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ZINC EFFECTS ON OXIDATIVE PHYSIOLOGY OF ORAL BACTERIA

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Abstract. Zinc is used as an anti-gingivitis agent in oral care products and affects multiple targets in oral bacteria. We found that it acts also to suppress respiration of oral streptococci and of *Fusobacterium nucleatum*, an organism associated with development of gingivitis. Zinc as either the sulfate or citrate chelate was a potent inhibitor of respiration of the oral streptococci *Streptococus mutans* GS-5 or UA 159 and *S. sanguis* NCTC 10904. 50% inhibitory concentrations for intact cells in suspensions were below 0.3 mM with nearly complete inhibition of O_2 metabolism at higher zinc levels. 1.0 mM Zn^{2+} inhibited O_2 utilization by *F. nucleatum* ATCC 25586 by some 50%, but almost 40% of O_2 metabolism by the anaerobe was not Zn sensitive. NADH oxidase plays the major role on O_2 utilization by oral streptococci and is also important for *F. nucleatum*. In cell extracts of streptococci, the 50% inhibitory concentration (IC_{50}) of Zn^{2+} for NADH oxidase activity ranged from ca. 2.1 mM for *S. sanguis* NCTC 10904 to 3.0 mM for the GS-5 strains of *S. mutans*. The enzyme in extracts of *F. nucleatum* was somewhat more Zn sensitive with an IC_{50} value of 1.4 mM. *S. sanguis* is a major generator of H_2O_2 in dental plaque. Zn^{2+}acted to reduce peroxide production assessed with use of horseradish-peroxidase and leuco-crystal violet. 0.1 mM $ZnSO_4$ was more than 50% inhibitory for cells in suspensions or mono-organism biofilms. Zinc is primarily bacteriostatic rather than bactericidal, and inhibition was at least partially reversible after zinc removal. Zinc was inhibitory also for the protective enzymes thiolperoxidase (IC_{50}= 0.1 mM), hypothiocyanite reductase (IC_{50} = 0.1 mM) and glutathione reductase (IC_{50} = 0.4 mM). Zinc acted mainly as a pro-oxidant for oral bacteria inhibiting NADH oxidase, considered to be protective against oxidative stress, and also other protective enzymes. This view is supported by findings of zinc enhanced peroxide killing of the organisms.

Abbreviations. Reactive oxygen species (ROS), NADH oxidase (NOX), NADH hypothiocyanite reductase (NHOR), glutathione reductase (GR), thiolperoxidase (TSA), catalase (CAT), pseudocatalase (PCAT), superoxide dismutase (SOD), 50% inhibitory concentration (IC_{50}).

1. INTRODUCTION

Bacteria in dental plaque are subjected to oxidative stress caused by reactive oxygen species (ROS), including hydrogen peroxide, produced as a result of plaque oxygen metabolism or contained in oral care products (Marquis, 1995). Oxygen itself has only minimal capacity directly to cause oxidative damage to cells, although it can be damaging for iron-sulfur clusters in some organisms (Imlay, 2003). However, metabolism of oxygen generally results in production of a variety of toxic oxygen species such as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2). Hydrogen peroxide and superoxide radical

can take part in the Fenton reactions to yield the highly toxic hydroxyl radical ($OH^{\cdot}$). The ROS can react with proteins, nucleic acids and lipids with resulting oxidative damage. A variety of protective enzymes act during oxidative stress to eliminate ROS or to repair oxidative damage, including superoxide dismutase, NADH peroxidase, glutathione reductase, thiolperoxidase and NADH hypothiocyanide reductase. NADH oxidase also is considered to be a protective enzyme if water rather than H_2O_2 is the end product.

Zinc is used in oral healthcare, mainly as zinc citrate, as an anti-gingivitis agent against bacteria such as *Fusobacterium nucleatum* and *Prevotella intermedia*, and also against caries caused by mutans streptococci. Zinc also has been found to be effective against oral anaerobes involved in periodontal diseases. Levels of zinc in dental plaque have been found to be about 2.5 mmole/g or 2.5 mmole/ml after use of zinc-containing toothpaste (Creeth, 1993). Moreover, zinc is considered to be a major antioxidant for animal cells (Powell, 2000; Zago and Oteiza, 2001). Mechanisms for its antioxidant actions include interactions with sulfhydryl groups to protect them against oxidation and competition between zinc and reduced transition metal cations resulting in reduced production of hydroxyl radicals. Zinc can act as an antioxidant also for bacterial cells. For example, Gaballa and Helmann (2002) found that increased intracellular levels of zinc protected cells of *Bacillus subtilis* against agents such as thiol-oxidizing diamide. Zinc also protected the cells against damage by hydrogen peroxide but protection depended very much on catalase, which was upregulated in association with a peroxide-induced *PerR* regulon. However, an antioxidant role of zinc in oral bacteria has not been identified .

The focus of our study is on zinc effects on the oxidative metabolism of oral streptococci associated with dental caries and of *Fusobacterium nucleatum* associated with gingivitis.

2. MATERIALS AND METHODS

2.1. Zinc uptake by *S. mutans* UA 159 in suspension

Cells were harvested from early stationary-phase cultures and washed once with salt solution containing 50 mM KCl and 1mM $MgCl_2$. The cells were resuspended in 20 mM Tris-HCl or acetate buffer at the indicated pH values. At intervals, 1 ml of cell suspension was mixed with silicone oil and centrifuged. Zinc in the pellets were extracted with 0.3 M HCl solution and assayed by means of atomic absorption spectrophotometry.

2.2. Oxygen metabolism by oral bacteria

For suspensions, cells were prepared as for zinc uptake experiments and resuspended in 20 mM Tris-HCl buffer, pH 7.0, containing 0.1% glucose. Oxygen levels were measured with a standard oxygen electrode (VWR) as described by Caldwell and Marquis (1999). Biofilms of *S. mutans* UA 159 were formed on glass slides in medium containing 3% tryptone, 0.5% yeast extract and 1% sucrose. The intact biofilms were washed with salt solution and placed in 20 mM Tris-HCl buffer, pH 7.0, plus 0.2% glucose. Oxygen metabolism was measured with a standard oxygen electrode as for cells in suspensions.

2.3. H_2O_2 production by *S. sanguis* ATCC 10904

Cells in suspensions and biofilms were prepared as for respiration assays in 20 mM Tris-HCl buffer, pH 7.0. H_2O_2 levels were assessed with use of horseradish peroxidase and leuco-crystal violet (Motolla et al, 1970).

2.4. Killing of *S. mutans* UA 159

Cells for the killing assay were suspended in 1% peptone at pH value of 7.0. The treated cells were sampled at intervals and diluted immediately in 1% peptone broth before plating on Difco tryptic soy agar as described by Dunning et al. (1998).

2.5. Enzymes assays

NADH oxidase (Pool and Claiborne., 1986.), SOD (McCord and Fridovich., 1969), CAT and PCAT (Thibodeau and Keef, 1990), NHOR (Courtois and Pourtois, 1996), GR (Bazzichi et al., 2002), TSA (Park., 2000).

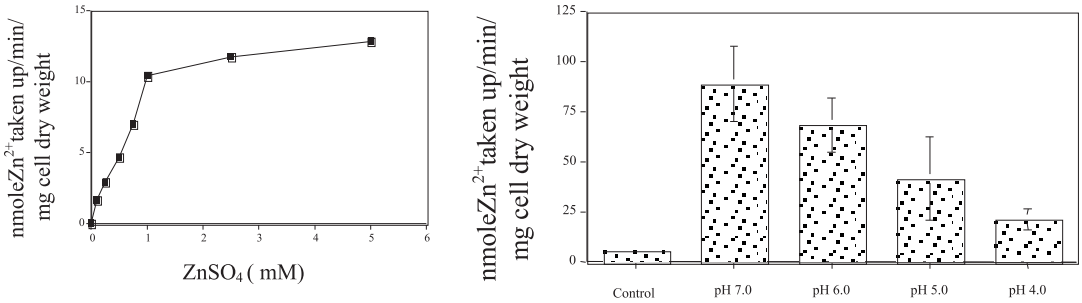
3. RESULT AND DISCUSSION

3.1. Uptake of zinc by oral bacteria

It is noted that zinc is transported into cell cytoplasm where it reacts with multiple targets and consequently, results in the damage of the cells. Zinc is usually taken up into the cells by vehicles like chelators or transport systems. We have studied the uptake of zinc by *S. mutans* UA159 using AAS. Initial experiments were performed with cells of *S. mutans* UA159 grown in suspension cultures with excess glucose. The data in Fig.1A, showed that zinc was taken up by the organism by a saturable process. In the concentration range from 0 to about 1 mmolar, the rate of uptake at pH 7 increased linearly with increasing initial concentration of zinc sulfate. Then, at higher concentrations, the uptake diminished relative to further increases in initial concentration of zinc sulfate to level off at about 2.5 μmol Zn^{2+} taken up per minute per mg cell dry weight. The uptake of zinc from zinc citrate did not differ appreciably from that of zinc sulfate (data not shown). The uptake was pH sensitive with greater uptake at pH 7.0, compared with that at pH values of 6.0, 5.0 or 4.0 (Fig. 1B). Zinc has generally been found to have greatest antimicrobial activity at pH values close to neutrality and to have somewhat diminished activity at more acid pH values. At least part of this decline in antimicrobial activity is likely to be due to reduced uptake of the metal.

3.2. Zinc inhibition of respiration by oral bacteria

Oral streptococci are capable of high levels of respiration (oxygen metabolism) but do not have electron transport systems in their membranes to be able to carry out oxidative phosphorylation. The need for respiration unconnected to oxidative phosphorylation is still somewhat of a mystery, although it may have to do with cell needs for oxidized NAD^+ to act as an electron acceptor, for example, in glycolysis. Other oral bacteria would be expected to have at least moderate respiratory capacities, including obligate anaerobes. As shown by the graphs presented in Fig. 2, zinc sulfate inhibited respiration of intact cells of *S. mutans* UA159, *S. sanguis* NCTC 10904, *S. mutans* GS-5 or *F. nucleatum*



(a) (b)

Fig. 1. Zinc uptake from zinc sulfate by *S. mutans* UA159 in suspension. (a): Uptake is a saturable process. (b): Uptake is sensitive to pH.

ATCC 25586. The mutans streptococci (Fig. 2A) appeared to be the most sensitive with 50% inhibitory concentrations of about 0.3 mM and with only a low level of respiration largely insensitive to zinc. Respiration of *S. sanguis* showed a more complex response to zinc with part of respiration as sensitive as that of the mutans streptococci, part with intermediate sensitivity and a significant component with low zinc sensitivity. Respiration of *F. nucleatum* (Fig. 2B) showed both zinc sensitive and zinc insensitive components. About 60% of O_2 utilization by this organism was inhibited by Zn while the remaining 40% was not. This may account for the higher IC_{50} (ca.1 mM) for respiration rate of this organism. The data show that mutans streptococci are the most sensitive with 50% inhibitory concentration (IC_{50}) of ca. 0.3 mM, while IC_{50} of *F. nucleatum* was 1.0 mM. The same patterns for inhibition of respiration were obtained with zinc citrate (data not shown). The cells on biofilms were also sensitive to zinc sulfate with IC_{50} of about 75 mM, much higher compared to suspension cells (Fig. 2C).

NADH oxidase plays the major role in O_2 utilization by oral streptococci (Higuchi et al, 1999) and also is important for *F. nucleatum*. This enzyme is very active in this anaerobe and thought to be the key enzyme for the growth of *F. nucleatum* in oxygen conditions as well as for producing a reducing environment for growth of obligately anaerobic organisms like *P. gingivalis* and *P. intermedia* (Diaz *et al,* 2002). The data (Fig 3.) showed that NADH oxidase of oral bacteria was sensitive to zinc with IC_{50} of about 1.4 mM for *F. nucleatum*, 2.1 mM for *S. sanguis* and 3.0 mM for *S. mutans*. The cells in biofilms were also sensitive to zinc, but higher zinc concentrations were required for the same level of inhibition compared to cells in suspensions (data not shown). The results suggest that the repressed respiration of cells in suspensions and biofilms is due mainly to inhibition of NADH oxidase. Respiration is source of toxic products such as ROS. ROS is considered to be toxic for host cells. The finding that zinc inhibits the respiration of oral bactreria indicated that zinc reduced ROS and therefore is an anti-oxidant in terms of inhibition of respiration. However, respiration is in part dependent on zinc-sensitive NADH

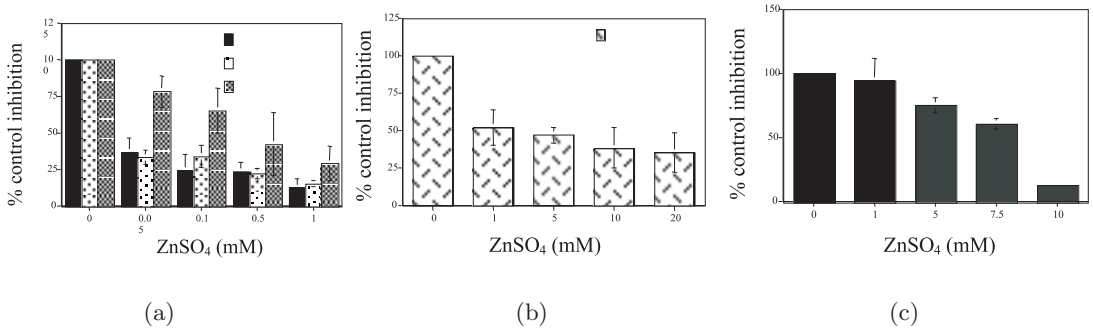


Fig. 2. Zinc inhibition of respiration of oral bacteria. (a): Streptococci; (b): *F. nucleatum* ATCC 25586; (c): Biofilms of *S. mutans* UA159. 100% respiration = 6.4, 3.7, 2.1, 1.7 0.66 nmoles O2 utilized/min/mg cell dry weight, respectively for *S. mutans* GS-5, *S. mutans* UA 159, *S. sanguis* NCTC 10904 and *F. nucleatum* ATCC 25586 and biofilms of *S. mutans* UA159.

oxidase, which function in recycling NADH back into metabolism as NAD^+, which can then serve as a receptor for reducing equivalents from glycolysis. Therefore, the sensitivity of NADH oxidase could cause metabolic problems.

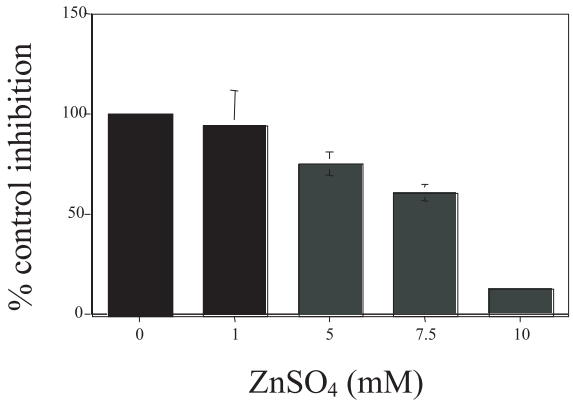
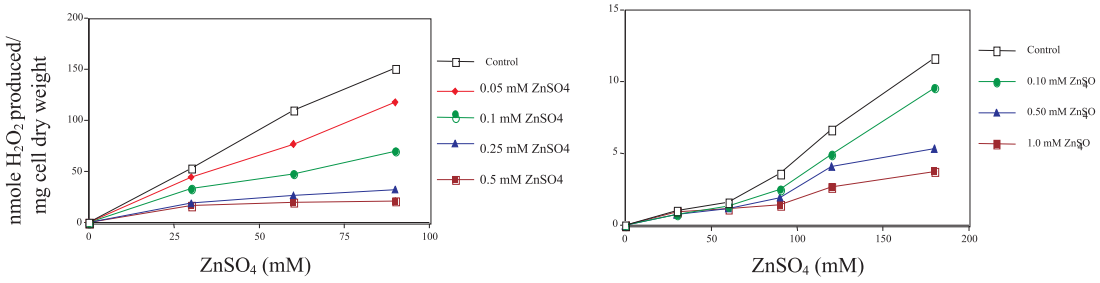


Fig. 3. Zinc inhibition of NADH oxidase. 100% activity = 0.056, 0.052, 0.032, 0.041 units/mg proteins, respectively for *S. mutans* GS-5, *S. mutans* UA 159, *S. sanguis* and *F. nucleatum*

3.3. Zinc inhibition of H_2O_2 production by oral bacteria

Streptococci possess two types of NADH oxidase: H_2O_2-yielding NADH oxidase (is denoted NOX-1) and H_2O yielding NADH oxidase (is denoted NOX-2). In *S. mutans* GS-5, NOX-2 is a dominant enzyme and NOX-1 is an AhpCF (alkylhydroperoxide reductase) meaning that it can both produce and degrade H_2O_2. H_2O_2 production offers a competitive

advantage for bacteria in dental plaque and net H_2O_2 producers such as *S. sanguis*, *S. gordonii*, *S. mitis.* We found that Zn reduced the H_2O_2 production by *S. sanguis* 10904 in both planktonic and biofilm cells (Fig 4A). *S. sanguis* is a major H_2O_2 generator in dental plaque, and 0.1 mM zinc was more than 50% inhibitory for H_2O_2 production by cells in suspension. Biofilms were more resistant to zinc with IC_{50} value of ca. 0.5 mM (Fig. 4B). The inhibition of NADH oxidase of *S. sanguis*, as shown in Fig. 3, is likely to be the basis for reduced H_2O_2 production by this organism.



(a) (b)

Fig. 4. Zinc inhibition of H_2O_2 production by *S. sanguis* ATCC 10904.
(a): Suspensions; (b): Biofilms

3.4. Zinc inhibition of protective enzymes in oral bacteria

During oxygen metabolism, oral streptococci release reactive oxygen species including superoxide radical and hydrogen peroxide, which can cause oxidative stress for the bacteria. To protect the cells from the oxidative damage, a variety of protective enzymes are synthesized. Table 1 presented major protective enzymes in oral bacteria. We examined the effect of zinc on the main enzymes including NHOR, GR, TSA, CAT, PCAT and SOD. NOX also considered to be a protective enzyme against O_2 toxicity because the final product is water. The data in Table 1 indicated that not all protective enzymes including two important enzymes CAT and SOD, were targets of zinc.

3.5. Zinc enhancement of H_2O_2 killing of oral bacteria

Fig. 5 showed that zinc enhanced H_2O_2 killing. However, very high levels of zinc were required for enhancement, and there are some questions regarding the physiological significance of this type of enhancement. When cells in suspensions were challenged with H_2O_2 in presence of Zn, the killing was somewhat enhanced compared to those that were exposed to H_2O_2 alone. The potential in enhancing toxicity of H_2O_2suggest that zinc is a pro-oxidant and enhance oxidative stress in the bacteria. Data on inhibition of protective enzyme activity presented above supported this finding.

Table 1. *Zinc inhibition of protective enzymes against oxidative damage*

Enzymes	Organisms	Inhibition level	IC_{50}(mM)
NADH oxidase	Streptococci	+	2.1 - 3.0
	F. nucleatum	+	1.4
NADH hypothiocyanite reductase (NHOR)	*S. sanguis*	+	0.1
Glutathione reductase (GR)	*S. mutans*	+	0.4
Thiolperoxidase (TSA)	*S. mutans*	+	0.1
Catalase (CAT)	*A.viscosus*	-	
Pseudocatalase (PCAT)	*L. plantarum*	-	
Superoxide dismutase (SOD)	Streptococci	-	

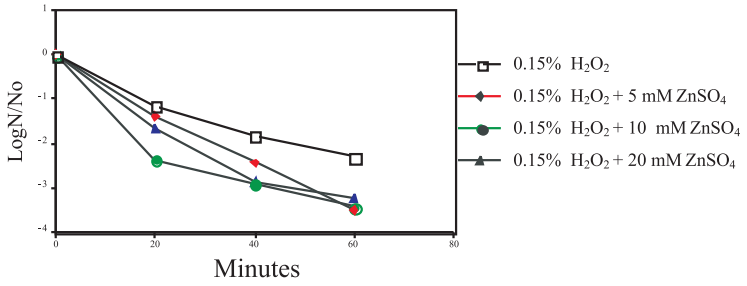


Fig. 5. Zinc enhancement of oxidative killing of *S. mutans* UA 159 in suspension.

4. CONCLUSIONS

1. Zinc is a potent inhibitor of respiration, especially that involving NADH oxidase in oral streptococci and is also an inhibitor of oxygen metabolism of the oral anaerobe *F. nucleatum.*

2. Zinc is an inhibitor for a number of enzymes considered to be protective against oxidative damage and appears to have an overall pro-oxidant action on oral bacteria.

3. Zinc is concentrated from the environment by *S. mutans* UA 159 in a pH dependent manner that mimics the pH dependence of anti-bacteria action with high potency at pH 7.0 and declining potency at the pH value was lowered to 4.0.

4. Zinc has multiple targets for inhibition of oral bacteria, and as shown here, these targets include enzymes involved in oxidative metabolism.

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