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Effects of copper and zinc ions on the germicidal properties of two popular pharmaceutical antiseptic agents cetylpyridinium chloride and povidone-iodine†

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The effects of copper and zinc ions on the rate of killing of Gram-negative bacterium *Pseudomonas aeruginosa*, Gram-positive bacterium *Staphylococcus aureus* and fungal yeast *Candida albicans* by antiseptic agents cetylpyridinium chloride and povidone-iodine (Betadine) were investigated. In the 48 test cases copper and zinc ions clearly potentiated the antiseptic agents in 28 (58.3%) cases and exhibited an improved (not clear potentiation) activity in 15 (31.3%) cases. In five (10.4%) cases there was no change in the antiseptics' antimicrobial activity. In general zinc potentiated the antiseptic agents more than copper. If an 'improved activity' was the only criterion for this study, then a more rapid antimicrobial effect was observed in 43 out of the 48 test cases, *i.e.*, 90%.

Keywords: *Copper; zinc; cetylpyridinium chloride; povidone-iodine; antimicrobial; antiseptic*

Copper and zinc are two trace metals for which there are daily recommended allowances (RDA). They are also well known for their antimicrobial properties. Copper is commonly used as an antimicrobial agent in swimming pools and elsewhere whereas zinc is used pharmaceutically in creams, ointments, eye drops, *etc*. to combat various types of infection. In the past various trace metals were used quite extensively as antiseptic agents, but they have had to make way for more popular synthetic drugs such as antibiotics and other elaborate antimicrobial agents. These antibiotics and antiseptic agents are well known to cause allergies and also to allow resistance to develop in some micro-organisms. They are also very expensive. To overcome microbial resistance, doses are often increased to levels which simply aggravate the problems already mentioned. Alternatively combinations of antimicrobial agents are not too infrequently employed. The consequence is all too often a higher incidence of untoward effects. On the other hand copper and zinc combinations such as the sulfates are very well tested and allergies are quite unknown to members of the health professions. In this study an attempt was made to establish if there exists antimicrobial interaction between these metal ions and two other popular antiseptic agents which are used quite widely because of their wide antimicrobial spectra and low incidence of side effects. The antiseptic agents selected were the quaternary ammonium compound cetylpyridinium chloride and the organic iodine compound povidone-iodine. The effects of the metal ions on these antiseptic agents were tested against a typical Gram-positive bacterium (*Staphylococcus aureus*), Gram-negative bacterium (*Pseudomonas aeruginosa*) and a fungal yeast (*Candida albicans*). All the microbial species were strains isolated from hospitalised patients with conditions which would not respond to the usual antibiotic therapy for infections caused by these micro-organisms.

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Experimental

The concentration necessary for each of cetylpyridinium chloride and povidone-iodine to kill an inoculum of 1×10^6 micro-organisms within 40 min, but not before 30 min, was determined. This value will be referred to as the minimum microbicidal concentration$_{30/40}$ or MMC$_{30/40}$. Increasing amounts of the metal ions were then added to each of the antiseptic agents and the time to kill the same number of micro-organisms noted. The micro-organisms were standardised spectrophotometrically to contain 1×10^6 micro-organisms per 0.0001 dm^3 of the culture medium.

MMC$_{30/40}$ determinations for the antiseptic agents

(1) Serial dilutions were made in Normal Saline (the reaction medium) for each of the antimicrobial test substances. These were sterilised at 115 °C for 30 min in an autoclave. (2) Each dilution was inoculated with 1×10^6 of the appropriate micro-organisms and kept at 37 °C in an incubator. (3) At 10 min intervals and for 40 min subcultures were made into Tryptone Soya Broth which contained 3% m/v Tween 80 as neutralising agent (the recovery medium). A level of 3% m/v Tween 80 has been shown by several researchers[1–5] to neutralise many antiseptic agents without inhibiting or killing the micro-organisms themselves. In preliminary tests this concentration was found to have no inhibitory effects on the test micro-organisms used in this study. The subcultures were then incubated at 37 °C for 24 h. (4) After incubation the dilutions were visually checked for growth (optical density).

Minimum lethal concentrations for the metal ions

This test was necessary to avoid the erroneous assumption that a particular antiseptic–metal ion combination killed a microbial population whereas the microbicidal effects may have been effected by the metal ions present and not necessarily a combination of metal ions and antiseptic agents. The test was essentially the same as for the antiseptic agents, but the total time period for subculturing was 48 h. The assumption was that the minimum amount which killed only after 48 h would not be able to kill within a time period of 40 min. The ions were used as sulfate salts (copper sulfate and zinc sulfate). These salts were soluble at the concentrations tested.

Interactions

(1) Solutions in Normal Saline containing the MMC$_{30/40}$ for the antiseptic agents plus 5, 10, 50 or 100×10^{-3} g dm^3 of the metal ions were prepared for each antiseptic agent, for each metal salt and for each micro-organism. These solutions were sterilised at 115 °C for 30 min. The stock solutions containing the antiseptic agents and the metal salts were sterilised to eliminate the presence of possible resistant extraneous micro-organisms and/or spores. (2) The dilutions containing the antiseptic agents and

metal salts were inoculated with 1×10^6 of the appropriate micro-organism. (3) The inoculated dilutions were kept at 37 °C in an incubator. (4) At 10 min intervals 0.0001 dm^3 samples were subcultured into the recovery medium and the latter reincubated at 37 °C for 24 h. (5) The subcultures were then visually examined for turbidity indicating microbial growth. The reason for choosing a reaction temperature of 37 °C is because the selected antiseptic agents are used on the skin and in body cavities where the average temperature comes close to 37 °C.

Results and Discussion

All the micro-organisms appeared to be sensitive to both Betadine (which contains 1% m/v of povidone-iodine) and cetylpyridinium chloride (Table 1 and 2).

The antimicrobial effects of the metals appeared to be particularly high against *Staphylococcus aureus*. This Gram-positive bacterium has been found in numerous investigative studies to be very fragile and sensitive to most antimicrobial agents such as antiseptics, preservatives, disinfectants, *etc*., currently on the market. The exception, however, is antibiotics. Strains resistant to several antibiotics are constantly emerging in hospitals where many medical staff members are healthy nasal carriers thereof. There are currently numerous reports on the resistance of *Staphylococcus aureus* to antibiotic agents. The apparent resistance of the Gram-negative microbial species (*Pseudomonas aeruginosa*) is not unexpected. The resistance of this bacterium, especially in organic tissues, is very well documented. It is a bacterium which can survive on simple inorganic chemicals, being able to convert them to more elaborate organic requirements. Its ability to mutate is equally well documented in the literature. The reason why this micro-organism appears to be resistant to zinc ions, but not equally resistant against copper ions, could be attributed to the fact that zinc preparations are over-the-counter pharmaceuticals for which a prescription is not required. They are often applied to the skin or mucosa of the eye for purposes of tissue regeneration and combatting infections, whereas copper-containing preparations are not that often used. This constant exposure of *Pseudomonas aeruginosa* to zinc may be the cause of the development of resistance. *Pseudomonas aeruginosa* is found on the human skin where it often infects wounds. Auto-infections of the eye (pink-eye infection) is also very common.

The information in Table 3 is important to avoid misinterpretation of the observed killing effect by metal ion–antiseptic agent combinations (Table 4 and 5). It should be clearly established that the combination of agents killed the inoculum and not the metal ions present as the latter also exert an antimicrobial effect on their own.

Copper

5×10^{-3} g dm^{-3}: This level of copper ions would not be able to kill any of the micro-organisms within a 40 min period (see above for minimum lethal concentrations). The killing effect of cetylpyridinium chloride against the yeast remained unchanged, but in the case of Betadine strong potentiation was evident. Cetylpyridinium chloride retained its original killing effects on *Pseudomonas aeruginosa*. Both antiseptic agents were potentiated against *Staphylococcus aureus*.

10×10^{-3} g dm^{-3}: In all instances the micro-organisms were killed over the whole testing period of 40 min. Since this level of copper could not have killed *Pseudomonas aeruginosa* and *Candida albicans*, a potentiated effect is obvious. The improved killing effect noted for *Staphylococcus aureus* could have been due to the antimicrobial properties of copper on its own against this micro-organism (minimum lethal concentration being 6×10^{-3} g dm^{-3}) and potentiation is therefore not clearly indicated.

50–100 $\times$ 10^{-3} g dm^{-3}: These levels of copper, ions in combination with the antiseptic agents, did not allow growth to take place at 5 min or longer. Once again, the improved effect is not necessarily potentiation as the minimum lethal concentrations for copper against all the micro-organisms are lower than 50×10^{-3} g dm^{-3}.

Table 1 Minimum microbicidal concentrations$_{30/40}$ ($MMC_{30/40}$) at 37 °C for Betadine and cetylpyridinium chloride against three pathogenic micro-organisms*

	Antiseptic agent									
	Betadine (1% m/v povidone-iodine)					Cetylpyridinium chloride				
	Concentration tested (% m/v)	Time/min				Concentration tested (% m/v)	Time/min			
		10	20	30	40		10	20	30	40
Pseudomonas aeruginosa	3.600	–	–	–	–	0.056	–	–	–	–
	3.500	–	–	–	–	0.055	+	+ ▸	+	–
	3.488	+	–	–	–	0.054	+	+	+	(+)
	3.486	+	+	–	–	0.053	+	+	+	(+)
	3.484	+	+ ▸	+	–	0.052	+	+	+	(+)
	3.482	+	+	+	+	0.051	+	+	+	+
Staphylococcus aureus	5.038	–	–	–	–	0.00040	–	–	–	–
	5.036	+	+	+	–	0.00038	+	–	–	–
	5.034	+	+	+	–	0.00036	+	+	–	–
	5.032	+	+ ▸	+	–	0.00034	+	+ ▸	+	–
	5.030	+	+	+	+	0.00032	+	+	+	+
Candida albicans	3.5	–	–	–	–	0.0025	–	–	–	–
	3.4	+	+	+	–	0.0020	+	+	–	–
	3.3	+	+	+	–	0.0018	+	+	–	–
	3.2	+	+ ▸	+	–	0.0016	+	+	–	–
	3.1	+	+	+	+	0.0015	+	+ ▸	+	–
						0.0014	+	+	+	+

* + = Growth, – = no growth, ▸ = $MMC_{30/40}$ = minimum concentration which kills after 40 min, but not after 30 min.

Zinc

5×10^{-3} g dm^{-3}: This level of zinc ion on its own did not kill any of the micro-organisms in the absence of other antiseptic agents. Any improved effect noticed would therefore be due to potentiation. Cetylpyridinium chloride retained its original killing effects against *Candida albicans*. The effect of cetylpyridinium chloride on *Staphylococcus aureus* also remained the same. Potentiation was strong in all other test cases.

10×10^{-3} g dm^{-3}: A potentiated effect was found for all the antiseptic agents against *Candida albicans* and *Pseudomonas aeruginosa*. Since this level of metal ion could have had a killing effect of its own against *Staphylococcus aureus*, the improved activity throughout does not necessarily indicate potentiation.

Table 2 Summary of the minimum microbicidal concentrations$_{30/40}$ (MMC$_{30/40}$) at 37 °C for Betadine and cetylpyridinium chloride against three pathogenic micro-organisms*

	Minimum contact killing concentration$_{30/40}$		
Antimicrobial agent	*P. aeruginosa*	S. *aureus*	C. *albicans*
Betadine	3.484	5.032	3.200
Cetylpyridinium chloride	0.055	0.00034	0.0015

* *P. aeruginosa* = *Pseudomonas aeruginosa*, *S. aureus* = *Staphylococcus aureus*, *C. albicans* = *Candida albicans*; MMC$_{30/40}$ = Minimum concentration which kills after 40 min, but not after 30 min.

Table 3 Minimum lethal concentrations after 48 h at 37 °C for copper and zinc ions against three pathogenic micro-organisms*

	Minimum lethal concentrations/ $\times 10^{-3}$ g dm^{-3} Metal ions		Highest test concentration which did not kill/ $\times 10^{-3}$ g dm^{-3} Metal ions	
Micro-organism	Cu^{++}	Zn^{++}	Cu^{++}	Zn^{++}
Pseudomonas aeruginosa	36	1917	34	1906
Staphylococcus aureus	8	9	6	8
Candida albicans	26	39	24	38

* The above values are for the metal ions Cu^{++} and Zn^{++} (as present in the salts $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$).

50×10^{-3} g dm^{-3}: A better, improved killing effect was found throughout. This level of ion did kill *Candida albicans* and *Staphylococcus aureus* in previous studies, therefore any observed killing effect better than that obtained for the antiseptic agents on their own, should rather be described as improved than potentiated. Nevertheless, turbidity indicating microbial multiplication was only present for 5 min in the case of cetylpyridinium chloride against *Staphylococcus aureus*. In all of the remaining combinations potentiation was very strong as evidenced by the total absence of turbidity over the whole testing period.

100×10^{-3} g dm^{-3}: In all the cases growth was absent from 5 min onwards. This indicates the same very strong potentiating effect as when 50×10^{-3} g dm^{-3} of the zinc ion was present. The growth observed for cetylpyridinium chloride against *Staphylococcus aureus* was completely absent.

Several authors[6–8] noticed that copper can carry certain substances which are extracellularly nontoxic into the microbial cell where they become intracellular toxins because of the fact that their interaction sites are inside and not on the cell. This effect was found to be more pronounced in Gram-positive micro-organisms as compared with Gram-negative bacteria. In this study, however, a better effect was noticed for the Gram-negative bacterium and this mechanism is perhaps unlikely. Several other mechanisms could possibly account for the increased killing effects found. Several investigators[7,9,10] remarked that copper can deplete microbial cells of magnesium and the latter is essential for protective cell wall formation. The metal is also known to bind with phosphate groups.[11] A much better improvement of the overall killing effect was noticed for the Gram-negative bacterium as compared with the Gram-positive species. One should also bear in mind the possibility that the oversupply of any one metal may not only deplete the microbial cell of other metals essential for normal cell membrane structure and function as well as enzyme activity, but that it may also alter the cell's need for other metal ions and in the process render the cell sensitive to damaging substances such as antiseptic agents or even metal ions themselves.[9,12–14] It may be possible that excess copper could affect the zinc requirement for dehydrogenase enzymes and the hydrolysis of phosphates and peptides, all of which are found on the cell wall surface. Such an effect may render the cell more permeable to antiseptic agents and intracellular solutes. Lastly, two research groups[15,16] proposed that polyvalent metal ions can cause effective charge neutralisation on microbial cells and this may lead to improved attachment and penetration of some antiseptic

Table 4 Effects at 37 °C of copper and zinc ions on the minimum microbicidal concentrations$_{30/40}$ (MMC$_{30/40}$) at 37 °C of Betadine and cetylpyridinium chloride*

	Copper								Zinc							
	5				10				5				10			
	Time/min															
Metal ion concentration/ $\times 10^{-3}$ g dm^{-3}	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40
Antimicrobial agents																
Betadine—																
P. aeruginosa (3.484)	+	(+)	(+)	–	–	–	–	–	–	–	–	–	–	–	–	–
S. aureus (5.032)	+	(+)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C. albicans (3.200)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cetylpyridinium chloride—																
P. aeruginosa (0.055)	(+)	(+)	(+)	–	–	–	–	–	–	–	–	–	–	–	–	–
S. aureus (0.00034)	(+)	–	–	–	–	–	–	–	+	+	+	–	+	–	–	–
C. albicans (0.0015)	+	+	(+)	–	–	–	–	–	+	+	(+)	–	+	+	–	–

* + Growth; – = no growth; (+) = faint growth; *P. aeruginosa* = *Pseudomonas aeruginosa*, *S. aureus* = *Staphylococcus aureus*, *C. albicans* = *Candida albicans*; MMC$_{30/40}$ (appear in brackets) = minimum concentration which kills after 40 min, but not after 30 min; Note: Concentrations of 50 and 100×10^{-3} g dm^{-3} of the metal ions allowed no growth in all test cases.

agents to the microbial cell. It is likely that more than one mechanism is involved and that the improved antimicrobial effects observed are the result of a combination of mechanisms. Zinc ions exhibited no antagonistic effect on the antimicrobial agents and an improved activity was observed in most of the combinations. Excellent potentiation was noted in several cases. The killing effects of zinc were mostly the same as those observed for copper. In the case of copper, a better killing effect was noted for 42 out of 48 cases, while an improved killing effect was found for zinc in 45 out of 48 cases. No antagonistic effects were observed for these two metal ions. All micro-organisms seemed to be affected adversely by the presence of copper and zinc ions. Zinc has been described as a carrier for oxine to obtain an improved antifungal effect.[17,18] It was found in these studies that zinc improved the antifungal effects of the quaternary ammonium compounds, but not of Betadine. It is obvious that some sort of physical and chemical interaction between zinc and the antiseptic agent is of importance for this mechanism to apply. Zinc can displace metals such as magnesium[7,10,19] and copper[9,12–14] from microbial cells. The effect of magnesium depletion (as well as other metal ions) from a microbial cell was discussed earlier. According to this mechanism, Gram-negative bacteria should be more sensitive than Gram-positive bacteria. This was found for the quaternary ammonium compounds against the bacteria (not the yeast), but not at all for benzyl alcohol and Betadine. The charge neutralisation effect described for copper may hold true for zinc as well. If zinc depleted the microbial cells or other essential metals, copper could be one of them. Copper is involved in the activity of some enzymes such as oxidases.[14] According to Eagon and Asbell,[19] zinc can inhibit some step of the energy transfer cycle which is involved in the transport of substrates catalysed by magnesium. It can induce conformational changes in the tertiary structures of cell membrane proteins involved in substrate transport.

Conclusion

All the types of antiseptic–metal ion interactions observed can be described as follows, taking into account that (1) the antiseptic agents should allow microbial growth for a period of up to 30 min, but not 40 min, and that (2) metal ion concentrations above the minimum lethal levels as previously determined for a 48 h incubation period could have produced, although unlikely over a 40 min test period, an antimicrobial effect of their own.

If the metal ion level is below the minimum lethal concentration and the possibility of an antimicrobial effect from the metal therefore unlikely, one can use the following as an indication of the degree of interaction: – – – – = excellent potentiation (EP); + – – – = good potentiation (GP); ++ – – = moderate potentiation (MP).

If the metal ion level is above the minimum lethal concentration and the possibility of an anti-microbial effect

Table 5 Interaction effects at 37 °C of copper and zinc ions on the minimum microbicidal concentrations$_{30/40}$ (MMC$_{30/40}$) of Betadine and cetylpyridinium chloride*

	Metal ion concentration/× 10^{-3} g dm^{-3}							
	Copper				Zinc			
Antimicrobial agents	5	10	50	100	5	10	50	100
Betadine—								
P. aeruginosa (3.484)	U	EP	EP	EP	EP	EP	EP	EP
S. aureus (5.032)	MP	IA	IA	IA	EP	IA	IA	IA
C. albicans (3.200)	EP	EP	IA	IA	EP	EP	IA	IA
Cetylpyridinium chloride—								
P. aeruginosa (0.055)	U	EP	EP	EP	EP	EP	EP	EP
S. aureus (0.00034)	GP	IA	IA	IA	U	GP	GP	EP
C. albicans (0.0015)	U	EP	IA	IA	U	MP	EP	EP

* A = antagonism, U = unchanged effect, MP = moderate potentiation, GP = good potentiation, EP = excellent potentiation, IA = improvement; *P. aeruginosa* = *Pseudomonas aeruginosa*, *S. aureus* = *Staphylococcus aureus*, *C. albicans* = *Candida albicans*; MMC$_{30/40}$ appear in brackets = minimum concentration which kills after 40 min, but not after 30 min.

Table 6 Summary of the effects of copper and zinc ions on the microbial killing rates of Betadine and cetylpyridinium chloride*

		Betadine		Cetylpyridinium chloride		
Copper ions	Potentiation	6		5		
	Improved action	5		5		
	Unchanged	1		2		
Zinc ions	Potentiation	7		10		
	Improved action	5		0		
	Unchanged	0		2		
						Total
Copper and Zinc	Potentiation	13	+	15	=	28 (58.3%)
	Improved action	10	+	5	=	15 (31.3%)
	Unchanged	1	+	4	=	5 (10.4%)
* Summary: increased killing rate (potentiation + improved action):		23	+	20	=	43 (89,6%)
	No effect:	1	+	4	=	5 (10,4%)
					Total:	48 (100%)

* (Note: Quantities of 5, 10, 50 and 100 × 10^{-3} g dm^{-3} of each ion were tested on the above antiseptic agents' abilities to kill *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*).

from the metal thus possible, although unlikely, over a 40 min testing period: – – – or + – – or ++ – would indicate an improved antimicrobial action (IA).

If there is microbial growth for up to 30 min, but not at 40 min: +++ – = unchanged effect (U).

Growth throughout the 40 min testing period: ++++ = antagonism (A).

The various types of interaction can now be described as one of the following: Excellent potentiation = EP, – – – –; Good potentiation = GP, + – – –; Moderate potentiation = MP, ++– –; Improved action = IA, – – – –/+ – – –/++ – –; Unchanged effect = U, +++ –; Antagonism = A, ++++. This scaling of responses is used in Table 5.

Table 6 gives a summary of the antimicrobial activities of combinations of the various antiseptic agents with copper and with zinc ions. For copper ions, in general, an excellent improvement in the killing activity of the antimicrobial agents was observed in most cases. No true antagonism was observed. It can be seen that a more rapid microbial killing rate was observed in about 90% of test cases. True potentiation was seen in about 58% of cases and an improved action in about 31% of cases.

Antimicrobial agents such as povidone-iodine (*e.g.*, Betadine, Podine) and cetylpyridinium chloride (*e.g.*, Savlon, Cetavlon) are non-specific cytotoxic agents which may cause untoward effects like skin or mucosal irritation, rashes and allergies, even at tested in-use concentrations in certain individuals. Copper and zinc ions in the form of the metal salts tested are well known for their antimicrobial properties as pharmaceutical agents against (bacterial, fungal, viral, protozoal) infections of the skin, eyes, *etc*. If small quantities of these metal salts can be employed in pharmaceutical formulations containing povidone-iodine and cetylpyridinium chloride, smaller quantities of the latter two agents may be used in pharmaceutical preparations in order to minimise the risk of unexpected side effects.

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