Morphology Changes of *E. coli* in Ag-HAp Observed by TEM

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ABSTRACT

The antimicrobial effects of HAp and Ag-HAp was observed using periprosthetic infection bacteria such as *Pseudomonas Aeruginosa, Staphylococcus Epidermidis, Escherichia coli* (*DH5α*). Ag-HAp showed good antimicrobial effects. TEM study of *E. coli* with and without Ag treatment in HAp was experimented in order to find the mechanism of Ag in antimicrobial effects. It was observed that the shape of Ag-treated *E. coli* was changed, the cells walls became inhomogeneous. The vacuoles at cytoplasm formed into *E. coli* and finally it was discovered by EDAX that there were many dark granules which contain the Ag element inside the cells.

I. INTRODUTION

Hydroxyapatite(HAp) has been used as implant materials because of its good biocompatibility[1] and was similar to human bone[2]. In addition to its biocompatibility, the cation exchange rate of HAp is very high with heavy metals or harmful ions such as Pb^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Ag^{+}, Co^{2+} etc [3,5]. Ag-HAp was known how to have a bactericidal effects, however the mechanism was not known to react between bacteria and Ag ions.

To observe the antimicrobial effects, three kinds of bacteria which caused periprosthetic infections (*Pseudomonas Aeruginosa, Staphylococcus Epidermidis, Escherichia coli* (*DH5α*)) were used in Ag-HAp and HAp. The bactericidal mechanisms of *E. coli* with Ag-HAp was compared those without Ag-HAp treatment by TEM. Ag-HAp was prepared by wet chemical process and antimicrobial effects of Ag against bacteria was investigated by viable count methods[3,4].

II. EXPERIMENT

1. Preparation of antimicrobial ceramics

   Ag-HAp were made by wet chemical process[3]. 0.01 M $AgNO_3$ was completely dissolved in the exact amount of distilled water and 0.167 M $Ca(OH)_2$ was suspended in the solution. 0.1 M H_3PO_4 was slowly dropped to 0.167M $Ca(OH)_2$ suspension and the solution was stirred with magnetic bar during the chemical reaction. The pH was monitored and the reaction was terminated at pH 9.1, which could produce the stoichiometrical HAp with Ca/P ratio of 1.67[4] .The precipitate formed during reaction was filtered and dried in the drying oven at 120℃. After drying, the antimicrobial ceramics(AC) was grinded with mortar and pestle in order to obtain fine powder.

2. Investigation of antimicrobial effects

   The following microorganisms were used : *Pseudomonas Aeruginosa, Staphylococcus Epidermidis, Escherichia coli* (*DH5α*) , among which *E.coli* and *P. Aeruginosa* are gram negative, whereas *S. Epidermidis* is gram positive. The bacteria were chosen because gram negative bacteria are responsible for more than 80% of all infections, with *E. coli* being responsible for more infections than all other genera combined. [11]

   The Ag-HAp powder and control sample HAp were immersed into the phosphate buffer saline solution(PBS) and shaken at 37℃ for 24 hours. The PBS solution contained about 1×10^5 cells/ml *E. coli, P. Aeruginosa, S. Epidermidis* respectively.

   0.1ml of the treated PBS solution was inoculated in the 20ml LB agar plate to cultivate the microorganisms at 37℃ for 24 hours, after that the number of colonies was counted. All glass ware were sterilized in the autoclave at 120℃ for 30 min before experiments.

3. Observation of treated *E. coli* with Ag-HAp by TEM

   After *E. coli* with Ag-HAp treatment and without Ag-HAp treatment in 10ml PBS solution was centrifuged at the speed of 400rpm for 15min (BECKMAN model J2-21), paraformaldehyde and glutaraldehyde were used for pre-fixation and osmium tetraoxide (OsO_4) for post-fixation, each for 2 hours. After each fixation, the sample were rinsed in Millonig's Phosphate Buffer solution. The sample were dehydrated in a graded series of acetone (50-100%), each for 1 hour. And then the samples were embedding in Epon. The embedding blocks were cut into ultrathin sections using ultramicrotome. The ultrathin sections were put on the copper grids, and were strained with uranyl acetate and lead citrate. As a final stage of the preparation, the

grids with the films on them were coated with carbon. The samples were observed in a JEOL-2000FX TEM at an accelerating voltage 70kV and the dark granules inside of *E. coli* was observed by EDAX.

III. RESULTS AND DISCUSSION

In the results of antimicrobial teat, the bacteria such as *Pseudomonas Aeruginosa, Staphylococcus Epidermidis, Escherichia coli* (*DH5α*) were normally grown in HAp. However, it was hardly grown in Ag-HAp [Fig. 1-3].

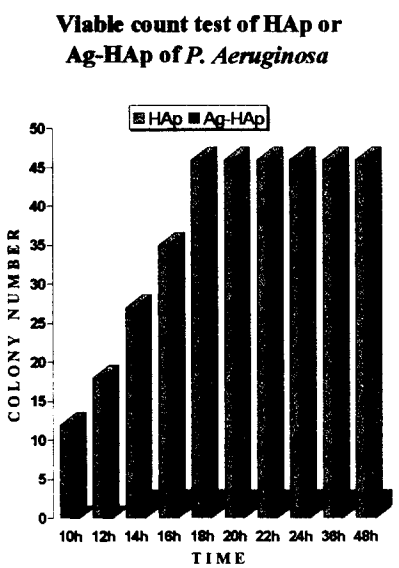

Fig 1

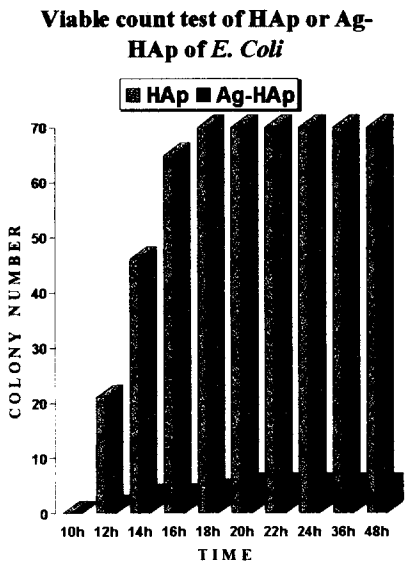

Fig.2

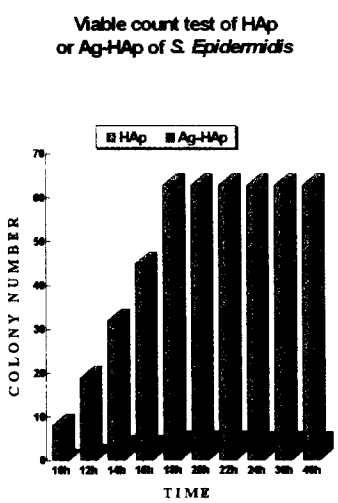

Fig. 3

The *E. coli* with Ag-HAp trearment and without Ag-HAp treatment were observed by TEM. Normal *E. coli* without Ag-HAp treatment was observed with intact cell membranes. The cell walls were homogeneous in thickness and the dense cytoplasm distributed homogeneously inside the cells [Fig. 4]. The cell walls of *E. coli* with Ag-HAp treatment was damaged and the thickness of cell wall became inhomogeneous. The thickness was 230nm at the thick point, whereas it was 16nm at the thin point. The cytoplasm of inside *E. coli* with Ag-HAp treatment was damaged,

distributed inhomogeneously and it was observed vacuoles in cytoplasm of inside *E. coli* [Fig. 5].

Fig .4 Normal *E. coli*

Fig. 5 Ag-HAp treated *E. coli*

It was found dark granules inside *E. coli* [Fig. 6], which was analyzed by EDAX. There were two weak peaks of the element Ag with peaks of Cu from copper grid and peaks of Pb from the lead citrate for staining process [Fig. 7]. It proved that Ag did exist inside the *E. coli*. It implies that silver ions penetrated the cell walls and result in the changes both in cell wall and cytoplasm. It was known that lipopolysaccharide (LPS) was attached to the outer membrane layer of *E. coli*. When the cells are reacted with endotoxin of gram negative bacteria, a heat-stable toxins from LPS is released[6,7]. The toxic properties of the outer membrane layer of the bacteria are responsible for some of the symptoms of infections[8].

Fig. 6 Ag-HAp treated *E.coli*

Ag-HAp treated *E. coli* lost their wave-shaped outer membrane layers associated with their toxicity, suggesting that the Ag-HAp treated bacteria lost their toxicity for human and mammals. It was known that vacuoles was formed by expansion and degenerating changes of organelle[8-10]. Thus it is thought that vacuoles was formed by degenerating changes of organelle in

cytoplasm inside *E. coli* with Ag-HAp treatment.

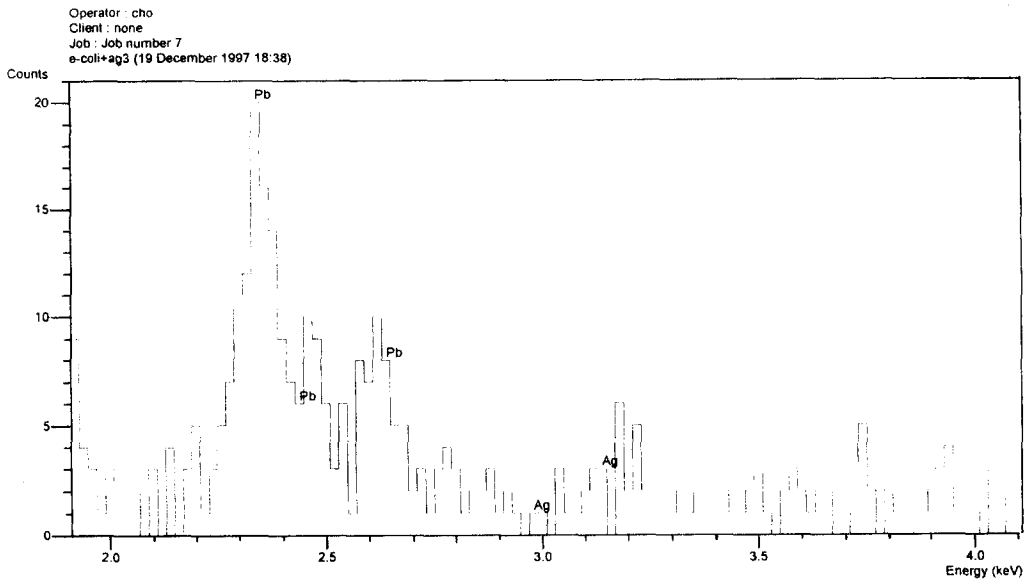

Fig. 7 EDAX analysis of the dark granules inside the damaged cells

IV. CONCLUSIONS

It is proved that Ag-HAp has a good antimicrobial effects. In observing the *E. coli* with Ag-HAp treatment, it was investigated that silver ions penetrated and changed microstructure of *E. coli*. The wave-shaped outer membrane layer was damaged and the vacuoles was formed by changes of cytoplasm.

ACKNOWEDGEMENT

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