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EVALUATING ANTIBACTERIAL AND ANTI-BIOFILM PROPERTIES OF COPPER AND ZINC IN BETA TRICALCIUM PHOSPHATE AGAINST STAPHYLOCOCCUS EPIDERMIDIS

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Abstract

Staphylococcus epidermidis is a common pathogen responsible for peri-implantitis, an infection localized around dental implants. Its virulence is largely attributed to biofilm formation, which enhances resistance to antibiotics and immune defenses. The challenge of treating *S. epidermidis* is further compounded by its resistance to several antibiotics, including methicillin. This study aimed to evaluate the antibacterial and anti-biofilm properties of a copper-zinc (Cu-Zn) metal alloy and its efficacy when integrated with beta-tricalcium phosphate (β -TCP) as a graft medium against *S. epidermidis* using in vitro methods. This research employs *Staphylococcus epidermidis ATCC* 12228. Antibacterial activity was assessed using Direct contact test and viability count via Colony-Forming Unit (CFU) analysis, while biofilm formation was evaluated using the tube adherence method. Increasing copper and zinc concentrations enhanced antibacterial efficacy, with Cu-Zn- β -TCP suspensions showing significantly greater antibacterial activity ($p = 0.01$) and improved anti-biofilm properties compared to Cu-Zn suspensions without β -TCP. The incorporation of copper and zinc effectively inhibited bacterial growth and biofilm formation of S. epidermidis. Furthermore, the Cu-Zn alloy demonstrated higher efficacy when used in conjunction with β -TCP as a graft medium, suggesting its potential for managing peri-implantitis.

Keywords: *Cu-Zn-β-TCP; Colony Forming Unit; Biofilm formation; Staphylococcus epidermidis*

1. Introduction

Staphylococcus epidermidis is a facultative anaerobic bacterium which causes clinical infections.(Namvar et al., 2014) The virulence factor of *S. epidermidis* lies in its ability to form biofilms, which protects the bacteria from the body's immune system and antibiotics.(Koch et al., 2020; Lee & Anjum, 2024)

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Originally, findings of *S. epidermidis* is considered to be a contaminant.(Săndulescu et al., 2023) However recent research showed traces of *S. epidermidis* in 68% sample taken from an orally healthy person and 81% sample taken from an orally healthy person with implants. (O'Connor et al., 2018) The presence of *S. epidermidis* complicates the longevity of dental implants such as bone grafts due to the risk of developing peri-implantitis from *S. epidermidis*.(Carvalho et al., 2023) Complications of peri-implantitis include bone loss and removal of the current implant to prevent further damage.

Treatment of *S. epidermidis* becomes difficult due to high resistance to antibiotics primarily methicillin, erythromycin, Gentamicin, and other antibiotics (Eladli et al., 2019). In the case of peri-implantitis, treatment would require eradication of *S. epidermidis* and its biofilm altogether to achieve success. *S. epidermidis* biofilm protects the bacteria from antibiotics therefore a more invasive procedure in which the implant would have to be surgically removed or replaced is required.(Siciliano et al., 2023).

Beta-tricalcium phosphate (β-TCP) is a synthetic bone substitute compound commonly used as a bone graft material due to its osteoinductive, osteoconductive properties, and good biocompatibility with bone.(Li et al., 2023). Chemical doping, a procedure of adding metal cations with oligodynamic properties to enhance the functional capabilities of β-TCP. These functional capabilities include increased antibacterial and structural ability, osteoinduction, osteogenicity, and others.(Bohner et al., 2020) Usage of metals with oligodynamic properties such as copper and zinc can be an alternative in the emergence of antibiotic resistance in bacteria.(Situmorang et al., 2019).

Copper (Cu) and Zinc (Zn) are metals known for their antibacterial and anti-biofilm properties. Research by Ghezzi et al. stated that copper layers have antibacterial and anti-biofilm properties against *Staphylococcus aureus*.(Ghezzi et al., 2023) Research by Noach et al. stated that zinc has antibacterial and anti-biofilm properties by inhibiting the cellular processes of various bacteria, including *S. aureus*.(Noach et al., 2023). The use of bone grafts with synthetic materials that have osteoinductive and osteoconductive properties, such as β-TCP, combined with metals that possess antibacterial and anti-biofilm properties, such as Cu and Zn, can provide a solution for treating and preventing peri-implantitis cases caused by *S. epidermidis*.

2. Method

S. epidermidis ATCC 12228 was inoculated into nutrient agar plates and incubated in 37^{o}C for 24 hours to for the working culture.

Table 1. Composition of Cu, Zn, and β-TCP within Cu-Zn-β-TCP suspension group

Group	Cu (mg)	Zn (mg)	β-TCP (mg)	Cu %	Zn %	β-TCP%
A	7	7	686	1	1	98
B	14	14	672	2	2	96
C	21	21	658	3	3	94
D	28	28	644	4	4	92
E	35	35	630	5	5	90

Copper, zinc, and β-TCP powders were prepared in culture tubes. Each group of suspensions and materials was prepared in triplicate to enhance data reliability and accuracy. The composition of each group is outlined in Table 1. Nutrient agar and Brain Heart Infusion (BHI) broth were prepared in Erlenmeyer flasks. The BHI broth was dispensed into culture tubes, with each tube containing 10 mL of BHI broth supplemented with 1% sucrose.

All culture tubes and Erlenmeyer flasks were sterilized through autoclaving at 121 ^{o}C for 30 minutes. Following sterilization, the nutrient agar was poured into individual Petri dishes.All the culture tubes and

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erlenmeyer flask were then autoclaved in 121°C for 30 minutes. After the sterilization process, the nutrient agar was poured into individual petri dishes.

To start the antibacterial testing, *S. epidermidis* was inoculated from the working culture into culture tubes filled with 10ml BHI broth until reaching density of 0,5 McFarland. Each suspension of Cu-Zn and Cu-Zn-β-TCP as shown in Table 1 and Table 2 was added into culture tubes filled with 10ml BHI broth which was prepared before and homogenized with vortex mixer. Both Cu-Zn and Cu-Zn-β-TCP suspensions were then incubated in 37°C for 24 hours. After 24 hours, 1 µL isolate were taken from each suspension group. Each isolated were cultured into separate nutrient agar plates and incubated at 37°C for 24 hours. After 24 hours, each plate were analyzed using colony counter.

For the antibiofilm testing, take tubes used from the antibacterial testing. Both Cu-Zn and Cu-Zn-β-TCP suspensions were then discarded from the tube and the culture tubes were rinsed with distilled water, continued by staining with 5 mL safranin. After 15 minutes, safranin was discarded and the tubes were rinsed using distilled water 2 times. Safranin stained biofilms were then observed with the naked eye. Under observation with the naked eye, a positive result for biofilm formation will show a layer of red stained layer in the tube wall indicating a biofilm is formed.(Niveditha et al., 2012)

Control groups were established to serve as benchmarks for comparison. The positive control group was prepared by inoculating *S. epidermidis* into BHI broth supplemented with 0.5 µg/mL of oxacillin. The negative control group was prepared by inoculating *S. epidermidis* into BHI broth without additional treatment. The media control group consisted of BHI broth without the inoculation of *S. epidermidis*.

The objective of the study was to identify the Cu-Zn composition that maximizes antibacterial and antibiofilm activity. Specifically, the goal was to determine the composition yielding 0 CFU and the absence of biofilm formation, indicating complete bacterial elimination and biofilm inhibition. Data were analyzed using a one-way ANOVA test followed by post-hoc Bonferroni analysis.

3. Result and Discussion

Table 3. Average of Colony Forming Unit (CFU) of Cu-Zn and Cu-Zn- β-TCP suspensions after 24 hours

Group	Cu-Zn	Cu-Zn-β-TCP	(-)ve Control	(+)veControl	Media Control
A (7mg Cu + 7mg Zn)	>300	281.7	>300	0	0
B (14mg Cu + 14mg Zn)	>300	56.3			
C (21mg Cu + 21mg Zn)	297.7	0			
D (28mg Cu + 28mg Zn)	73	0			
E (35mg Cu + 35mg Zn)	0	0			

- Negative control consists of β-TCP blanks
- Positive and Negative control measurements were performed in triplicate

The only anomaly found throughout the results in the results is in group D of Cu-Zn suspension. A 0 CFU data is found in 1 group and other groups have 164 and 55 CFU. The explanation for this anomaly remains hard to justify. However, several possibilities include uneven suspension which was used for inoculation, dry agar plates surface, and factors like researchers fault during preparation.

The goal for this experiment is to determine whether adding Cu and Zn can cause a complete inhibition of bacterial growth and biofilm formation. A complete inhibition of bacterial growth is depicted by a 0 CFU count as shown with positive control group (oxacillin) in Table 3. CFU count of Group A and B in Table 3. did not meet the criteria of 0 CFU count therefore both groups are not relevant for data analysis. Both datasets of group E have 0 CFU count and therefore data analysis is not needed to determine significant difference between both Cu-Zn suspension and Cu-Zn-β-TCP suspension of group E.

Data analysis of group C and D is done with one way ANOVA test. Group C achieved a p-value of 0,0017 and group D achieved a p-value of 0,204. With these results we can determine that group c have significant difference between Cu-Zn suspension and Cu-Zn-β-TCP and group D does not. A *Post-hoc*

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analysis using Bonferroni test is done to confirm the statistically significant difference in group C. The p-value achieved from the Bonferroni test is 0,0174 therefore confirming the results of group C. With these results, It can be concluded that Cu and Zn does exhibit antibacterial effects on *S. epidermidis* and such effects show better efficiency when combined with β-TCP graft base. From these results also can be concluded that a composition of 6% Cu-Zn within 94% β-TCP graft base showed the best antibacterial capabilities.

Antibacterial properties

The research results showed that Cu and Zn exhibit antibacterial properties against *S. epidermidis*. Increased efficacy is found in groups with higher composition of Cu-Zn marked by decreasing number of CFU. A study indicates that copper coating displayed antibacterial effect by releasing Cu ions into bacterial membrane causing cell leakage or even rupture and reduce CFU numbers in *S. epidermidis.*(Vibornijs et al., 2021) Another study implicated a proportional decrease of CFU in accordance to increased exposure Cu^{2+} ions (Salah et al., 2021).

Antibacterial properties of Zinc depend on its ability to penetrate cell membrane. Zinc showed contact killing ability equivalent to 5% of copper's contact killing ability (Mathews et al., 2015). Therefore in this research, zinc rely on copper to deteriorate bacterial membrane and release Zn^{2+} ions alongside Cu^{2+} ions to exude antibacterial effect. Results also showed that Cu and Zn exhibit higher efficacy of antibacterial activity when placed within β-TCP media. Possible reasoning behind these findings is adhesion of Cu and Zn onto the bottom of the culture tube which reduced direct contact between Cu and Zn particles with the bacterial itself in Cu-Zn suspensions. These findings were not found in Cu-Zn-β-TCP suspensions where Cu and Zn particles bind into β-TCP particles instead of the culture tube wall.

A study indicates that direct contact between Cu particle and bacterial membrane caused severe damage to cell envelope and created a passage for ions to enter bacterial cell to cause further damage leading into cell death.(Mathews et al., 2013)

Biofilm Formation

Positive result criteria in tube adherence test is shown with the appearance of red layer of biofilm due to safranin staining in culture tube wall which indicates biofilm formation described by findings on negative control group (β-TCP only group) shown in Table 6. Negative result in tube adherence test is demonstrated by both positive control group and media control group in Table 6. Findings showed that culture tube wall remained clear and showed no signs of biofilm formation. Staining residue at the surface of culture tube and appearance of a ring-like structure on tube wall are common and doesn't indicate biofilm formation.

The results shown in Table 6 indicated that Cu and Zn possess anti-biofilm properties against *S. epidermidis.* Biofilm formation in Cu-Zn suspension groups declined in accordance with an increase of Cu and Zn within the suspension and reached maximum biofilm inhibition in the 35mg+35mg Cu-Zn proportion. The biofilm assay also indicates the synergistic mechanisms of Cu-Zn-β-TCP in *S. epidermidis* biofilm eradication or inhibition. Cu-Zn-β-TCP suspension group achieved minimum biofilm formation with much lower composition of Cu and Zn compared to Cu-Zn suspension group, that is the 2% proportion.

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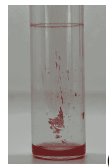
Table 4. Formed Biofilm of S. epidermidis against Cu-Zn and Cu-Zn-β-TCP after 24 hours

Group	A	B	C	D	E
Cu-Zn	 (+)	 (+)	 (+)	 (+)	 (-)
Cu-Zn- β-TCP	 (+)	 (-)	 (-)	 (-)	 (-)
Positive Control		Negative Biofilm (Slight formation)			
Negative Control		Positive Biofilm			
Media Control		Negative Biofilm			

Anti-biofilm Mechanism

Copper and zinc complex inhibits biofilm formation by limiting bacterial quorum sensing activities.(Padaga et al., 2024) *Staphylococcus* family possesses a unique quorum sensing system known as agr quorum sensing. Inhibition in agr quorum sensing activities will result in fewer production of phenol soluble modulin (PSM) which plays an important part in biofilm formation.(Otto, 2004) PSM creates channels within biofilm structure which is used to transfer nutrition to every cell inside the biofilm. High activities of PSM will cause biofilm clusters to detach and spread infections (Periasamy et al., 2012).

4. Conclusion and Suggestion

This study illustrated antibacterial and anti-biofilm capabilities of copper and zinc combined as a complex. Results of the research showed that Cu-Zn complex has higher antibacterial and anti-biofilm effectivity when integrated into β-TCP media which indicates possibility of usage as peri-implantitis prevention method. This study also suggested that copper and zinc showed the best antibacterial capabilities with a concentration of 6% within 94% β-TCP. Further studies are needed to ensure usage safety of Cu-Zn complex as a doping material within β-TCP or other bone graft subtitutes.

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