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Potentiation of Bacterial Killing Activity of Zinc Chloride by Pyrrolidine Dithiocarbamate

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Zinc has antimicrobial activity and zinc salts including zinc chloride ($ZnCl_2$) have been used for the control of oral malodor. In this study, we hypothesized that pyrrolidine dithiocarbamate (PDTC), a zinc ionophore, may enhance antimicrobial efficacy of $ZnCl_2$. The bactericidal effectiveness of $ZnCl_2$ alone (0.5-8 mM) or in combination with PDTC (1 or 10 μM) was evaluated by *in vitro* short (1 h) time-killing assays against *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. Only a slight viability decrease was observed with $ZnCl_2$ or PDTC alone after 1-h incubation. By contrast, combination of $ZnCl_2$ and PDTC could achieve a more than 100-fold viability reduction compared with $ZnCl_2$ or PDTC alone in *F. nucleatum* and *P. gingivalis*. Therefore, PDTC greatly enhanced the bactericidal activity of $ZnCl_2$ against the oral malodor-producing bacteria. These results suggest that use of PDTC may be useful for enhancing bactericidal activity of anti-malodor regimens of zinc salts.

***Keywords*:** oral malodor, zinc, pyrrolidine dithiocarbamate, bactericidal

Halitosis or oral malodor is an unpleasant or offensive odor emanating from the oral cavity, leading to discomfort and psychosocial embarrassment. Oral malodor arises mainly from the production of volatile sulfur compounds (VSCs) resulting from the microbial degradation of amino acids in the diet, desquamated epithelial cells, serum, and saliva. VSCs originating from the oral cavity consist essentially of hydrogen sulfide and methyl mercaptan (Delanghe *et al*., 1999; Loesche and Kazor, 2002; Nakano *et al*., 2002). VSCs are mainly produced through putrefactive activities of bacteria residing at different sites in the oral cavity. In the absence of microorganisms, the odoriferous components are not generated (Persson *et al*., 1989, 1990). The putative malodorous species are mainly Gram-negative strict anaerobic bacteria, including *Fusobacterium nucleatum* and *Porphyromonas gingivalis.*

Since oral malodor is associated with bacterial overgrowth on the tongue, antimicrobial treatment approaches have been used to reduce the bacterial load. Toothpastes and mouthrinses with antimicrobial properties can reduce oral malodor by reducing the number of bacteria chemically. Often used active ingredients in these products are chlorhexidine, triclosan, essential oils, and cetylpyridinium chloride. Other chemical agents can reduce halitosis by chemically neutralizing odorous VSCs. Often used active ingredients of these products are metal ions and oxidizing agents (Roldan *et al*., 2003; van den Broek *et al*., 2008).

Metal ions with high affinity for sulfur are known to inhibit formation of VSCs. Zinc is the metal ion of choice for this purpose due to its low toxicity and to its other favorable properties, such as not causing dental staining (Young *et al*., 2001). A mouthrinse containing 0.05-0.1% (4-8 mM) zinc is widely used in dental clinics. It is known that zinc ions possessing anti-VSC effects have affinity for sulfur, forming sulfides with low solubility. The anti-VSC effects are probably also related to the antibacterial properties of the metal (Wåler, 1997). The antimicrobial mechanism involves the capability of zinc ions to inhibit glycolysis of microorganisms by oxidizing thiols groups in essential glycolytic enzymes (He *et al*., 2002).

Pyrrolidine dithiocarbamate (PDTC) is a water-soluble, low-molecular-weight thiol antioxidant and has been used widely as a potent inhibitor of nuclear factor-κB (NF-κB) in mammalian cell cultures to assess the role of NF-κB in signaling pathways (Munoz *et al*., 1996; Mochizuki *et al*., 2005). PDTC is also a zinc ionophore. Zinc ionophores are compounds that facilitate transmission of zinc ions across cell membranes by combining with the ions. Two thiol groups of PDTC confer heavy metal-chelating and free radical-scavenging properties to this compound (Camps and Boothroyd, 2001). In mammalian cells, PDTC has been shown to have many biological activities, including inhibition of inflammatory genes' expression. Moreover, we recently reported that PDTC inhibited bacterial growth (Kang *et al*., 2008).

In the present study, we hypothesized that PDTC may enhance antimicrobial efficacy of zinc. This study investigated whether PDTC increases bactericidal effectiveness of zinc chloride ($ZnCl_2$) against two important VSC-producing bacteria, *F. nucleatum* and *P. gingivalis.*

Materials and Methods

Bacteria and growth conditions

F. nucleatum ATCC 10953 and *P. gingivalis* ATCC 33277 were grown in

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tryptic soy broth or on tryptic soy agar at 37°C under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). Tryptic soy broth was supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml). Tryptic soy agar was additionally supplemented with sheep blood (5%).

Determination of minimum inhibitory concentrations (MICs)

Bacterial cultures of logarithmic-phase were added to culture medium containing a series of $ZnCl_2$ dilutions in wells of 96-well plates. The final inoculum concentration of 1×10^6 CFU/ml was controlled by measuring the optical density (OD) at 600 nm and extrapolating CFU/ml using preset standard curves. Successive twofold dilutions of $ZnCl_2$ were prepared in a 100 μl volume and 100 μl each of *F. nucleatum* or *P. gingivalis* was added to the prepared plates. After incubation for 24 h, microbial growth was measured using a microplate reader at 600 nm. The MIC was defined as the lowest dilution at which no growth was detected.

Time-kill assays

In vitro bactericidal activities were evaluated using time-kill assays. 10 ml of *F. nucleatum* or *P. gingivalis* was cultured in 50-ml tubes for colony counts. The approximate bacterial inoculum size was 1×10^6 CFU/ml. Bacterial cultures were treated with $ZnCl_2$ (1×-8× MIC), PDTC (1 or 10 μM), or $ZnCl_2$+PDTC. At 0 and 1 h of incubation, aliquots of 100 μl were obtained from the tubes and 10-fold dilutions were made and cultured on tryptic soy agar plates for 24-48 h. The number of colonies formed was counted.

Crystal violet assay assessment of cytotoxicity

Oral KB carcinoma cells were grown in DMEM containing 10% fetal bovine serum and 50 μg/ml gentamicin at 37°C in 5% CO_2. Cells were seeded at 1×10^5 cells per well in 48-well plates and allowed to adhere overnight. The following morning, cells were treated with $ZnCl_2$ and PDTC and incubated for 24 h. Cell viability was assessed by crystal violet assay that measures the number of viable adherent cells. After removing nonadherent cells by repeatedly washing the cultures with PBS, cells were fixed with 100% methanol and stained with 1% crystal violet solution at room temperature for 10 min. Plates were thoroughly washed with PBS, 33% acetic acid was added to each well and the OD of dissolved dye, corresponding to the number of viable cells, was measured in a microplate reader at 570 nm.

Results

MICs of $ZnCl_2$ against *F. nucleatum* and *P. gingivalis*

First, we determined MICs of $ZnCl_2$ against *F. nucleatum* and *P. gingivalis* using the broth microdilution assays. The MICs of $ZnCl_2$ were 1 mM for *F. nucleatum* and 0.5 mM for *P. gingivalis.*

Effect of PDTC on $ZnCl_2$ killing of *F. nucleatum* and *P. gingivalis*

Next, we determined whether $ZnCl_2$ is able to kill *F. nucleatum* and *P. gingivalis* within a short time period and addition of PDTC enhances the killing. One-hour time-kill assays were performed. *F. nucleatum* cultures were treated with $ZnCl_2$ (1× -8× MIC, 1-8 mM), PDTC (1 or 10 μM), or $ZnCl_2$+PDTC. After 1 h, colony counts were determined. A viability decrease of less than 1 log was observed with $ZnCl_2$ or PDTC alone after 1-h incubation. The addition of PDTC dose-dependently increased the bactericidal activity of $ZnCl_2$. Combination of $ZnCl_2$ (4-8 mM) and PDTC (10 μM) achieved a more than 100-fold viability reduction compared with $ZnCl_2$ or PDTC alone (Fig. 1). One-hour time-kill assays were also carried out against *P. gingivalis*. *P. gingivalis* cultures were treated with $ZnCl_2$ (1×-8× MIC, 0.5-4 mM), PDTC (1 or 10 μM), or $ZnCl_2$ +PDTC. The results of *P. gingivalis* were similar to those of *F. nucleatum* (Fig. 2).

Cytotoxicity of $ZnCl_2$ and PDTC

Effect of $ZnCl_2$ and PDTC on viability of KB cells was examined. KB cells were treated with $ZnCl_2$ (8 mM), PDTC (10 μM), or $ZnCl_2$+PDTC, and cell viability was assessed by crystal violet staining assays. The cell viability was slightly

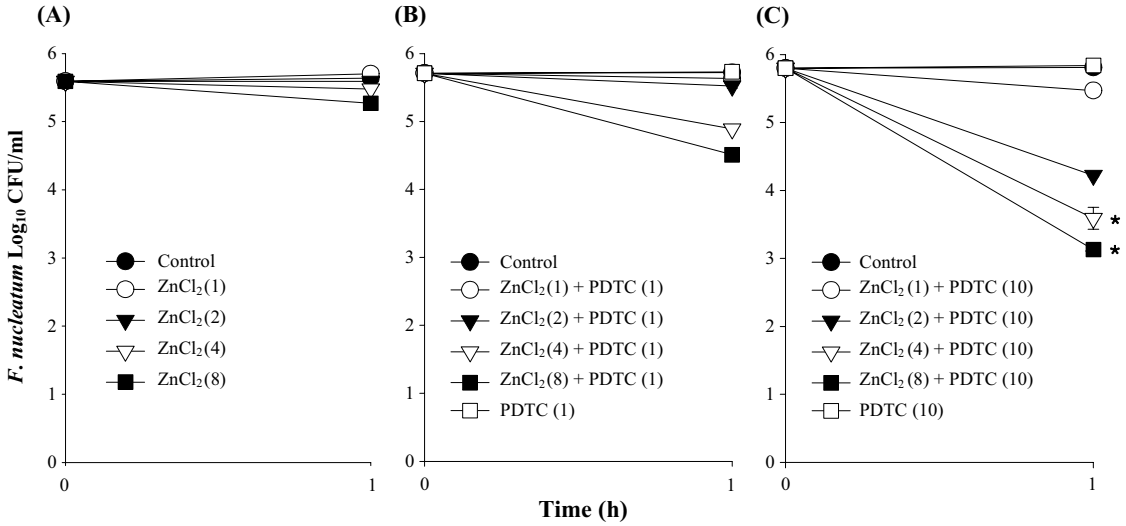

Fig. 1. Killing of *F. nucleatum* by $ZnCl_2$ and PDTC. *F. nucleatum* cultures (1×10^6 CFU/ml) were treated with $ZnCl_2$ (1-8 mM), PDTC (1 or 10 μM), or $ZnCl_2$+PDTC. After 1 h, colony counts were determined. Data are expressed as the Means±SD of a representative experiment performed in triplicate. Similar results were obtained in two other experiments. (A) $ZnCl_2$ alone, (B) $ZnCl_2$+1 μM PDTC, and (C) $ZnCl_2$+10 μM PDTC. * Synergistic combination (≥100-fold viability reduction compared with $ZnCl_2$ or PDTC alone).

reduced by $ZnCl_2$ or PDTC. Addition of PDTC did not increase the cytotoxicity of $ZnCl_2$ after all (Fig. 3).

Discussion

The present study showed that PDTC enhanced the bactericidal activity of $ZnCl_2$ against *F. nucleatum* and *P. gingivalis*. Synergistic effect of $ZnCl_2$/PDTC combination could be observed. Combination of $ZnCl_2$ (4×-8× MICs) and PDTC (10 μM) achieved a more than 100-fold viability reduction compared with $ZnCl_2$ or PDTC alone (Figs. 1 and 2). When drugs are tested in combination by the time-kill method, synergy is defined as a ≥100-fold decrease in the number of CFU achieved with a drug combination compared to that achieved with the most active drug tested alone (Citron and Hecht, 2003). We selected 1 h as the time period of time-kill assays because short time bactericidal effect should be more clinically relevant than long time effect considering the oral topical application of zinc.

The antimicrobial mechanism of zinc salts involves the capability of zinc ions to inhibit glycolysis of microorganisms by oxidizing thiols groups in essential glycolytic enzymes. It is therefore suggested that the potentiation of bacterial killing activity of $ZnCl_2$ by PDTC may be due to facilitation of entry of zinc ions into the inside of bacterial cells.

Although the major mechanism of zinc as a VSC inhibitor is thought to be that zinc ions with high affinity for sulfur inhibit formation of VSCs, the anti-VSC effects are probably also related to the antibacterial properties of zinc. In this respect, the result of this study is meaningful, indicating that addition of a small amount of PDTC to zinc products will increase the antimicrobial efficacy of the products. When $ZnCl_2$ alone cannot achieve a substantial killing of malodor-producing bacteria, addition of PDTC will potentiate the bactericidal activity of $ZnCl_2$.

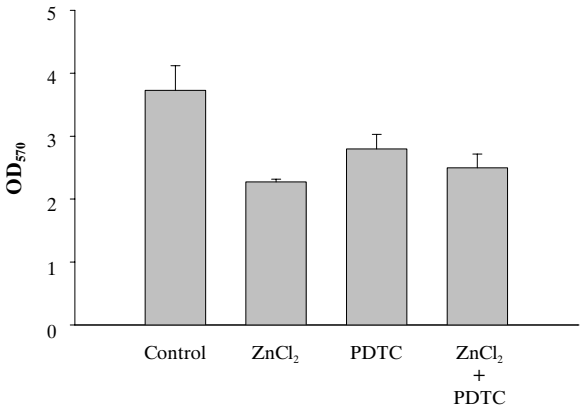

Fig. 3. Effect of $ZnCl_2$ and PDTC on viability of KB cells. 1×10^5 KB cells were seeded in 48-well plates. The next day, cells were treated with $ZnCl_2$ (8 mM) and PDTC (10 μM) and incubated for 24 h. Cell viability was assessed by crystal violet assays. Data are the Means±SD of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.

Although our results showed some cytotoxicity of $ZnCl_2$ (Fig. 3), 8 mM (0.1%) $ZnCl_2$ has been safely used in clinics for the reduction of halitosis. It appears that addition of a small amount of PDTC to zinc products will not increase the cytotoxicity of $ZnCl_2$ (Fig. 3). In fact, PDTC has been widely used in *in vitro* and *in vivo* experimental models (Lauzurica *et al*., 1999; Cuzzocrea *et al*., 2002).

Halitosis is a common condition and can be a crippling social problem. Even with the implementation of good oral hygiene, many patients continue to have halitosis of oral origin. In such instances, rinsing and gargling with an efficacious mouthrinses are advised. Zinc salts are being widely used for the purpose of the control of oral malodor. The results of the

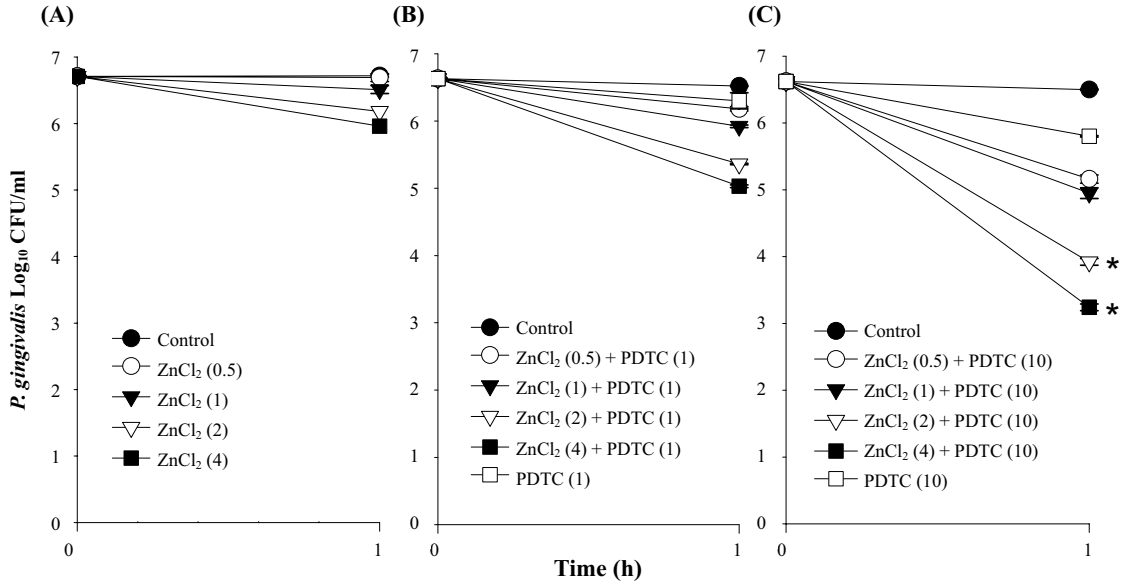

Fig. 2. Killing of *P. gingivalis* by $ZnCl_2$ and PDTC. *P. gingivalis* cultures (1×10^6 CFU/ml) were treated with $ZnCl_2$ (0.5-4 mM), PDTC (1 or 10 μM), or $ZnCl_2$+PDTC. After 1 h, colony counts were determined. Data are expressed as the Means±SD of a representative experiment performed in triplicate. Similar results were obtained in two other experiments. (A) $ZnCl_2$ alone, (B) $ZnCl_2$+1 μM PDTC, and (C) $ZnCl_2$+10 μM PDTC. * Synergistic combination (≥100-fold viability reduction compared with $ZnCl_2$ or PDTC alone).

present study suggested that addition of PDTC to the formulations of zinc-containing mouthrinses may be beneficial, potentiating the bactericidal effectiveness of zinc.

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