ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1986, p. 342–343
0066-4804/86/020342-02$02.00/0

Vol. 29, No. 2

Enhanced Toxicity of Copper for *Streptococcus mutans* under Anaerobic Conditions

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Received 5 September 1985/Accepted 19 November 1985

Copper inhibition of 11 strains (serotypes a through g) of *Streptococcus mutans* was increased by anaerobic incubation. Anaerobic toxicity was reversed by cuprous, but not by cupric, chelators. Susceptibility to aerobic copper inhibition was related to serotype; serotypes c, e, and f (biotype I) were most sensitive.

Certain cations reduce the acidogenicity of dental plaque; copper is one of the most active of these (11). In human volunteers, topical applications of copper to teeth inhibited acid production by plaque for several hours (10), and oral rinsing with a copper solution retarded both plaque formation and the development of gingivitis (17, 18). In experimental caries studies using animals orally inoculated with *Streptococcus mutans*, topical applications of copper significantly reduced caries scores and produced lower *S. mutans* counts (1). The cariostatic activity of copper may be related to its antibacterial activity, as the addition of a low concentration (0.16 μM) of copper to continuous cultures of *S. mutans* appears to be bactericidal (3). It has been suggested that copper may be useful clinically in the treatment of gingivitis as well as in the prevention of caries (17).

Present studies report the aerobic and anaerobic susceptibilities of 11 strains of *S. mutans* representing serotypes a through g (Table 1). Molten Todd-Hewitt agar (Difco Laboratories) at 45°C was inoculated with the test organism (10^5 CFU/ml, final concentration), and sterile paper disks (0.64 cm in diameter; Difco Laboratories) containing various concentrations of copper (added as a filter-sterilized solution of cupric sulfate) were placed on the agar surface after it solidified in plates. Duplicate plates were incubated (at 35°C) aerobically (95% air, 5% CO_2) and anaerobically (90% N_2, 5% H_2, 5% CO_2; modified Forma Scientific model 1024 anaerobic system) (9). After 24 h, zones of inhibition of growth around the disks were measured. Of the three copper concentrations tested, all strains were more susceptible under anaerobic conditions (Table 1). In the aerobic atmosphere, only three strains (LM7, Ingbritt, and GS5) were inhibited by 1.5 μmol of copper per disk; in the anaerobic chamber, this copper concentration inhibited all strains, producing zones of inhibition ranging from 20 to 29 mm. The anaerobically increased susceptibility to copper was confirmed in brain heart infusion and Todd-Hewitt broth cultures. For example, from an inoculum of 10^4 CFU/ml, the aerobic growth of *S. mutans* OMZ176 in both media was completely inhibited by 1.6 mM copper, while anaerobic growth in Todd-Hewitt broth and brain heart infusion broth was completely inhibited by 0.2 and 0.8 mM copper, respectively.

A sharp distinction was noted between the aerobic susceptibilities of strains Ingbritt, GS5, LM7, and OMZ175 of serotypes c, e, and f, representing biotype I (13), and the remaining strains (Table 1). This was most evident at 6 μmol of copper per disk, which produced a zone of inhibition with the biotype I strains that was approximately twice that noted with the other strains. High aerobic susceptibility to copper may be another biochemical characteristic shared by biotype I strains.

The anaerobically, enhanced toxicity of copper for *S. mutans* may be due to metabolic shifts induced by anaerobiosis. However, evidence presented below suggests that the toxicity of the reduced (cuprous) ion, which could be present in anaerobic media at higher levels, is primarily responsible for copper toxicity in *S. mutans*. Increased copper toxicity also was noted during incubation in a N_2 atmosphere (data not shown), suggesting that the reducing capacity of the culture medium (Todd-Hewitt or brain heart infusion) generated the cuprous ion. Rapid metal reduction by the media was indicated by the addition to freshly autoclaved media containing the cuprous chelator bathocuproine disulfonate (BCDS; Sigma Chemical Co. [19]) of cupric ion, which resulted in the immediate appearance of the red color of the BCDS complex. An oxidant (H_2O_2) reversed anaerobic copper inhibition. Paper disks containing H_2O_2 (3 to 96 μmol) and disks containing copper (3 μmol) were placed on seeded Todd-Hewitt agar plates, which then were incubated anaerobically. Although the H_2O_2 produced a zone of inhibition, "spurs" of growth were evident be-

TABLE 1. Toxicity of copper for strains of *S. mutans*

Strain	Serotype[b]	Zone of inhibition (mm)[a] with Cu (μmol/disk):					
		Aerobic			Anaerobic		
		1.5	3.0	6.0	1.5	3.0	6.0
LM7	e	10	13	24	27	31	33
Ingbritt	c	10	15	20	28	30	34
GS5	c	9	16	23	28	32	35
OMZ175	f	0	11	20	27	31	33
FA1	b	0	9	11	20	23	24
AHT	a	0	8	11	23	27	33
HS6	a	0	8	11	21	23	27
BHT	b	0	8	12	22	27	30
B13	d	0	0	11	29	34	35
6715-15	g	0	0	10	20	24	27
OMZ176	d	0	0	11	22	27	29

[a] Disks containing the indicated copper concentrations were placed on the surface of seeded Todd-Hewitt agar plates that were incubated (35°C) aerobically or anaerobically.

[b] See reference 16.

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TABLE 2. Effect of valence-specific chelators on anaerobic copper toxicity in *S. mutans* OMZ176[a]

Metal (0.4 mM)	Chelator	Copper valence specificity	Growth inhibition (%)[b]
Cu	None		49
Cu	BCDS	Cuprous	0
Cu	FerroZine	Cuprous	0
Cu	Desferal	Cupric	55
Cu	EDTA	Cupric	100
None	BCDS		0
None	FerroZine		0
None	Desferal		0
None	EDTA		100
Fe	EDTA		0
Fe	None		0

[a] Todd-Hewitt broth cultures were incubated anaerobically for 20 h at 35°C with shaking; growth was determined by dry weight measurements (3). Metal chelates were formed before addition to the medium. Chelator concentrations: BCDS and FerroZine, 0.8 mM; EDTA and Desferal, 0.4 mM.

[b] Calculated by comparison with controls (no additions); 0, growth equivalent to or greater than that of the control; 100, no apparent growth.

tween the peroxide- and copper-containing disks, suggesting that oxidation of copper lowered the toxicity of the metal. Finally, the addition of the copper complexes of the cuprous chelators BCDS and FerroZine (Sigma Chemical Co.) (4, 14) to the anaerobic cultures protected *S. mutans* from copper inhibition; the cupric chelator deferoxamine mesylate (Desferal; CIBA-GEIGY Corp.) (2) was ineffective (Table 2). Rapid reductive release of the cuprous ion from cupric Desferal has been observed (4), and this may account for the toxicity of this complex.

The copper complex of the cupric chelator EDTA (8, 12) also failed to prevent growth inhibition; however, this agent had multiple effects on *S. mutans* in that, unlike the other chelators, it completely inhibited growth when it was added in its metal-free form (Table 2). Reduction of cupric EDTA will yield not only the cuprous ion, but also the metal-free ligand, perhaps accounting for the increased toxicity of cupric EDTA (Table 2). The addition of the ferric complex of EDTA abolished EDTA inhibition of *S. mutans* (Table 2) by preventing the formation of the metal-free ligand. EDTA has a high affinity for both ferric and ferrous iron (8), so the reduction of iron should not generate iron-free EDTA. EDTA will protect other bacteria from copper toxicity (12). This suggests either the removal of copper from the complex by the *S. mutans* culture system or a unique toxicity of cupric EDTA for *S. mutans*. Earlier work (5) demonstrated EDTA inhibition of *S. mutans*, although only those results from aerobic cultures were reported.

Copper sulfate-containing mouthwashes are being considered because of their efficacy in preventing caries in rats, and in reducing plaque formation and the incidence of gingivitis in humans (17, 18). Dental plaque is considered an anaerobic environment (6, 15), and this report demonstrates copper to be more effective against *S. mutans* under anaerobiosis. The toxicity may be mediated primarily by cuprous ions. Strains of serotype c (biotype I) are the predominant human isolates (7); biotype I strains may be the most susceptible to copper inhibition. These observations may help explain the cariostatic effectiveness of this trace metal.

This work was supported by Public Health Service grant DE 04903 from the National Institute of Dental Research. C.T. was a Summer Research Fellow supported by Public Health Service Short Term Research grant DE 07119 from the National Institute of Dental Research.

We are grateful to G. D. Shockman, Temple University Health Science Center, Philadelphia, Pa., and to E. L. Thomas, St. Jude Children's Research Hospital, Memphis, Tenn., for supplying cultures. Desferal was a gift from CIBA-GEIGY Corp.

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