus affected only locally by the treatment, while the unicellular organism is affected globally. On the other hand, the defense of multicellular organisms against ClO_2 is usually supported by a circulatory system, which is able to continuously replace the amino acid and antioxidant losses of individual cells. In such a case, it is very easy to choose a concentration c and exposure time T such that, e.g. during a mouth rinse, a planktonic bacterium should be killed in seconds, but at the same time the person is not harmed in any way. We will discuss this selectivity in detail in Chapter 3 of this article.

C) Selectivity according to the metabolism of bacteria

Bacteria can defend themselves against chlorine dioxide both passively and actively. Passive protection with accumulated antioxidant reserves was already discussed in point A). However, bacteria can defend themselves even actively against chlorine dioxide, e.g. with the help of NADH continuously produced in their metabolic processes. This is because NADH can quickly reduce chlorine dioxide to chlorite ion [Bakmutova-Albert, et al., 2008]. Theoretically, it is conceivable that bacteria carrying out oxidative phosphorylation may have an advantage over bacteria carrying out pure fermentation, since the former can produce much more NADH molecules from a single glucose molecule than the latter. However, in the comparison, in addition to stoichiometry, the rate of metabolism also matters, and fermentation takes place faster than oxidative phosphorylation. The fact that anaerobes are common among the pathogenic bacteria in the mouth, and these can be much more sensitive to chlorine dioxide than aerobic bacteria, can provide an opportunity for further selection according to metabolism.

The fact that, according to the latest research, many bacterial species are capable of extracellular electron transfer (EET) can provide an opportunity for even more pronounced selectivity. A part of the metabolism of such bacteria is placed outside the cell. In this case, the electron transferred outside the bacterial cell by EET can already reduce a part of ClO_2 before it reaches the bacteria itself. This can greatly increase the bacteria's chances of survival. So far, however, there is no experimental evidence that EET plays an important role in oral biofilms. The effect of chlorine dioxide on the metabolism of bacteria will be discussed in the next article in our article series.

D) Habitat selectivity

The inner surface of the oral cavity is a complex habitat, which is why the exposure of the bacteria in different parts of the mouth to chlorine dioxide can be different during a rinse with mouthwash. It is particularly important whether a bacterium is planktonic or found in the biofilm, or within the biofilm in which part of it. The biofilm provides some degree of protection against chlorine dioxide, since the ClO_2 in the mouthwash cannot enter it by mechanical flow, but only by diffusion. For this reason, the bacterium that is further away from the surface of the biofilm will be exposed to less chlorine dioxide during mouth rinsing, thus increasing the chance of survival. This effect is enhanced by the fact that part of the chlorine dioxide reacts with the bacteria in the outer zone of the biofilm, as well as with the material of the biofilm.

In the case of biofilms in the oral cavity, it is also an important aspect whether they adhere to a hard or soft surface, and what is the shape of the surface. Because of these differences, ClO_2 can also select between different biofilms. In practice, this means that parameters c and T can be chosen such that, while in the course of a mouth rinse most of the bacteria in the dental biofilm are killed, a significant proportion of the bacteria in the biofilm on the tongue still survive. We intend to deal with the effect of chlorine dioxide on biofilms adhering to soft surfaces in detail in a later article.

E) Colonization resistance

We saw above that the bacteria living in the biofilm have a better chance of survival. The phenomenon of colonization resistance can explain why the pathogenic bacteria are displaced from a biofilm. It is known since the middle of the last century [Lloyd, et. al, 1977], that the bacterial community of a biofilm is able to prevent the incorporation of bacteria harmful to the host and/or the biofilm into the biofilm and their reproduction there. We will detail this effect in Chapter 4 of this article.

3. THE EFFECT OF CHLORINE DIOXIDE ON THE HUMAN BODY

Before discussing in detail the various selective effects of chlorine dioxide on the oral microbiome, we will briefly examine whether the use of a pure chlorine dioxide mouthwash can cause any side effects on the oral mucosa or the entire human body.

3.1. Local effect of chlorine dioxide on epithelial cells on the surface of the oral mucosa

In order for ClO_2 in mouthwash to pose a threat to a human cell, two conditions must be met:

- 1) Chlorine dioxide must enter the cell.
- 2) The ClO_2 concentration inside the cell must reach a critical threshold value. Each poison has a threshold value specific to the given toxin and the given cell, below which it can no longer cause poisoning symptoms in the cell.

The first condition is fulfilled, since ClO_2 is not only soluble in water, but also in organic phases [Noszticzius et al., 2007; Pitochelli. 1998], therefore – being dissolved in the lipid phase of the cell membrane – it can easily diffuse through it. So ClO_2 can get inside the cell.

However, according to the second condition, getting in alone is not enough, because chlorine dioxide must also accumulate there to the extent that its concentration eventually exceeds the poisoning threshold. However, this condition is quite difficult to fulfill, since a protective peptide, glutathione, is present in a fairly high concentration in human cells (and in the cells of all multicellular organisms, i.e. in the cells of animals and plants as well) [Forman et al, 2009]. Glutathione is a so-called radical scavenging compound [Galano and Alvarez-Idaboy, 2011]. Its role is to capture the free radicals that are produced in the cells during metabolism and that could otherwise cause damage there.

Since chlorine dioxide is also a free radical, glutathione reacts rapidly with chlorine dioxide entering the human cell [Ison et al., 2006] and neutralizes it. Other reducing agents in the cells are able to reduce oxidized glutathione (glutathione disulfide) back to glutathione up to a certain limit [Lu, 2009]. Furthermore, through circulation, a constant supply of the most diverse components (e.g. amino acids necessary for glutathione synthesis) as well as other antioxidants that react quickly with chlorine dioxide is available for human cells. As a result of all this, during a mouth rinse, the ClO_2 concentration in the cells can remain far below the poisoning threshold.

3.2. The global effect of chlorine dioxide on the entire human body

An important question is whether so much chlorine dioxide can pass through the mucous membrane that, if distributed throughout the body, could have a harmful effect on other organs. Based on the theoretical considerations described in the previous paragraph, the answer is no, since the concentration of chlorine dioxide is extremely low even at the point of entry. The most direct experimental confirmation of the answer is the investigations of Lubbers and his colleagues [Lubbers et al., 1982; Lubbers and Bianchine 1984]. The background to this research is that chlorine dioxide has been used in the USA since the 1940s to disinfect drinking water instead of elemental chlorine. The fact that ClO_2 -containing drinking water is harmless to mammals was initially supported only by animal experiments, but in the 1980s there was a need to test directly on humans whether such drinking water has any health-damaging effects. Therefore, Lubbers and colleagues performed human tolerance tests with aqueous solutions of chlorine dioxide. During their experiments, their test subjects drank aqueous solutions that contained only chlorine dioxide in the concentration range of 0.1–24 ppm. The subjects tolerated these solutions well: consumption of two 500 ml doses of 24 ppm chlorine dioxide solution per day did not cause any adverse effects.

The recommended dose of the pure ClO_2 based mouthwash is 25 ml of a 15 ppm solution. Based on Lubbers' results, we can state that if *consuming* that large amount of chlorine dioxide applied in their experiments did not cause any problems, then *rinsing* with a mouthwash containing much less chlorine dioxide should not pose a threat to the human body either.

We can also state that since ClO_2 already reacts with glutathione at the point of entry into human tissues, there is no need to worry that the circulation could make chlorine dioxide systemic. In other words, the use of chlorine dioxide mouthwash can cause neither local nor global side effects at the cellular level¹.

3.3. Where does hyperpure chlorine dioxide exert its antimicrobial effect and how can it help the human immune system?

As mentioned in the previous paragraphs, the rapid glutathione – ClO_2 reaction in human cells and tissues can keep the chlorine dioxide concentration far below the toxic level. However, this extremely low ClO_2 concentration cannot even damage, let alone kill, the microbes residing in the cells or in the space between the cells. In other words, microbes inside human tissues are completely protected from the effects of chlorine dioxide. At the same time, the microbes that appear inside the tissues are exposed to the attack of the human immune system, which can assert itself there in full force.

In addition to theoretical considerations, laboratory animal experiments also confirmed that chlorine dioxide is too reactive to be present in significant concentrations inside living tissues [Abdel-Rahman, et al., 1979; ATSDR, 2004]. It should be noted that this important circumstance is not mentioned in the otherwise thorough review articles discussing the antimicrobial effect of chlorine dioxide (e.g. [Andrés, et al., 2022; Peredo-Lovillo, et al., 2023]. However, a recent publication [Rubio-Casillas, Campra-Madrid, 2021] once again draws attention to the fact that ClO_2 is very quickly reduced to chlorite ion (ClO_2^-) when it enters the human body. This means that ClO_2 itself cannot exert a significant antimicrobial effect inside the human cells and tissues. We will have to take this fact into account when determining the location of the antimicrobial effect of chlorine dioxide in the oral cavity.

The place of the antimicrobial effect is therefore to be found outside of the human tissues. This place is a narrow zone bordering the surface of the tissues, where there are no longer any human cells, but where pathogenic microbes might appear. Such microbes can still be dangerous for the human body because they are very close to the human tissues. For example, their toxins may reach the human tissues. This place is a **transition zone** between living tissues and the environment (Figure 1). In the common case when the outer surface of the human body is a mucous membrane, the transition zone is a liquid film covering the epithelial cells of the mucous membrane, which might consist of several layers.

¹ Experience also supports this, as hyperpure chlorine dioxide solutions have been on the market in Hungary since 2008, and no harmful side effects have been revealed in the time since then.

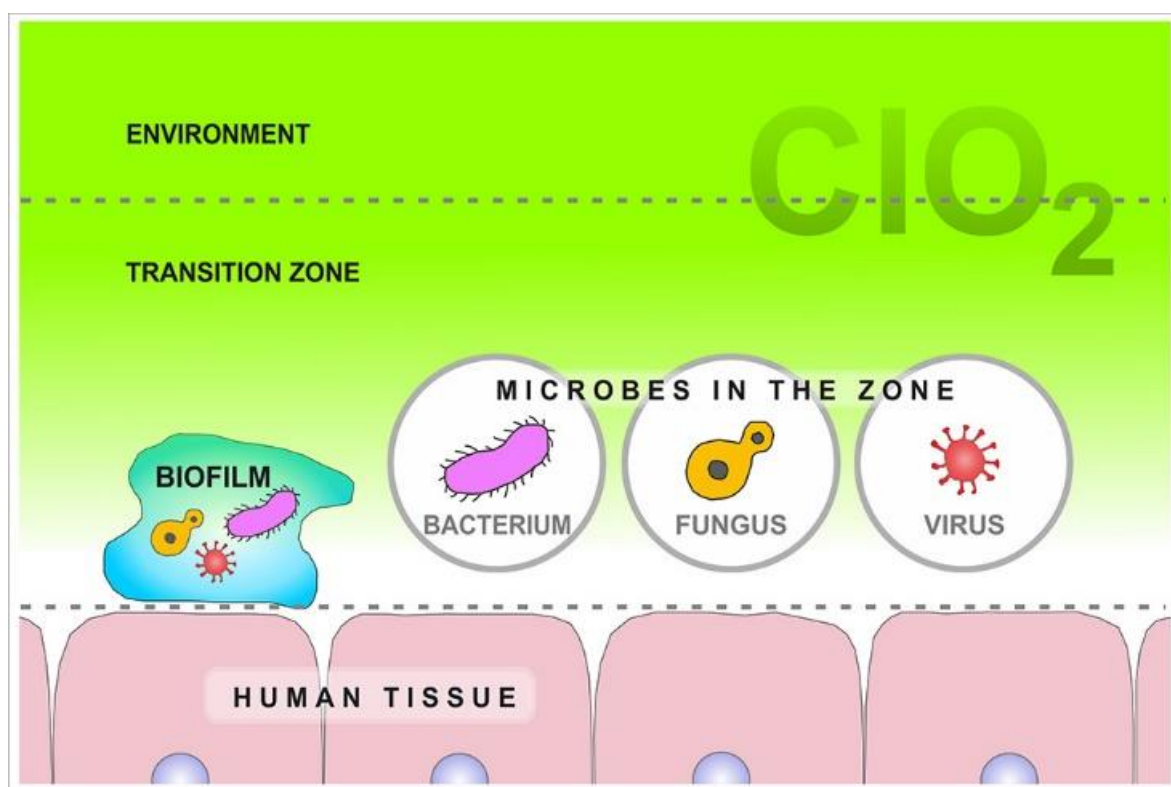


Figure 1. The transition zone separating human tissues (e.g. the mucous membrane of the oral cavity) from the environment (e.g. the space of the oral cavity that does not contain human cells).

The lower and upper limits of the transition zone are indicated by dashed lines. Chlorine dioxide can penetrate this zone from the environment (e.g. mouthwash), and it can exert its antimicrobial effect here helping the human immune system. However, due to glutathione, the ClO_2 concentration as well as its effect in the living tissue is negligible there. Going from top to bottom in the Figure, the green color indicating ClO_2 fades because human cells continuously consume the ClO_2 that penetrates by diffusion, and thus the ClO_2 concentration decreases in the transition zone as it approaches the tissues.

The transition zone is often filled with some kind of viscoelastic fluid or hydrogel, or a mixture of the two. Examples of this include saliva, tears, and mucus, in each of which, in addition to water, mucin is an important component, as well as various electrolytes, cellular substances, and debris. Another example is wound exudate, which forms the microenvironment of wounds [Junker, et al., 2013].

Human cells are typically no longer found in the transition zone, but the conditions (dark, warm and moist environment, as well as nutrients) favor the proliferation of microbes and the formation of biofilms. Chlorine dioxide entering the transition zone can prevent this, because in this zone ClO_2 can exert its effect on the microbes almost as unhindered as if the experiment were carried out in a Petri dish. By killing microbes in the transition zone, ClO_2 can help the human immune system, which is not as effective in the transition zone as it is inside the tissues.

This help is particularly important in the case of biofilms, because while the white blood cells of the human immune system cannot or can only get in there with great difficulty [Papayannopoulos, 2019], ClO_2 can in principle penetrate biofilms without any difficulty and can thus kill the biofilm microbes. ClO_2 , as already mentioned, is both water- and lipid-soluble, and thus, as a small molecule, it can penetrate the hydrophilic and lipophilic zones of the biofilm matrix just as easily.

4. THE ROLE OF THE BACTERIAL COMMUNITY OF THE BIOFILM IN SELECTIVITY

4.1. Colonization resistance

In Chapter 2.2, we have shown that, according to experimental evidence, chlorine dioxide mouthwashes can kill bacteria selectively. How can this be explained? We can start from the fact that, according to the experimental observations, there is an oral biofilm that ensures the survival of the bacteria in it. If this biofilm could use some mechanism to choose which bacteria it accepts and which it rejects, this could explain the selectivity. But is there such a mechanism?

The fact that oral biofilms protect pathogenic bacteria less or not at all can be explained by the theory of colonization resistance [He, et. al., 2014; Radaic, Kapila, 2021]. In the case of oral biofilms, He et al. were the first to scientifically examine the issue [He, et. al., 2010; He et. al., 2014], who reached an interesting result. In the system they investigated, a "sensor" bacterium (*S. saprophyticus*) detects the invading foreign bacterium and informs a "mediator" bacterium (*S. infantis*). The mediator bacterium, in addition to removing the previous inhibition on a "killer" bacterium connected to it (*S. sanguini*), also encourages the latter to produce hydrogen peroxide, which can kill the invading bacterium. The bacterial community forming the biofilm works like an immune system, it prevents the integration of bacteria harmful to the biofilm or the host organism into the biofilm, thereby selecting between beneficial and harmful bacteria.

4.2. Colonization resistance-enhancing effect of antibacterial mouthwashes

The effect of this selection can be enhanced by antibacterial mouthwash, which kills pathogenic bacteria trapped outside the protective biofilm more effectively. If this is the case, the selection will depend less on the type of antibacterial mouthwash. Indeed, Hercegh et al. found [Hercegh et al., 2023] that not only chlorine dioxide but also chlorhexidine-based mouthwash selectively kills *S. mutans*. All of this supports the assumption that the selective inactivation of pathogenic bacteria is made possible by colonization resistance, and antibacterial mouthwashes only amplify this effect.

4.3. Maintaining oral hygiene by exploiting the phenomenon of colonization resistance

In recent years, the importance of a healthy oral flora, which is "provided to us by evolution," has been increasingly emphasized [Baker, Edlund, 2019]. Among the earliest settlers of a healthy oral flora, various non-pathogenic *Streptococcus* species play an important role, such as *S. mitis*, *S. sanguinis*, and *S. gordonii* [Baker, Edlund, 2019]. In a state of eubiosis, these and many other coexisting bacteria form the oral microbiome, and the number of pathogenic microbes is negligible. However, sometimes the balance is disrupted, and this can cause the proliferation of pathogenic bacteria. According to the authors, in order to restore the natural flora, it is worth using targeted antibiotics that specifically affect only one pathogenic microbe, such as *Streptococcus mutans*. This is not an easy task in the case of *S. mutans*, because in addition to the pathogenic bacterium to be eliminated, there are also several beneficial bacteria that belong to the same *Streptococcus* family. However, researchers from the University of California [Eckert, et al., 2014] managed to develop a method, which produces antimicrobial peptides able to target specific bacteria. This method was used by Guo and his colleagues [Guo, et al., 2015] to selectively eliminate the pathogenic bacterium *Streptococcus mutans* in an in vitro oral bacterial community derived from human saliva. The results provide interesting scientific conclusions about how the structure of a microbial community is transformed when a single pathogenic member of the community is selectively eliminated.

However, it may be of greater importance for everyday practice if it were proven in general that an antimicrobial mouthwash can selectively kill all pathogenic bacteria in the oral cavity *simultaneously*. In this case, to restore the natural oral flora, and to maintain it (i. e. for preventive purposes) the use of hyperpure chlorine dioxide-based mouthwashes besides probiotics may also be justified, since both serve the same purpose.

5. HABITAT-BASED SELECTION

5.1. Hard and soft surfaces in the oral cavity. Habitat-dependent biofilms.

About 20% of the human oral cavity is made up of hard surfaces (such as teeth, implants, and dentures), and the remaining 80% is made up of soft surfaces of the mucosa [Gurenlian 2007]. Hard surfaces do not shed or renew themselves, while soft surfaces are continuously exfoliating and renewing.

The hard and soft surfaces are separated from the outside world by a transition zone, consisting of two layers: the very thin pellicle, which is formed from saliva and covers the entire surface of the oral cavity [Hannig, 2002; Chawhuaveang et al., 2021], and the liquid saliva (mucus) layer that moistens the pellicle. Bacterial biofilms form in the saliva layer, while attaching to the pellicle [Bertolini et al., 2022; Al-Ahmad et al., 2007]. The composition and structure of the bacterial colonies that form biofilms also depend on the habitat [Mager, et al., 2003; Socransky and Haffajee, 2005; Mark Welch et al., 2020].

5.2. Effect of ClO₂ on bacteria in the biofilms of hard surfaces. Model calculations

5.2.1. Plaque: the dental biofilm that forms on hard surfaces

The most studied oral biofilm is undoubtedly the plaque, which is the dental biofilm formed on the hard surface of the tooth, which plays an important role in the development of tooth decay, gingivitis and periodontal disease [Socransky and Haffajee 2002 Al-Ahmad et al., 2007; Bertolini et al., 2022].

There are two types of plaque: supragingival and subgingival. However, in this paper, we will only examine the effect of chlorine dioxide on supragingival plaque. Subgingival biofilm only plays a role in gingivitis or periodontal disease, but is practically absent in a healthy mouth, as shown, for example, in the paper by Könönen et al [Könönen, et al., 2019]. Furthermore, in the case of subgingival plaque, the biofilms that form on the soft surface of the gums also play a role, which we do not want to deal with now.

The part of the dental plaque that remains despite regular brushing gradually turns into "dental calculus", better known as tartar, which, unfortunately, can only be removed by a dentist. Calcium and phosphate ions found in saliva play the main role in the mineralization of the plaque [Jin and Yip, 2002]. These ions penetrate the biofilm and react with each other there to form an insoluble mineral precipitate.

5.2.2. ClO₂ penetration into the dental biofilm: model calculation

For the sake of clarity, we present a schematic drawing (Figure 2) showing how chlorine dioxide penetrates the supragingival biofilm based on our model calculations.

Figure 2.a) shows the biofilm symbolically, similar to the representation of e.g. Jakubovics et al. [Jakubovics et al., 2021]. In the Figure, the bacteria are disproportionately enlarged and in reality they are more densely located. The bacteria actually make up about 15–20% of the volume of the biofilm [Socransky and Haffajee, 2002].

Hundreds of types of bacteria can coexist in such a biofilm, of which only a few pathogenic bacteria can cause problems, but only if they proliferate excessively. The bacteria are held together by a hollow gel-like matrix (light blue), the material of which is secreted by the bacteria. However, for the sake of simplicity, Figure 2 shows only a few types of bacteria.

First, bacteria (white and yellow spheres) settle on the pellicle (pink), and then they are joined by other bacteria of the gradually developing biofilm (orange rods and red spheres, the latter representing pathogenic bacteria).

5.2.3. Fundamentals of the model calculation

The purpose of the calculation is to determine whether the majority of bacteria in dental plaque can be killed by a single chlorine dioxide rinse. The assumptions and approximations used in our model are as follows:

- i. The majority of bacteria in a given location are considered inactivated a few seconds after the chlorine dioxide concentration there exceeds a certain value.

- ii. The amount of ClO_2 used to kill bacteria or to react with the pellicle is negligible in the case of dental plaque.
- iii. For ClO_2 concentration profile calculations, the model relies solely on Fick's laws of diffusion [Crank, 1975], and does not take into account the reactions of ClO_2 in the gel matrix.
- iv. The model assumes an ideal initial concentration distribution, which is created instantaneously. This would mean that chlorine dioxide would appear with a sharp boundary in Figure 2. b) corresponding to $t=0$. However, in reality, the gradient is continuous, as shown in the Figure.
- v. Contrary to what is depicted in Figure 2, the model disregards the heterogeneous (cavernous) structure of the biofilm and assumes a homogeneous gel matrix medium as an approximation.

The last two of the approximations can cause a significant difference in the calculation result only at the very beginning of the flushing in a relatively short (4 s) initial period.

Since we neglect the fact that the amount of chlorine dioxide decreases in the reactions with the bacteria or with the matrix, therefore the rate-determining step of the process is the diffusion of ClO_2 . The dynamics of diffusion is always determined by some characteristic distance in addition to the diffusion coefficient D . In order to estimate the time during which chlorine dioxide can safely inactivate the majority of bacteria in the biofilm, we need approximate values of the characteristic geometric dimensions of dental plaque. We want to overestimate the time required for the inactivation of bacteria, so we take an upper estimate for the thickness of the plaque and the pellicle, and a lower estimate for the value of the diffusion coefficient D .

5.2.4. Values of the parameters in the model

- 1) In the case of plaque, we calculate the thickness of the mature plaque (together with the pellicle), which is 50 μm [Al-Ahmad et al., 2007]. It should be noted that in published experiments [Al-Ahmad et al., 2007] the thickness of the plaque was only around 15 μm after 1 day, and the thickness of 50 μm was only reached after 1 week.
- 2) The diameter of the bacterial spheres and the cylindrical rods is considered to be 1 μm .
- 3) The thickness of the pellicle is also assumed to be 1 μm . According to literature data [Chawhuaveang et al., 2021], the final thickness of the “mature” pellicle is 0.1–1 μm . The pellicle reaches this thickness in 1.5–2 hours.
- 4) The model assumes that the diffusion constant D of chlorine dioxide in the gel matrix medium does not differ significantly from the value measured in a crosslinked gelatin gel [Noszticzus et al., 2013]: $D = 260 (\mu\text{m})^2\text{s}^{-1}$. The justification for this is discussed in detail in Supplement [S]. However, we know that the value of D in plaque is probably larger than the value used in the calculations, i.e. the diffusion is faster in reality than assumed, which strengthens our conclusion. We assume that the pellicle has the same diffusion coefficient.
- 5) We assume that chlorine dioxide does not diffuse into tooth enamel and does not react with it.²
- 6) In the model, the concentration required to inactivate the majority of bacteria is 5 ppm, and the majority of bacteria are considered inactivated if the ClO_2 concentration at that location has reached this limit for 3 seconds. As mentioned, according to Bernarde, at 30 °C, a 5 ppm chlorine dioxide solution reduces the number of active bacteria by 6 orders of magnitude in 2.1 s, and even more in 3 s. So after 3 s, we can be sure that in a place where the ClO_2 concentration is 5 ppm or more, the majority of bacteria (specifically at least 99.9999%) are inactivated.
- 7) In our calculations, we model the second rinse according to the recommended usage. We assume that the first rinse has already removed the majority of the substances in saliva that can react with chlorine dioxide, so during the second rinse, the chlorine dioxide concentration at the outer edge of the plaque is constant: 15 ppm for the rinse duration of 15 s [Solumium Coral user instructions].

² It should be noted that ClO_2 can actually diffuse into tooth enamel, albeit very slowly. However, ClO_2 does not react with the material of the tooth, only with the organic dyes that have somehow got there before. This reaction is also used for teeth whitening [Ablal et al., 2013].

5.2.5. Model calculation results

The details of the model calculation are described in Supplement [S]. The most important result of the calculation is that rinsing with a mouthwash containing 15 ppm chlorine dioxide for 15 seconds, as recommended, kills the majority of bacteria in the dental biofilm according to this model.

Figure 2 shows the process of chlorine dioxide penetration based on the calculations.

After about 4 seconds of transient behavior a so-called regular stage is reached [S], when the ClO_2 concentration c in all points of the plaque approaches the final asymptotic state with the same exponential time dependence. The asymptotic state is the final equilibrium state, in which the ClO_2 concentration is 15 ppm at all points of the plaque.

During the regular stage the concentration in the plaque can be approximated by the following formula:

$$c(z, t) = c_0 - \frac{4c_0}{\pi} \cdot \exp\left(-\frac{t}{\tau_{Hn}}\right) \cdot \sin\left(\frac{\pi z}{2d}\right),$$

where z is the distance from the upper surface of plaque, t is the time elapsed since the start of rinsing, c_0 is the chlorine-dioxide concentration of the rinsing solution (15 ppm) and τ_H is the time constant of the exponential function (the “H” index refers to the hard surface). The time constant τ_H can be calculated from the plaque thickness d and the diffusion coefficient D of chlorine dioxide measured in the gel matrix using the following formula:

$$\tau_H = \frac{4 \cdot d^2}{\pi^2 \cdot D}.$$

For a plaque with a thickness of $d = 50 \mu\text{m}$, the time constant calculated as above is $\tau_H = 3.90 \text{ s}$. So the concentrations everywhere inside the plaque approach the asymptotic 15 ppm very quickly, even in the lower boundary, where the difference is relatively largest. Here, at the end of the rinsing ($t = 15 \text{ s}$) the ClO_2 concentration is 14.6 ppm. This shows that diffusion is a very fast process for such small distances ($d \leq 50 \mu\text{m}$).

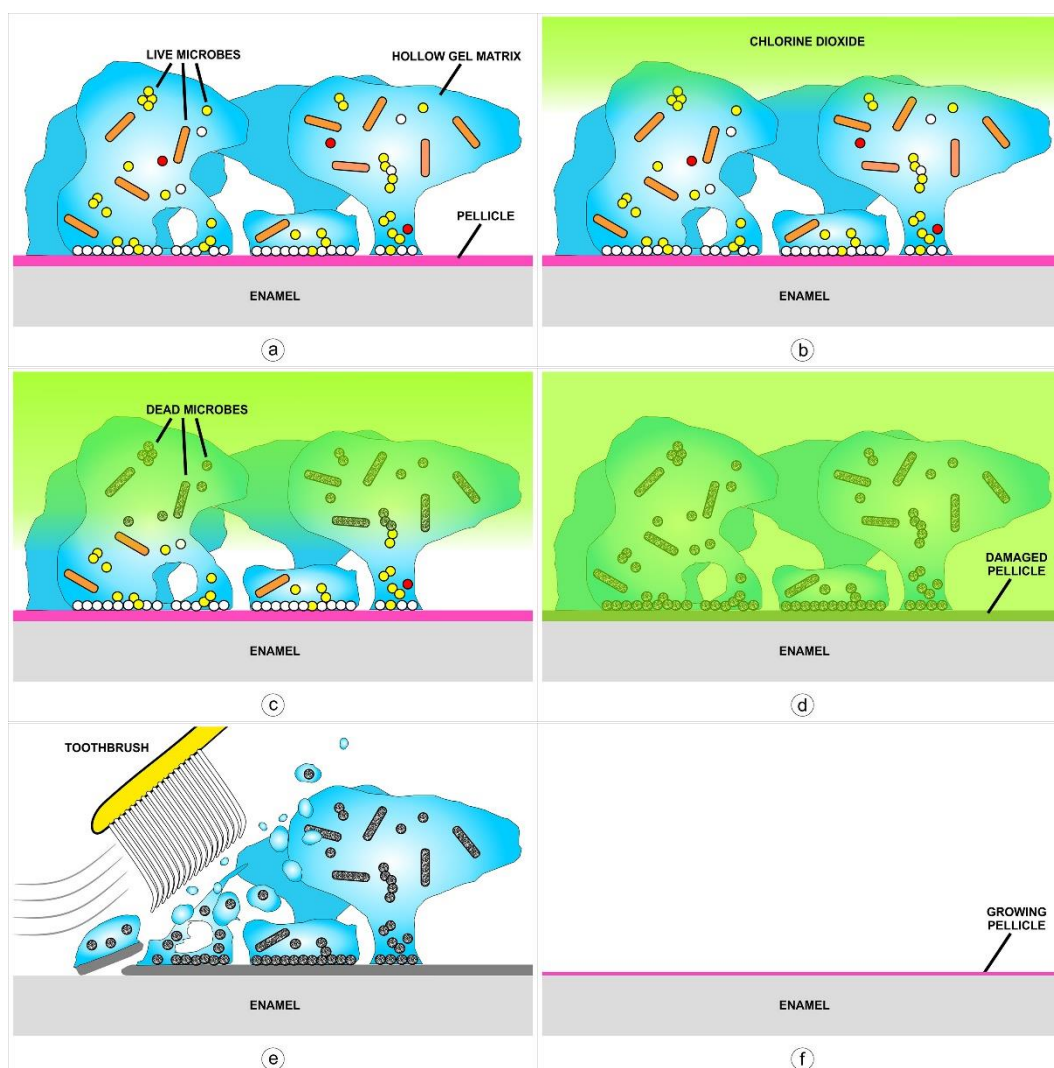


Figure 2. Effect of chlorine dioxide ($[\text{ClO}_2]=15$ ppm) rinsing and subsequent brushing on dental plaque

a) $t < 0$ s: cross-sectional view of the plaque before rinsing with chlorine dioxide. Above the pink pellicle, the bacteria are located in the light blue gel-like matrix. Pathogenic bacteria are indicated by red spheres.

b) $t = 0$ s: start of rinsing. Chlorine dioxide mouthwash (yellowish-green) appears in the oral cavity above the plaque.

c) $t = 1$ s: ClO_2 has penetrated deeply into the biofilm by diffusion. In the middle of the biofilm, the chlorine dioxide concentration at this time is approx. 4.11 ppm, but only 0.850 ppm at the bottom of the biofilm (at the pellicle).

d) $t = 4$ s. The chlorine dioxide concentration in the middle of the biofilm is 10.2 ppm, and at the bottom of the biofilm it has already increased to 8.16 ppm. Since at this point in time the ClO_2 concentration already exceeds the 5 ppm limit everywhere, by the end of the next 3 seconds ($t = 7$ s) practically all bacteria will have died, and the pellicle will be transformed and damaged as a result of the reaction with chlorine dioxide.

e) Completing the oral care by brushing the teeth, which removes the biofilm matrix together with the dead cells and the damaged pellicle. Most of the ClO_2 disappears within 20-30 seconds after the end of rinsing, even without brushing.

f) The formation of a new pellicle starts immediately on the tooth enamel, which is able to protect the tooth enamel after 3-9 minutes [Hannig, 2002]. This is already the beginning of a new cycle, at the end of which, after half a day, the mature biofilm shown in Figure a) is formed again.

5.2.6. Limitations of the model calculation

As mentioned (5.2.5.), the most important practical result of the model calculation can be considered that a 15-second rinse with a 15 ppm chlorine dioxide mouthwash can kill the majority of dental biofilm bacteria. According to the caption of Figure 2.d), this actually occurs after 7 seconds. However, the model contains several assumptions/approximations (see points i.-v. of paragraph 5.2.3.), and not all of these are fulfilled ideally in reality. The question is how much difference this can cause between the behavior predicted by the model and the actual behavior. The main reason for the discrepancy is that we do not take into account the effect of the chemical reactions of ClO_2 (approximations ii and iii). Chemical reactions continuously consume chlorine dioxide during diffusion, so it would provide a more accurate description if we used reaction-diffusion equations [De Wit, 1999] instead of diffusion equations. This is currently not possible because we do not know the relevant chemical reactions and thus their kinetic equations. Therefore, it is usual to proceed as we did, that is omit the reaction term from the reaction-diffusion equation, which can give a good approximation in many cases. For example, in the case of chlorhexidine (CHX) diffusing in dental plaque, Stewart [Stewart, 2003] uses such a model to calculate the time it takes for the CHX concentration in the center of a hemispherical plaque ($R = 260 \mu\text{m}$) on the tooth surface to reach 90 percent of the CHX concentration in the rinsing solution. Assuming a rinsing solution at 30°C (near body temperature), this time is calculated to be 298 s.

In our case, the picture may be slightly modified if approximation ii) is not good enough, i.e. the amount of ClO_2 required to kill the bacteria cannot be neglected. This can slow down the penetration of ClO_2 into the biofilm, especially when the bacterial density is high. We will discuss such a case in a later part of this series, where we will discuss the specific biofilm found on the tongue, where bacteria almost completely fill the biofilm [Mark Welch, et al., 2020]. However, in the case of plaque, the density of bacteria (the aforementioned 15–20% by volume) is much lower, and the hollow structure of plaque also facilitates the penetration of ClO_2 . Therefore, the conclusion that the chlorine dioxide rinse mentioned in the example kills most of the plaque bacteria can be maintained. This is all the more possible, since the potential slowing down of ClO_2 penetration by chemical reactions can be more than compensated for by the fact that the real thickness of the plaque is much smaller than assumed in the calculation (see the first point of paragraph 5.2.4.).

However, the situation could fundamentally change if bacteria capable of EET (i.e. Extracellular Electron Transfer) colonize the biofilm [Tokunou et al., 2022, Paquete et al., 2022, Glasser et al., 2017]. In that case, the reaction with chlorine dioxide can no longer take place only inside the bacterial cells, but in the entire volume of the gel matrix, since EET can deliver the electrons reacting with chlorine dioxide everywhere. This is somewhat similar in effect to bacterial cells filling the entire volume of the gel matrix. In such a case, the model must definitely take into account that chlorine dioxide not only diffuses, but also reacts simultaneously, at every point in the volume of the gel matrix. As a result, the penetration of ClO_2 into the gel matrix can be slowed down extremely, and it cannot be ruled out that due to the slowdown, a significant portion of the plaque bacteria may survive the chlorine dioxide mouth rinse.

Although the specific electron-conducting ability of dental plaque has not been proven [Tokunou et al., 2022], there is no doubt that certain pathogenic bacteria found in dental plaque, such as *Streptococcus mutans*, are capable of exhibiting electro-activity [Naradasu et al. 2020].

The validity of approximation iii) may also be undermined if chlorine dioxide can react with the biofilm matrix itself or with the pellicle. The former can also slow down the penetration of ClO_2 into the biofilm. Such reactions certainly exist, since proteins containing one of the three reactive amino acids (cysteine, tyrosine, and tryptophan) may occur in the matrix in small concentrations. However, these reactions can not only slow down the penetration of ClO_2 into the biofilm, but if they reach a certain level, they can have a significant impact on some property of the biofilm, e.g. the adhesion of the biofilm to tooth enamel or the cohesion of the biofilm (its ability to stay together) can change significantly. The possibility that the matrix material itself is damaged cannot be ruled out if it contains significant amounts of ClO_2 -reactive proteins.

5.2.7. Are there ClO₂-reactive proteins in the dental biofilm matrix?

A thorough review of the biofilm matrix in general was recently published [Flemming et al., 2023], and a review published two years ago specifically addresses the dental biofilm matrix [Jakubovics et al., 2021]. According to these reviews, the biofilm matrix – including dental plaque – contains a wide variety of extracellular polymers. In addition to the long-known polysaccharides, it also contains proteins, amyloids (also proteins), lipids, and even extracellular DNA. Of the listed components, ClO₂ can react with a measurable rate only with proteins, and of the proteins, only with those that contain one of the three reactive amino acids mentioned above. According to the 2023 review [Flemming et al., 2023], amyloids are composed of a wide variety of amino acids, so it cannot be ruled out that the previously mentioned reactive amino acids are also found in them. Moreover, 3 aromatic amino acids, namely phenylalanine, tyrosine and tryptophan, occur particularly frequently in amyloid proteins [Stanković, et al., 2020], the latter two of which are ClO₂-reactive amino acids. However, we were unable to find any data on whether these reactive amino acids are actually found in the plaque amyloids, nor on whether the three reactive amino acids play a prominent role anywhere in the biofilm matrix or occur in significant quantities.

5.2.8. The role of the pellicle and the ClO₂-reactive proteins it contains

One of the important roles of the pellicle is to make the surfaces of the oral cavity slippery. Without the pellicle, these surfaces would likely be hydrophobic [Gibbins et al., 2014]. Hydrophobic surfaces cannot be wetted by water (or more precisely, saliva) and thus cannot become slippery. The outer surface of the pellicle, on the other hand, is hydrophilic, and in this way the lubricating effect of saliva can prevail.

The pellicle covers both hard and soft surfaces of the oral cavity. Although the composition of the enamel pellicle over hard surfaces and the mucosal pellicle covering soft surfaces differs slightly, both types of pellicle contain at least two proteins with which chlorine dioxide can react: the mucin MUC5B and statherin [Perez-Vilar et al., 2004]. MUC5B contains all three reactive amino acid residues (cysteine, tyrosine, and tryptophan) [Hannig and Hannig, 2017], and statherin is a tyrosine-rich peptide [Schlesinger and Hay, 1977]. The reactive amino acids in the thin pellicle layer can react in a few tenths of a second in the case of a 5 ppm chlorine dioxide solution.

After their reaction with ClO₂, the spatial structure of the mentioned peptides obviously changes. We don't know if the pellicle will still stick to the underlying hard or soft surface in the same way afterwards as it did before the reaction.

It would create an interesting application opportunity if it were proven that, as a result of its reaction with chlorine dioxide, the adhesion of the pellicle on hard surfaces would weaken to these surfaces or to the biofilm matrix. This would mean that after chlorine dioxide treatment, dental plaque would be much easier to remove from tooth enamel, e.g. by brushing. This interesting possibility is suggested by the work of Anna Herczeg and his co-authors [Hercegh et al., 2013b], who measured the effect of hyperpure chlorine dioxide in vitro on dental biofilm grown on a "microtiter" plate. While live bacteria adhered more strongly to the microtiter plate, bacteria killed by chlorine dioxide were easily detached from it even after mild washing. This suggests that the connections (e.g., various nanoscale fibers originating from the bacteria) by which the killed bacteria were attached to the biofilm matrix were also damaged, and thus the washing water could more easily wash them out of the matrix.

5.2.9. Why is it worth using a pure ClO₂ based mouthwash before brushing your teeth?

Dead cells are important starting points for the mineralization of the entire biofilm, i.e. the formation of tartar [Jin and Yip, 2002]. Tartar can only be removed by a dentist, but regular brushing significantly reduces the number of dead bacteria and can even remove most of the pellicle from the tooth enamel [Hannig, 2002]. However, brushing cannot always and everywhere achieve 100% results. Based on the previous paragraphs, we have seen that biofilm and pellicle treated with chlorine dioxide mouthwash are probably easier to remove by brushing than the original untreated one due to the structural changes in the proteins, so it may be worth using the mouthwash first and only then brushing your teeth.

However, there are two other arguments suggesting that it is more beneficial to use chlorine dioxide mouthwash not after brushing, but rather in the reverse order.

i) Most toothpastes contain a variety of beneficial components, including components that inhibit plaque formation and gingivitis [Sanz et al., 2013], and this beneficial effect is reduced if the exposure time of the toothpaste is shortened by using mouthwash immediately after brushing.

ii) After brushing, some toothpaste remains on the surface of the teeth, in the interdental spaces, etc. for a while and this, as mentioned, can have a beneficial effect. However, if mouthwash is used immediately after brushing, the remaining toothpaste can inhibit the antibacterial effect of chlorine dioxide. This effect can be strong because while the recommended ClO_2 concentration is only a maximum of 30 ppm, the concentration of many components in toothpaste exceeds this by orders of magnitude³.

5.2.10. Comparison of ClO_2 with chlorhexidine

Chlorhexidine (CHX) is a widely used antiseptic. In dentistry, it is primarily used as an active ingredient in antibacterial mouthwashes [Deus and Ouanounou, 2022], and is considered as the “gold standard” for antiplaque activity [Balagopal and Arjunkumar, 2013]. Stewart [Stewart, 2003] was the first to perform a model calculation for the diffusion of CHX in plaque. Based on literature data, he estimated the effective diffusion coefficient of CHX in plaque at 30 °C to be $D_e(\text{CHX}) = 0.84 \cdot 10^{-6} \text{ cm}^2\text{s}^{-1}$. As can be seen, this is almost four times smaller than the value we estimated for chlorine dioxide: $D_e(\text{ClO}_2) = 3.2 \cdot 10^{-6} \text{ cm}^2\text{s}^{-1}$. In other words, ClO_2 – if we consider only diffusion – can penetrate plaque faster than CHX.

Another important difference is that, while in the case of CHX, a longer time and a higher concentration are required for the bactericidal effect to develop (see e.g. [Stinner et al., 2011]), chlorine dioxide – as we have seen before – can kill the bulk of bacteria extremely quickly even in small concentrations.

From the point of view of the selective inactivation of pathogenic bacteria, it does not matter whether we use high-purity chlorine dioxide or chlorhexidine-based mouthwash, as it was experimentally proven by Hercegh et al. [Hercegh, et al., 2023]. However, there are other aspects as well. For example, chlorhexidine mouthwash cannot be used for as long as you like, but chlorine dioxide mouthwash can be used, because it is much gentler on human tissues. In principle, it cannot cause systemic side effects, it does not stain the teeth and tongue, and according to studies, it is more pleasant to use [Hercegh, et al., 2023].

5.3. Research suggestions related to mouthwash use

Killing the majority of bacteria living in plaque can inhibit plaque formation and thus complement the effect of brushing. After a normal brushing, there are always places where a small part of the plaque, with bacteria living in it, can remain. On the one hand, late-colonizing bacteria can immediately attach to such places, and on the other hand, starting from these places, they can help the formation of new plaque in areas cleaned of plaque.

It is known that antibacterial mouthwashes containing chlorhexidine (CHX) have anti-plaque and anti-gingivitis effects [Löe, et al., 1970; Afennich, et al., 2011]. The latest clinical studies [Hercegh et al., 2023] have shown that the antibacterial effect of a mouthwash containing hyperpure chlorine dioxide is at least as strong as that of a mouthwash containing (CHX). Based on all this, it can be assumed that a mouthwash containing only chlorine dioxide may also have an anti-plaque and anti-gingivitis effect. It would therefore be desirable to compare hyperpure chlorine dioxide mouthwash with the “gold standard” CHX from these aspects as well.

Research on the use of hyperpure chlorine dioxide as a mouthwash has already begun: Zsolt Lohinai and colleagues [Hercegh et al., 2013a; Hercegh et al., 2013b; Hercegh et al., 2023; Szabó, et

³ We note that each of the few toothpastes we tested contained more or less components that reacted with chlorine dioxide, but we did not conduct systematic research on this.

al., 2023], and László Kőhidai and colleagues [Láng et al., 2021; Kőhidai et al., 2022] have been conducting experimental and clinical studies for years.

Perhaps one of the most important goals of experimental research would be to answer the question in which habitat and by what mechanism beneficial bacteria in the mouth can survive chlorine dioxide mouthwash in such a surprising fraction.

CONCLUSIONS

The primary goal of this review was to collect data/information already available in the scientific literature that may be important in relation to the oral use of high-purity chlorine dioxide. Based on the research results and information collected, some conclusions useful for practice can be drawn, and working hypotheses can be established that may provide new directions and help for further scientific research. Here, we would like to highlight four conclusions/hypotheses:

- 1) The active ingredient chlorine dioxide exerts its antimicrobial effect in a transition zone between human tissues and the environment, thereby supporting the human body's immune system. In the oral cavity, this transition zone is the saliva covering the mucous membrane, in which the amount of human cells is negligible, but where various microbes can occur, as shown in Figure 1. The low-concentration chlorine dioxide used can effectively kill the microbes in the transition zone, but at the same time, it has virtually no effect on human tissues, because the small amount of chlorine dioxide that gets inside the mucosal cells is neutralized extremely quickly by the glutathione present there.
- 2) It is a widely held belief, although not scientifically proven, that chlorine dioxide mouthwashes are risky because ClO_2 kills all bacteria, both beneficial and harmful (i.e. pathogens). However, this statement is only true for planktonic bacteria, not for bacteria that are aggregated in biofilms. The majority of bacteria in the oral cavity are found in biofilms. In the case of planktonic bacteria, only much less than 0.0001% of the bacteria would survive a mouthwash, but various experiments show that the average survival of oral bacteria is 10–30%. This suggests that there must be a habitat in the mouth where a biofilm helps the bacteria to survive.
- 3) An even more interesting observation is that ClO_2 kills certain pathogenic bacteria (e.g. *Streptococcus mutans* or *Fusobacterium nucleatum*) better than average, i.e. selectively. This can be explained by the theory of colonization resistance. The observation is of great practical importance. If it were proven in general that any pathogenic bacteria can be killed selectively in the oral cavity with antimicrobial mouthwash due to colonization resistance, then both the restoration of the natural oral flora and its maintenance, i.e. prevention, could be achieved by using hyperpure chlorine dioxide-based mouthwashes.
- 4) According to Socransky et al., the oral microbiome consists of several habitats and biofilms. There is no experimental data yet on which habitats microbes can survive chlorine dioxide mouthwash. In the absence of experimental data, we used a diffusion model to investigate the possibility of survival in dental plaque. Plaque was chosen as a first step because it is the most studied oral biofilm and the hard surface of tooth enamel can be easily modeled. According to our model calculation (Figure 2), the majority of plaque bacteria cannot survive a 15-second mouthwash with 15 ppm ClO_2 . However, teeth and other hard surfaces make up only 20% of the oral cavity, the remaining 80% being soft surfaces covered with epithelial cells. It is suspected that the majority of the surviving bacteria found in saliva after chlorine dioxide rinsing originate from the dorsal and lateral surfaces of the tongue, as this is also the case in normal conditions without chlorine dioxide mouth rinsing [Socransky and Haffajee, 2005]. However, it would be important to support and verify this assumption with experiments.

AUTHOR CONTRIBUTIONS

Conceptualization: Zoltán Noszticzius, Kristóf Kály-Kullai, Ákos Zsembery, László Rosivall

Formal Analysis: Kristóf Kály-Kullai, Zoltán Noszticzius

Investigation: Marianna Megyesi, Thuy B. Lawson

Methodology: Kristóf Kály-Kullai, Zoltán Noszticzius

Project Administration: Zoltán Noszticzius

Writing – Original Draft Preparation: Zoltán Noszticzius, Kristóf Kály-Kullai, Mária Wittmann

Writing – Review & Editing: Kristóf Kály-Kullai, Ákos Zsembery, Arvin Shahbazi, László Rosivall, Marianna Megyesi, Thuy B. Lawson, Mária Wittmann, Zoltán Noszticzius

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