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Prevalence of Monovalent Copper Over Divalent in Killing *Escherichia coli* and *Staphylococcus aureus*

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Abstract

This study opens the investigation series focused on antimicrobial effects of copper (Cu) compared to silver (Ag), which is currently used to treat wound infection in burn victims as well as in chronic wounds. Noticeably, in its ionized state, Cu is more commonly present as Cu^{2+} rather than as Cu^{+}, while electronic configuration similarity of Cu^{+} and Ag^{+} indicates that actually it may be the active state. To test this hypothesis, effect of Cu^{+} and Cu^{2+}, using Ag^{+} ions and metallic copper as controls on *Escherichia coli* and *Staphylococcus aureus* bacteria, was examined under anaerobic conditions. Cu^{+} was produced by two different methods, and its effect on microorganism growth was tested using a syringe and Petri dish methods. It was found that the presence of Cu^{+} causes a dramatic depletion in the viability of both microorganisms. Metallic copper did not have any effect on the viability, whereas Cu^{2+} and Ag^{+} ions had much lower activity than Cu^{+} ions. Minimal inhibitory concentration of Cu^{+} for *E. coli* was twice lower than that of Cu^{2+}. The obtained results show that Cu^{+} proves to be a potent antimicrobial agent.

Introduction

Copper (Cu) is an essential trace element for most living organisms; however, in high concentration, it can exert biocidal effect while exploiting various mechanisms, most of them are yet not fully understood [6]. First mentioned in an Egyptian papyrus dated back to 2600–2200 BC, the antimicrobial properties of copper have been well recognized and documented throughout history [4].

Over recent decades, Cu has regained scientific interest due to its possible applications in the healthcare setting. One of the most common and established medical applications of copper is the addition of ionized copper-silver in hospital water systems as an effective method to control Hospital-acquired *Legionella* infection [18]. Recently, numerous laboratory studies have demonstrated the effective impact of Cu biocidal surfaces on reducing the spread of various bacteria [10, 19, 21], yeasts [20] and viruses [12], a conclusion that was later supported in clinical trials [3, 7, 11].

The mechanisms behind copper toxicity are still a subject of ongoing research, but a number of contributing factors have already been established. A major factor is the ability to act as a catalyst for oxidative damage to tissues through cyclic redox reactions, alternating between Cu^{+} and Cu^{2+}. The reactive oxygen species generated in these reactions directly damage essential cell components like nucleic acids, proteins, and lipids [5, 19]. Intracellular-free Cu ions also contribute to cell death through protein inactivation [2, 8]. The significance of the effect of copper on DNA degradation is still controversial, with different mechanisms of toxicity observed in various types of microorganisms [13]. In several studies that involved enterococci, DNA degradation as a result of impact of Cu ionic species and further generation of superoxide was recognized a key factor in cell death process [19]. In contrast, other studies that explored toxic effect exerted by copper on gram-negative bacteria such as *Escherichia coli* suggested that depolarization of the cytoplasmic membrane is the main target, and degradation of DNA occurs only after cell death [9].

The two most common ions formed by copper are the Cu^{+} (cuprous) and Cu^{2+} (cupric). The cuprous ion is chemically

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more active and less stable of the two oxidation states and is easily oxidized in an aqueous environment. It is possible to maintain high concentration of cuprous in aqueous environment by adding reagents which form a very stable complex with the cuprous ion, like acetonitrile [15] or benzoic acid [17].

Silver (Ag^+) ions are used to treat wound infection in burn victims as well as in chronic wounds. The electronic configuration similarity of Ag^+ and Cu^+ raised our suspicion that namely Cu^+ may be an active state. In order to test this hypothesis, we compared the antibacterial effect of Cu^+, Cu^{2+}, Ag^+ ions and metallic copper (Cu^0) on gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus* bacteria.

Materials and Methods

Syringes Technique

Gram-negative bacteria *E. coli* ATCC MG 1655 and gram-positive bacteria *S. aureus* ATCC 25923 were incubated at room temperature under anaerobic conditions in syringes containing LB broth growth medium. To ensure anaerobic conditions, argon gas (UHP) was bubbled through a sterile filter into a syringe filled with growth medium. Prior to microorganisms insertion Cu^+ was formed in situ, starting with de-aerated aqueous solutions containing a mixture of $CuSO_4$ as the source for Cu^{2+} ions, metallic copper, and acetonitrile as a stabilizing ligands, according to the reaction [14]:

$$\begin{gathered} Cu^{2+} + Cu^0 + nCH_3CN \longrightarrow H_2O\, 2\left[Cu^+(CH_3CN)n\right]^+ \\ K_{1\%CH_3CN} = 1 \times 10^2 \end{gathered} \tag{1}$$

Noticeably, each Cu^{2+} results with two Cu^+ ions.

Acetonitrile concentration of 0.5% v/v (0.1 M) was used according to preliminary tests showing it to be the minimal effective concentration. As a reference, bacteria were incubated under the same conditions in a growth medium containing: metallic copper ($4\times2\times0.1$ cm) alone, acetonitrile alone (reference), $CuSO_4$ or $AgNO_3$. Bacteria were counted at different time intervals by colony-forming units (CFU) technique, plating bacteria from serial dilutions onto LB agar and incubating overnight at 37 °C.

Petri Dish Technique

Bacteria were incubated at 37 °C in a Petri dish with LB agar containing 20 mM ascorbic acid and 0.05–3.5 mM $CuSO_4$ under oil, to prevent air oxygen penetration. As a control, bacteria were incubated under the same conditions without the ascorbic acid, or without $CuSO_4$. In this series of experiments, ascorbic acid acted as the reducing agent, according to the reaction:

$$2Cu^{2+} + \text{ascorbic acid} \rightarrow 2Cu^+ + \text{dehydro ascorbic acid} \tag{2}$$

The viability of bacteria was detected by colony-forming units (CFU) count.

Results

Effect of Cu^+, Cu^{2+}, Cu^0 on Gram-Negative Bacteria *E. coli*

The viability of *E. coli* was tested in anaerobic conditions using syringe technique. All experiments used at least four syringes and executed at least three repetitions, among them: (a) 0.25–0.5% v/v acetonitrile aqueous solution contained metallic copper and $CuSO_4$ that react in the presence of acetonitrile to produce two Cu^+ ions prior to bacteria insertion in accordance with Eq. (1), (b) 0.25–0.5% v/v acetonitrile aqueous solution contained $CuSO_4$ only, that in aqueous solution dissolves to produce Cu^{2+} ions, (c) 0.25–0.5% v/v acetonitrile aqueous solution contained metallic copper, (d) 0.25–0.5% v/v acetonitrile aqueous solution was used as a reference, not containing copper.

After 24 h of incubation in a syringe, the solution was placed on LB agar and viability of the bacteria was detected by the presence (+) or absence (−) of colonies on LB agar.

Table 1 summarizes the results of 16 various experiments with different concentrations of Cu^+, Cu^{2+}, metal copper, and control groups ("reference"). Note that, Cu^+ is stable only under the conditions presented in column A in Table 1. It is shown that bacterial growth is inhibited only under conditions where enough Cu^+ is present (column A, rows 2, 4 in Table 1), whereas under similar concentrations of Cu^{2+}

Table 1 Effect of Cu^+, Cu^{2+}, Cu^0 versus control on *E. coli* viability under anaerobic conditions

Initial concentration $CuSO_4$, mM	CH_3CN, % v/v	(A) $CuSO_4$ and metal copper (Cu^+)	(B) Only $CuSO_4$ (Cu^{2+})	(C) Only metal copper	(D) Reference (no copper)
0.3	0.5	+	+	+	+
0.4	0.5	0	+	+	+
4.0	0.25	+	+	+	+
4.0	0.5	0	+	+	+

All experiments were done in triplicates

"0"—absence of colonies, "+"—uncountable colonies (more than 300) (columns A and B, rows 1, 3 in Table 1) bacteria thrive. The same can be said about the results obtained for metallic copper (Cu^0) and the reference (columns C and D, accordingly).

Minimal Inhibitory Concentration of Monovalent and Divalent Copper Ions for *E. coli*

To determine the minimal inhibitory concentration (MIC) of Cu^+ and Cu^{2+}, four syringes 0.5% v/v acetonitrile aqueous solution with decreasing ions concentrations from 8 to 2 mM were prepared. Two controls were used, a positive one not containing copper, and a negative, without *E. coli* inoculation. After 24 h of incubation presence of *E. coli* colonies was checked, the colonies were counted where possible (data not shown). MIC of Cu^+ was found to be around 4 mM, whereas MIC of Cu^{2+} was nearly twice higher, ranging from 6 to 8 mM.

In order to strengthen our assumption, we arranged additional experiment using ascorbic acid as reducing agent (instead of metal copper with acetonitrile) and different techniques of growing bacteria on Petri dishes with LB agar. 20 mM ascorbic acid embedded in the agar reduces divalent copper ion to monovalent copper ion in accordance with Eq. (2). Table 2 demonstrates that ascorbic acid and copper ion concentration (0 to 4 mM $CuSO_4$ embedded in the agar) affect *E. coli* viability 24 h after incubation. The results indicate that the bactericidal effect of reduced copper ions (Cu^+) is several times more potent than that of Cu^{2+}.

Kinetics of Bactericidal Effect of Copper and Silver Ions on *E. coli*

Basing on the chemical similarity of copper and silver, and their clinical use as antibacterial agents as well, we decided to compare their antibacterial effect over time (Fig. 1a). Survival of test bacteria used five syringes and executed at least three repetitions, (a) (Cu^+ in the figure)—0.5% v/v acetonitrile aqueous solution contained metallic copper and 2 mM $CuSO_4$ that react in the presence of acetonitrile to produce two Cu^+ ions prior to bacteria insertion in accordance with Eq. (1), (b) (Cu^{2+} in the figure)—0.5% v/v acetonitrile aqueous solution contained 4 mM $CuSO_4$ (only) that in aqueous solution dissolves to produces Cu^{2+} ions, (c) (Cu metal in the figure)—0.5%v/v acetonitrile aqueous solution contained metallic copper, (d) (reference in the figure)—0.5% v/v acetonitrile aqueous solution was used as a reference not containing copper, and (e) (Ag^+ in the figure)—0.5% v/v acetonitrile aqueous solution contained 4 mM $AgNO_3$.

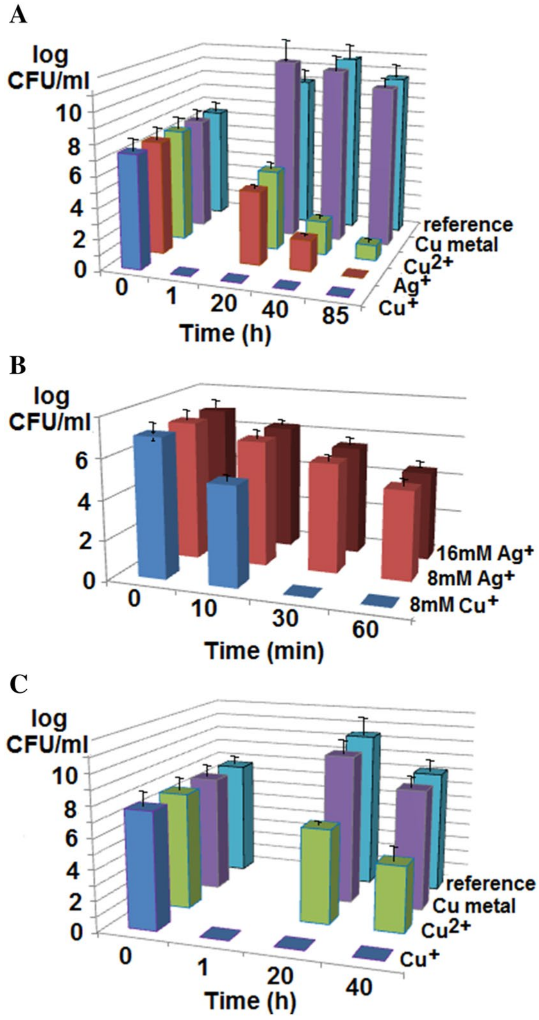

Fig. 1 Viable cell count of bacteria (Δ log10 CFU/ml) over time using syringes technique: **a, b** *E. coli*, **c** *S. aureus*. All experiments were done in triplicates

Each syringe was sampled at time intervals of 1, 20, 40, and 85 h from inoculation. Time–kill curve is presented by plotting the viable cell count of bacteria (Δ log10 CFU/ml) over time (Fig. 1a). The syringe with Cu^+ ions demonstrated a significant reduction of the initial bacteria inoculums by 7 log10 CFU/ml at 1 h of incubation in anaerobic environment no viable bacteria cell was found. A more gradual decline in the viable cell count along time was observed in the Cu^{2+} syringe, and it should be noted that even after 85 h, live bacteria still remained in the syringe (1 log10 CFU/ml). A similar pattern of decline was seen in the syringe that contained

Table 2 Effect of ascorbic acid (reducing Cu^{2+} to Cu^+) on *E. coli* viability on a LB agar in a Petri dish

	Control	Cu^+		Cu^{2+}	
Ascorbic acid	20 mM	20 mM	20 mM	–	–
$CuSO_4$	0 mM	0.01 mM	0.05 mM	3.0 mM	4.0 mM
E. coli viability	++	++	0	++	0

All experiments were done in triplicates

"0"—absence of colonies, "++"—bacterial lawn silver ions (Ag^+), but after 85 h no viable cells were found. The syringes with metal copper and the negative controls (reference syringes) showed no toxic effect on bacteria survival during the experiment timeline.

To provide further proof to our hypothesis, we doubled the Ag^+ concentration. Figure 1b shows the results of 1-h incubation of *E. coli* in syringes with 8 mM Cu^+ (0.5% v/v acetonitrile aqueous solution contained metallic copper and 4 mM $CuSO_4$ that react in the presence of acetonitrile to produce 8 mM Cu^+ ions) versus 8 mM and 16 mM Ag^+ (0.5% v/v acetonitrile aqueous solution contained 8–16 mM $AgNO_3$). This experiment indicates that even compared with doubled Ag^+ concentration, Cu^+ exhibits more potent antibacterial effect.

Kinetics of Bactericidal Effect of Copper and Silver Ions on Gram-Positive Bacteria *S. aureus*

Figure 1c demonstrates the growth kinetics of gram-positive bacteria *S. aureus* in anaerobic conditions using four syringes and executed at least three repetitions. (a) (Cu^+ in the figure)—0.5% v/v acetonitrile aqueous solution contained metallic copper and 2 mM $CuSO_4$ that react in the presence of acetonitrile to produce two Cu^+ ions prior to bacteria insertion in accordance with Eq. (1), (b) (Cu^{2+} in the figure)—0.5% v/v acetonitrile aqueous solution contained 4 mM $CuSO_4$ (only) that in aqueous solution dissolves to produce Cu^{2+} ions, (c) (“Cu metal” in the figure)—0.5% v/v acetonitrile aqueous solution contained metallic copper, (d) (reference in the figure)—0.5% v/v acetonitrile aqueous solution was used as a reference not containing copper. It is noticeable that Cu^{2+} had very little bacteriostatic effect whereas exposure to Cu^+ caused depletion of bacterial count within 1 h of incubation.

Discussion

The antimicrobial effect of copper (Cu) and silver (Ag) has long been recognized. Although its mechanism of action is unknown, copper is used as a disinfectant in the healthcare setting, while silver ions are currently used to treat wound infection in burn victims as well as in chronic wounds.

In this study, we were able to show the potency of Cu^+ over Cu^{2+} copper ion in inhibiting gram-positive and gram-negative microorganisms. Although the mechanism of copper antimicrobial effect is not yet fully understood, possible routes such as oxidation of various cell proteins, nucleic acids and lipids, damaging DNA, and various essential cell reactions have been proposed [2, 5, 8, 13, 19]. Recently, Santo et al. have shown that the antimicrobial effect of metallic copper on *Staphylococcus haemolyticus* was exerted via membrane damage rather than the damage of DNA or other intracellular components [16]. Our findings may be well compatible with such mechanism, since as we found in our kinetic experiments, Cu^+ eliminated all microorganisms within 1 h of incubation, thus supporting a possible fast cell membrane damage rather than slower DNA or metabolism blockage. Cu^+ was found to be a more potent antimicrobial agent than Ag^+ which is a popular antimicrobial ion used in many modern types of wound dressings [1]. This finding may lead to further development of novel prospective antimicrobial dressings. The results of this study were verified using two different methods of bacterial growth and, more importantly, by creating Cu^+ using two different chemical reactions, one with metallic copper and acetonitrile and the other with ascorbic acid. It is worth mentioning that both materials are nontoxic, which makes this finding a promising starting point for further progress towards clinical application. More detailed investigation of the effect of Cu^+ on other organisms as well as its possible toxicity and applicability is needed.

Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest

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