Turkish Journal of Clinics and Laboratory

To cite this article: Kirmusaoglu S. Bactericidal and antibiofilm activities of copper against biofilm producer pathogens colonized on orthopedic implants. Turk J Clin Lab 2018; 9(1): 13-18.

Original Article

Bactericidal and antibiofilm activities of copper against biofilm producer pathogens colonized on orthopedic implants

Ortopedik implantlar üzerinde kolonize olan biyofilm üreten patojenler üzerinde bakırın bakterisidal ve antibiyofilm aktivitesi

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ABSTRACT

Aim: New and alternative antimicrobial and antibiofilm agent discovery has gained attention since antibiotic resistance was easily developed. It has been accepted that metals have antimicrobial activity. Abiotic surfaces such as orthopedic implants that are impregnated with copper can prevent colonization of biofilm producer pathogens, and can detach biofilms produced on implants. In this study, the effects of copper against planktonic bacteria and biofilm embedded bacteria adhered on kirschner wire orthopedic implant were studied.

Material and Methods: MICs, MBCs and bMBC of copper against main biofilm producer pathogens such as methicillin resistance *Staphylococcus aureus* (MRSA), methicillin sensitive *Staphylococcus aureus* (MSSA), methicillin resistance *Staphylococcus epidermidis* (MRSE), methicillin sensitive *Staphylococcus epidermidis* (MSSE), *Escherichia coli (E. coli)*, *Klebsiella pneumonia (K. pneumonia), Pseudomonas aeruginosa (P. aeroginosa), Proteus mirabilis (P. mirabilis)* colonized on kirschner wire orthopedic implant were determined.

Results: MICs, MBCs, and bMBCs of copper against pathogens were ranged from 0.063 to 0.75 mg/mL. This study revealed that 0.75 mg/mL of copper inhibit all isolates analyzed in this study. The most tolerant pathogen was MRSA. The activities of copper against biofilm embedded bacteria and planktonic bacteria were found to be the same.

Conclusion: Indwelling medical devices such as orthopedic wires, prosthetics can be impregnated by copper to overcome colonization and production of matured biofilm on indwelling devices, consequently, implant associated infections.

Key words: Biofilm, biofilm embedded pathogens, copper, implants, antibiofilm, antimicrobial.

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Recevied 24.03.2017 accepted:11.04.2017
Doi: 10.18663/tjcl.300359

ÖZ

Amaç: Antibiyotik direnci kolaylıkla geliştiğinden beri, yeni, alternatif antimikrobiyal ve antibiyofilm ajan keşfi dikkat çekmektedir. Metallerin antimikrobiyal aktivitelere sahip olduğu kabul edilmiştir. Bakır ile emdirilmiş ortopedik implantlar gibi abiyotik yüzeyler, biyofilm üreten patojenlerin kolonizasyonunu önleyebilir ve implant üzerinde oluşturulan biyofilmleri ayırabilir. Bu çalışmada, bakırın planktonik bakteriler ve kirschner teli ortopedik implantı üzerine yapışan biyofilme gömülü bakterilere karşı etkisi çalışıldı.

Gereç ve Yöntemler: Kirschner teli ortopedik implant üzerinde kolonize olan metisilin dirençli Staphylococcus aureus (MRSA), metisilin duyarlı *Staphylococcus aureus* (MSSA), metisilin dirençli *Staphylococcus epidermidis* (MRSE), metisilin duyarlı *Staphylococcus epidermidis* (MSSE), *Escherichia coli (E. coli)*, *Klebsiella pneumonia (K. pneumonia)*, *Pseudomonas aeruginosa (P. aeroginosa)*, *Proteus mirabilis (P. mirabilis)* gibi ana biyofilm oluşturan patojenler üzerinde bakırın MIK, MBK ve MBEK değerleri belirlendi.

Bulgular: Bakırın, patojenlere karşı MIK, MBK ve MBEK değerleri 0.063 - 0.75 mg/mL arasında değişmektedir. Bu çalışma, 0.75 mg/mL bakırın çalışmada analiz edilen tüm izolatları inhibe ettiğini göstermiştir. En dirençli patojen MRSA idi. Bakırın biyofilme gömülü bakteriler ve planktonik bakteriler üzerindeki aktivitesi aynıydı.

Sonuç: Ortopedik teller, protezler gibi yabancı medikal cisimler, yabancı cisimler üzerinde kolonizasyon ve olgun biyofilm oluşturulmasını önlemek için bakır ile emdirilebilir

Anahtar Kelimeler: Biyofilm, biyofilme gömülü patojenler, bakır, implantlar, antibiyofilm, antimikrobiyal.

Introduction

Biofilms that are sticky polysaccharides produced by biofilm producer bacteria lead microorganism to adhere biotic and abiotic substances such as host cells and indwelling medical devices, respectively. Biofilm infections become one of the main infection seen in hospitalized and immunosuppressed patients expose the patient to a higher risk of mortality worldwide [1, 2, 3]. Biofilm a place to embed sessile form of microorganism is more resistant to immune defence and antimicrobials such as antibiotics than planktonic microorganisms. Bacterial biofilms make treatment difficult and irresponsive to antibiotics by escaping immune defence of host and making infection recurrent and chronic [1, 2, 4]. Bacterial biofilms have a high risk to lead chronic infections such as indwelling device-associated infections, periodontitis, chronic wound infections, chronic urinary tract infections (UTI), chronic otitis media (OM), cystic fibrosis pneumonia, recurrent tonsillitis, chronic rhinosinusitis [1, 4] and valve-associated endocarditis [5]. Due to irresponsive treatment caused by the development of antibiotic resistance, consequently, functional loss of medical device (such as prosthetic implants, joints, stents, catheters) [6, 7], medical device may be removed out of the place localized [3] to overcome indwelling device-associated infection [1]. When implant-associated infection emerges in patients, the replacement of colonized indwelling device cause high risk of reinfection and bone destruction. Due to the development of antibiotic resistance, surgery fails and amputation may be done [8].

Microorganisms can adhere to surfaces that are made of stainless steel approximately after 28 days [9]. Due to this, in recent years, alternative treatment options such as natural compounds, new chemicals and metals such as copper, platinum and silver have being investigated and used as an antimicrobial compounds to treat resistant isolates caused by biofilms, and eradicate biofilms of microorganisms. Abiotic surfaces that are impregnated with copper such as urinary catheters can prevent colonization of pathogens such as *S. aureus*, MRSA, *Pseudomonas aeruginosa*, *E. coli* O157:H7, *Enterobacter aerogenes*. fungi and viruses on surfaces. Wound dressings containing copper, and paints that are incorporated with copper have been used to overcome rising of microorganism [10]. Inorganic metals such as copper that are toxic to bacteria are used as copper-based antimicrobials in topical balm, disinfectants, surface coatings, cleansers and biocides [11].

The effect of heavy metals such as copper against certain bacteria that were grown in media in vitro, rather than on the orthopedic implants have been studied in most studies. In this study, the activities of copper against bacterial growth and detachment of bacteria from biofilm produced on kirschner wire orthopedic implant were studied.

The aims of this study are to prevent biofilm production and detach mature biofilm produced by pathogens on abiotic medical surfaces such as kirschner wire orthopedic implant by using copper.

Materials and Methods

The bacteria.

Biofilm producer isolates of methicillin resistance *Staphylococcus aureus* MRSA (MRSA), methicillin sensitive *Staphylococcus aureus* MSSA (MSSA), methicillin resistance *Staphylococcus epidermidis* (MRSE), methicillin sensitive *Staphylococcus epidermidis*, methicillin sensitive *Staphylococcus epidermidis* (MSSE), *Escherichia coli* (E. coli), *Klebsiella pneumonia* (K. pneumonia), *Pseudomonas aeruginosa (P. aeroginosa)*, *Proteus mirabilis (P. mirabilis)* were used for this study.

Preparation of bacterial suspension

Bacterial suspensions were prepared and adjusted to 0.5 McFarland (1.10^8 cfu/ml). This bacterial suspensions were twenty fold (1/20) diluted to reach 5.10^6 cfu/ml. Bacterial suspension was adjusted by ten fold dilution (1/10) in such a way as the final concentration become 5.10^5 cfu/mL.

Assessment of MRSA and MRSE

Methicillin resistance of *S. aureus* and *S. epidermidis* was determined by cefoxitin by Kirby Bauer disk diffusion method and broth microdilution method according to the Clinical Laboratory Standards Institute criteria 2013 (CSLI). Bacterial suspensions of Staphylococcal strains were prepared in Tryptic soy broth (TSB), and adjusted to 0.5 McFarland (1.10^8 cfu/mL). The staphylococcal strains from bacterial suspensions were inoculated by the spread plate method to Mueller Hinton agar, and 30 µg cefoxitin disks were put on the inoculated plate. Zone diameters of cefoxitin were measured after incubation in 24 hours at 37°C. The zone measurements were categorized into sensitive (≥ 22 mm), or resistant (≤ 21 mm for cefoxitin) categories [12].

Preparation of copper concentrations

1 mg/mL of copper suspension was prepared with sterile distilled water (Merck, Germany). This suspension was double fold diluted (1/2) to reach concentrations extended from 0.063-1 mg/mL. Due to occurrence of long internals between 0.25, 0.5 and 1 mg/mL, the concentrations of 0.375 and 0.75 mg/mL were also prepared.

The determination of biofilm

Preparation of orthopedic implant

Kirschner wire orthopedic implant was used for screening antibiofilm activity of copper (1.8 mm diameter, SZO, China). Kirschner wires were cut into 1 cm pieces.

Qualitative assay for biofilm

Congo red agar method (CRA).

Isolates were inoculated on Congo red agar media (CRA) (Merck TM) as described by Freeman et al. (1989) to identify whether isolates were biofilm producer or not [13]. The CRA medium was constructed by mixing 0.8 g of Congo red and 36 g of sucrose (Sigma, Missouri, EUA) to 37g/L of BHI (Oxoid, Basingstoke, Hampshire, England). After incubation period that was 24 h at 37°C, morphology of colonies that undergone to different colours were differentiated as biofilm producers or not. Black colonies with a dry crystalline consistency indicated biofilm producers, whereas colonies retained pink were non-biofilm producers.

Tube method (TM). The biofilm formation of isolates were also detected by this method that is described by Christensen et al. (1985). Isolates were inoculated in polystyrene test tube which contained TSB and incubated at 24 h at 37°C [14]. The sessile isolates of which biofilms formed on the walls of polystyrene test tube were stained with saphranin for 1 hour, after planktonic cells were discharged by rinsing twice with phosphate-buffered saline (PBS). Then, saphranin stained polystyrene test tube was rinsed twice with PBS to discharge stain. After air drying of test tube process, the occurence of visible film lined the walls and the bottom of the tube indicates biofilm production [14].

Determination of MICs and MBCs of copper

Bacterial suspension was prepared and adjusted to 0.5 McFarland (1.10^8 cfu/mL) in Mueller Hinton Broth (MHB) containing 2% NaCl [15]. This bacterial suspension was twenty fold (1/20) diluted to gain 5.10^6 cfu/mL. 180 µl of each copper concentration and 20 µl of bacterial suspensions were dispersed to each well of microplate to obtain 5.10^5 cfu/mL as a final concentration (ten fold dilution (1/10)). Microplates incubated at 37°C for 24 hours. The lowest concentration of copper in which bacterial growth did not observed visually was determined as minimum inhibitory concentration (MIC) of copper, according to Clinical Laboratory Standards Institute (CLSI) [12].

After incubation of wells, 100 µl inoculum from the wells in which MIC was observed and two of concentrations were higher than MIC inoculated to PCAs to determine minimum bactericidal concentration (MBC) of copper. After incubation of inoculated PCAs at 37°C for 24 hours, the lowest concentration of copper in which bacterial colonies were not occured in PCA was determined as MBC of copper, according to Clinical Laboratory Standards Institute (CLSI) [12]. The studies were repeated in triplicates.

Formation of biofilm on implant and determination of bMBCs of copper

In summary, biofilm formation process on abiotic surfaces by bacteria was done. Quantification of biofilm embedded bacteria grew on abiotic surface, and biofilm embedded bacteria remained on abiotic remained on abiotic surface after addition of agent on abiotic surface on which mature biofilms formed determined by plate counting.

Bacterial suspension was prepared and adjusted to 0.5 McFarland (1.10^8 cfu/mL) in Mueller Hinton Broth (MHB) containing 2% NaCl [15]. This bacterial suspension was twenty fold (1/200) diluted to gain 5.10^5 cfu/mL. Kirschner wire orthopedic implants were placed into each test tubes containing 5.10^5 cfu/mL isolate and incubated at 37°C for 24 hours to lead bacteria to produce biofilm on kirschner wire. After incubation, kirschner wires on which biofilms were produced were discharged and rinsed with phosphate-buffered saline (PBS) (pH 7.2), then, transferred into each test tubes containing copper concentrations. After incubation at 37°C for 24 hours, kirschner wires discharged and placed into test tubes containing sterile MHB and vortexed for 2 minutes. Then, 100 µl samples of each test tubes vortexed were inoculated on plate count agars (PCA), and incubated at 37°C for 24 hours. After incubation at 37°C for 24 hours, the lowest concentration of copper in which colonies of biofilm embedded bacteria were not grown was determined as minimum bactericidal concentration (bMBC) of copper for biofilm that is also defined as minimum biofilm eradication concentration (MBEC) in this case [12], [16]. The studies were repeated in triplicates.

Results

The effect of heavy metals such as copper against certain bacteria have been studied in most studies. In this study, the activities of copper against bacterial growth and detachment of bacteria from biofilm produced on kirschner wire orthopedic implant were studied. Biofilms of MRSA, MSSA, MRSE, MSSE, *E. coli, K. pneumonia, P. aeruginosa*, and *P. mirabilis* were detached from kirschner wires completely by copper at the concentrations of 0.75, 0.25, 0.5, 0.375, 0.125, 0.125, 0.375 and 0.125 mg/mL, respectively (Table 1).

MICs, MBCs, and bMBC of copper against pathogens were ranged from 0.063 to 0.75 mg/mL. This study revealed that 0.75 mg/mL of copper inhibit all isolates analyzed in this study, whereas there were no bacterial growth and biofilm on kirschner wire orthopedic implants at 1 mg/mL of copper.

The MICs of copper against MRSA, MSSA, MRSE, MSSE, *E. coli, K. pneumonia, P. aeruginosa,* and *P. mirabilis* were 0.5, 0.25, 0.375, 0.375, 0.125, 0.125, 0.25, and 0.063 mg/mL, respectively. The MBCs of copper against MRSA, MSSA, MRSE, MSSE, *E. coli, K. pneumonia, P. aeroginosa,* and *P. mirabilis* were 0.75, 0.25, 0.5, 0.375, 0.125, 0.125, 0.375, and 0.125 mg/mL, respectively (Table 1).

In this study, the most tolerant pathogen was MRSA of which reason may be due to their resistance to methicillin and most antimicrobials [1], [17], [18]. MIC, MBC and bMBC of copper against MRSA were the highest according to other pathogens. This was followed by *S. epidermidis* (MRSE and MSSE), MSSA and *P. aeruginosa, E. coli* and *K. pneumonia*, respectively (Table 1).

P. mirabilis was the most sensitive pathogen to copper when compared with the others. It was followed by high sensitivity of *E. coli* and *K. pneumonia* to copper. MICs that were 0.063 and 0.125 mg/mL sufficient to eliminate *P. mirabilis*, and both of *K. pneumonia* and *E. coli,* respectively, whereas bMBCs that were 0.125 mg/mL sufficient to detach mature biofilms of *P. mirabilis, K. pneumonia* and *E. coli* produced on kirschner wires (Table 1).

Although, it is hard to eliminate biofilm embedded bacteria than planktonic ones, the effects of copper against planktonic and sessile bacteria that is also referred biofilm embedded bacteria and adhered on kirschner orthopedic wire were the same after 24 hours incubation (Table 1).

Table 1. MICs, MBCs and bMBCs of Copper

Pathogens	MICs (mg/mL)	MBC (mg/mL)	bMBC (mg/mL)
MRSA	0.5	0.75	0.75
MSSA	0.25	0.25	0.25
MRSE	0.375	0.5	0.5
MSSE	0.375	0.375	0.375
E. coli	0.125	0.125	0.125
K. pneumoniae	0.125	0.125	0.125
P. aeruginosa	0.25	0.375	0.375
P. mirabilis	0.063	0.125	0.125

Discussion

In this study, the most tolerant pathogen was MRSA due to their resistance to methicillin and most antimicrobials [1, 17, 18]. Researchers had revealed that copper-based antimicrobials that have multitoxicity effect not only effective against sensitive bacteria but also against multi drug resistant (MDR) microorganisms such as methicillin resistant *Staphylococcus aureus* MRSA [11].

Reyes-Jara et al. (2016) contributed a study which determines copper susceptibility of *E. coli,* coagulase negative *Staphylococci* (CNS), *S. aureus* and *Streptococcus uberis (S. uberis)* isolated in milk samples of bovine clinical mastitis. Reyes-Jara et al. (2016) found that *E. coli* was the most sensitive pathogen to copper. Reyes-Jara et al. (2016) revealed that at the concentration of 1000 ppm copper inhibited whole isolates examined. They found that coagulase negative *Staphylococcus* (CNS) that was the most resistant pathogen to copper was followed by *S. aureus*, and *E. coli* was the most sensitive pathogen to copper [19].

Koseoglu Eser et al. (2015) compared antimicrobial activity of copper coupon with stainless steel coupon against multi drug resistant bacteria (MDR) such as MRSA, *P. aeruginosa* and *Acinetobacter baumannii,* and revealed that copper coupon was more effective than stainless steel coupon [20].

It has also demonstrated that copper had an synergistic effect with other certain chemicals [21], [22], such as quaternary ammonium compounds (ex: benzalkonium chloride, cetalkonium chloride, cetylpyridinium chloride, myristalkonium chloride, and Polycide) and Amphotericin B are more effective to eliminate biofilms of certain bacteria such as *P. aeruginosa, E. coli, S. aureus, Salmonella enterica* serovar *Cholerasuis,* and *Pseudomonas fluorescens,* and *Candida albicans* a few times more than sole treatments, respectively [22].

Sole copper and of which combinations with biocides inhibits sessile forms of *P. aeruginosa* ten fold more than planktonic forms. They revealed that metal cations and oxyanions detach biofilms according to increasing concentration and process time [22].

Conclusion

Indwelling device associated infections that can be untreatable and recurrent can be prevented by metals instead antibiotics to avoid antibacterial resistance. Copper can be used as a bactericidal and antibiofilm substance solely or combinations with antimicrobial agents in solutions. Indwelling medical devices such as orthopedic wires, prosthetics can be impregnated by copper to overcome colonization and production of matured biofilm on indwelling devices, consequently, implant associated infections, and to avoid removal of implants colonized by biofilm producer pathogens out of the body.

Declaration of conflict of interest

The authors received no financial support for the research and/or authorship of this article. There is no conflict of interest.

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