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Full Length Research Paper

Comparhense study of sporicidal and sporstatic effect of CuO and AgO metal nanoparticles upon spore of *Clostridium botulinum* type E

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Nanoparticles due to high volume levels have high sporicidal and bacterial properties. In this study, we examined the sporicidal effect of metal oxide nanoparticles of silver and copper on the bacterium *Clostridium botulinum* type E. In this study, the bacterium, *C. botulinum* type E, and silver and copper oxide nanoparticles with average diameter of 20 nm were employed. The bacterium was identified and confirmed by PCR method. All colors and media culture used were of Merck Company. The bacterium was determined and confirmed by PCR and antibody was raised against *C. botulinum* type E. Peterson method was used for isolation and purification of spores. Ratios of MIC/MBC and phenol coefficient were calculated by microdilution method; and Syndics' sporicidal obtained amount of D value was determined by pure plate method. Strains of bacteria were confirmed by catalytic domain of *C. botulinum* type E. SEM and TEM images confirm nanoparticles size was about 20 nm. Powers of sporicidal effect of the nanoparticles were compared with other chemical sporicidals, like Glutaraldehyde. Phenol coefficient obtained was about 50 and the ratio of MIC/MBC good was about 1/2, respectively. D value for the critical concentration of silver nanoparticles was about 7 min. Results of this study showed that nanoparticles studied for its high ratio of surface to volume properties had high sporicidal effect and it is predicted that this nanoparticle can be used for environmental sterilization.

Key words: *Clostridium botulinum* type E, Ag, Cu oxide nanoparticles, sporicidal, material.

INTRODUCTION

Bacterium *Clostridium botulinum*

The strain, *Clostridium botulinum* bacterium includes Gram-positive, large-bacilli, sporogenous, microaerophilic bacteria andt has optimal growth in aerobic condition. This bacterium during growth produces a toxin, a very poisonous substance. Mortality ratio of this toxin can be 1 ng per kg of body weight in mice. The bacteria are mobile and lack capsule; and may be seen as single, in pairs or short chains in media (Pickett, 2008; Johanson, 2001).

This bacterium strain is able to produce four types of botulism: food, infant, wound and an uknown botulism. This toxin often enters into the bloodstream through mucosal surfaces such as colon, lung tissue or wound. It prevents the release of acetyl-choline in nerve endings, causing muscle paralysis, which in extreme cases, is often death (Simpson, 2004; Nishiki, 1994).

The specificity of the antigenic has been identified by 7 different types of bacteria that are tagged with letters A to

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G label. Types A, B and E are mainly for human diseases, and while types C and D cause diseases in most animals (Franciosa et al., 1994). Epidemiologic study conducted showed that most of the poisoning is from type E botulism. In Asia countries, including Iran, due to the desire to consume food products of marine, type E has high frequency. In 72% poisoning outbreak, caused by foods prepared at home and 9% of cases, the food is prepared in food factory (Cerf, 1977). Spores are resistant form of these bacteria. Spores are more oval or sub terminal. Because the diameter of the spore is larger than cell, spores often bind to the cell wall (Russell, 1982).

Dealing with spores' strains of *C. botulinum* is vital in the food industry, because it is a food poisoning factor. Different sporicidal and sporestatics have been introduced, which are mostly chemicals and their impacts on the structure of spores are also different (Ostiguy, 2008). The exact locations of the effects of these sporicidal on different layers are also different, as spores are made up of different layers. These layers are added to spore resistance against chemical and physical agents.

One of the greatest achievements of nanotechnology is the use of nanoparticles, especially metal oxide nanoparticles, in various fields of medicine, various industries such as agriculture, animal husbandry, depending on classification, appliances, cosmetics and health and military. This technology is to serve humanity by controlling disease-causing agents.

Bacterial pathogenesis (such as *C. botulinum*) damages too many different industries, humans and animals. Today, it is very costly to prevent and recover patients with these bacteria. Therefore, nanoparticles could be an alternative method for treating these patients (Huang, 2005).

The aim of this study is to carry out sporicidal characterization properties of metallic silver and copper oxide nanoparticles on the bacterium *C. botulinum*. These results were investigated by usual sporicidal agent in different conditions, such as temperature and pH. Nanoparticles will be used in future against bacteria causing disease in the food industry category.

METHODS AND METHODS

Strain produced and methods for its detection

Bacterium *C. botulinum* type E was prepared by Environmental Science Research Center of IHU. Bacteria were cultured in Cook Meat Media of Sigma Co. for 72 h in 32°C in aerobic Jar containing 15% nitrogen gas and carbon dioxide. Biochemical tests such as sugars and gelatin hydrolysis were performed to confirm the strain studied.

The first bacterial genome was purified with mini-preparation method to confirm the strain by PCR. Forward and reverse primers were designed against catalytic domain of *C. botulinum* type E. Sequence of the forward and reverse primer is shown as follows: Bioinformatics analysis was carried out for confirmation of the genome. All materials were prepared by PCR and primers from Sinogen Co.

Upstream primer:
5′ ATGCCAAAAATTAATAGTT 3′ Tm= 52/1

Downstream primers:
5′ CATTTTCCGTATTCCTTT 3′ Tm= 54/9

Purification of spores

Spores of bacteria were purified by Peterson method. Then, to achieve high purity of the spores, this method was corrected. For example, to reach the final rinse, pure water increased 5 to 10 times and the term primary cultures of bacteria also increased from 46 to 72 h in Peterson method. After staining with Malachite Green color, bacterium was studied under a light microscope model (Model UNBC-11) and necessary photos were taken with digital photographs. The t - student test was used to analyze the results ($p<0/05$).

Preparation of nanoparticles

Silver and zinc oxide nanoparticles were ordered from Nanoshell companies of USA. XRD diagram was taken by device Philips U234 model and TEM image was taken by H987 electron microscope photographs in the Faculty of Tehran University.

Sporicidal properties of nanoparticles

Sporicidal properties of nanoparticles were performed by calculating the MIC/MBC ratio. These methods were of microdilution method. Purification of spores was cultured in the presence of concentrations of 1, 0/5, 0/01, 0/05, 0/001 and 0/005 M of both nanoparticles for 32°C in an aerobically Jar. To obtain the MIC/MBC, colonies from 72-h culture in BHI medium were counted with a colony counter. Also the MIC/MBC ratio was calculated before and after the addition of nanoparticles, using the optical absorption spectrum changes. The formulas used for this work are listed below:

$$\left[\frac{(viableCFUat0hour - viableCFUat24hours)}{viableCFUat0hours}\right]\times 100\,\% \quad (1)$$

And for formulating and studying sporicidal power of nanoparticles, the following formula was used:

$$\text{bactericidal efficacy (\%)} = \frac{\text{alive number in reference group} \_ \text{alive number in experiment group}}{\text{alive number in reference group}} \times 100\% \quad (2)$$

Inhibitory and attraction power of nanoparticles was compared with phenol and formaldehyde that have a common sporicidal chemical. Phenol coefficient for silver oxide nanoparticles was calculated in Table 1.

RESULTS

Strains confirmation was done by PCR. Designed primers have been specific primers for the strain. PCR sequencing results are shown in Figure 1, which confirms the strain.

Table 1. Calculation the phenol coefficient.

Disinfectant	Dilution	Time (min)			Standard material (%)	Dilution	Time (min)		
		5	10	15			5	10	15
AgO nanoparticles	1:2000	-	-	-	Phenol 6	1:120	-	-	-
AgO nanoparticles	1:4000	+	-	-	Phenol 6	1:133	-	-	-
AgO nanoparticles	1:8000	+	-	-	Phenol 6	1:144	-	-	-
AgO nanoparticles	1:16000	+	+	+	Phenol 6	1:154	+	-	-
AgO nanoparticles	1:32000	+	+	+	Phenol 6	1:173	+	-	-
AgO nanoparticles	1:64000	+	+	+	Phenol 6	1:200	+	+	+

+ Means growth and - means No growth.

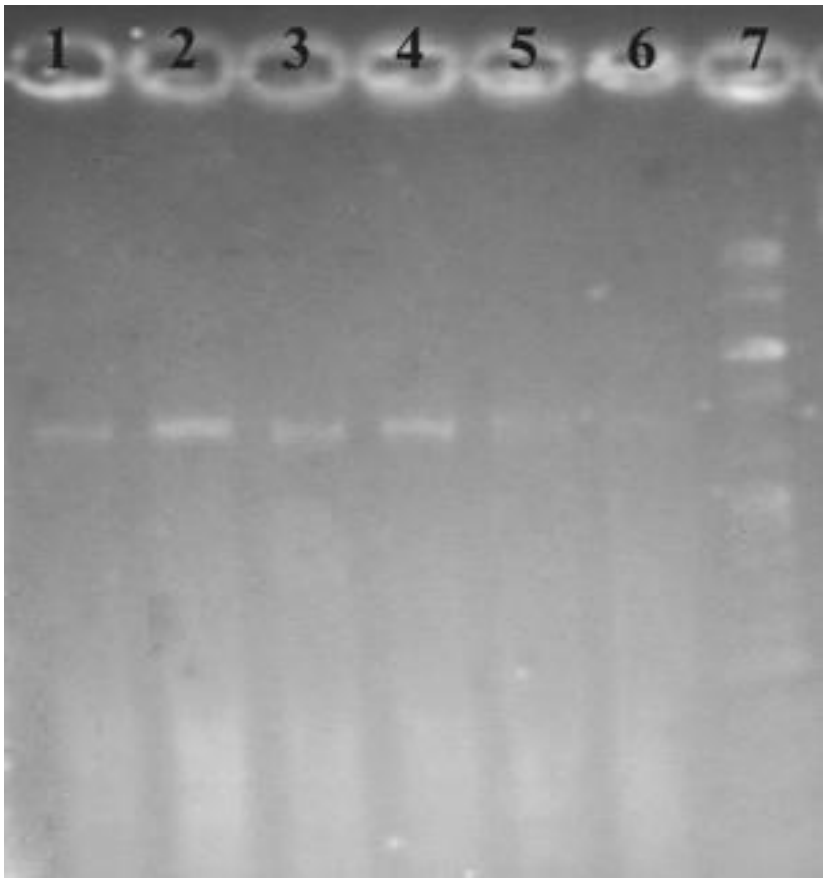

Figure 1. Image of gel to confirm the bacterial strains. The temperature gradient, Columns 4 and 6 is the highest expression. Columns from left to right include: Column 1: PCR product at Tm 50°, Column 2: PCR product at Tm 52°, Column 3: PCR product at Tm 54°, Column 4: PCR product at Tm 56°, Column 5: PCR product at Tm 58°, Column 7: PCR product at Tm 60°, Column 7: the ladder 10000 bp.

Purification of spores

Spores were purified using Peterson method, but for better quality, purification method was changed. Finally, purified spores were painted with Malachite Green dye. An example of stained spores is shown in Figure 2.

Confirmation of nanoparticles size

To confirm the size classification of nanoparticles SEM and TEM images were taken. Figure 3 showed that silver nanoparticles had a diameter of 20±5 nm, and copper nanoparticles, a 10-30 nm.

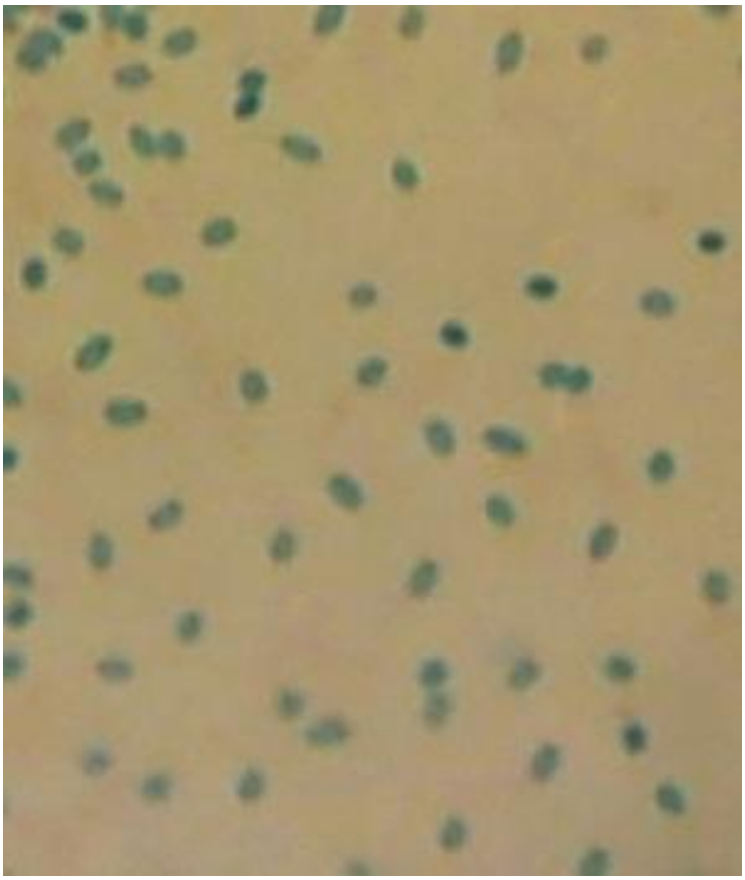

Figure 2. Purification spore and painted by Malachite Green method.

Sporicidal properties of nanoparticles

MIC / MBC ratio of nanoparticles

The minimum inhibitory concentration, minimum concentration to inhibit growth of bacteria and spores were for both copper and silver nanoparticles of the bacteria. Results for both copper and silver nanoparticles are shown in Table 2. As specified in this table, MC/MBC ratio for silver nanoparticles is 0/1, but this ratio cannot be calculated for the copper nanoparticles. Copper nanoparticles, not sporicidal property, in 1 molar concentration of Cu have a sporestatic property. Phenol coefficient for silver nanoparticles is 52, indicating that AgO nanoparticle is good sporicidal property (Table 2).

Phenol coefficient for silver nanoparticles = 8000/154 = 52

The second formula shows that sporicidal power of AgO nanoparticles in 0/01 M (the critical concentration) is about 79%. But this power for copper nanoparticles in 1 M (critical concentration) is about 49%. Therefore, sporicidal power of silver oxide nanoparticles is high. These nanoparticles are introduced into an appropriate sporicidal. D value was obtained for the sporicidal power of the silver oxide nanoparticles. Concentration of nanoparticles in the specified time and temperature specified that reduced 90% primary germ is D value. Therefore, the concentration ratio was investigated at 32°C for 0/01 M silver nanoparticles for 7 min.

D_{32} = 7 min

DISCUSSION

The strong connection of the outer membrane of nanoparticles is to prevent the transfer agent dehydrogenase, activity of periplasmic enzymes in spores, and to prevent RNA, DNA and synthesis of proteins. That ultimately leads to cell lyses. In this paper, the performance was shown for the spores of *C. botulinum* type E (native in Iran) (Raffi et al., 2008). Some factors such as the minimum time, the MIC/MBC, phenol coefficient, D value were calculated. This factor is necessary and appropriate for the treatment of infections caused by spore *Clostridium*. Nanoparticles such as nano-particles of

Table 2. Results of the MIC/MBC ratio.

Type of microbe	Type of nanoparticle	MBC concentration (M)	MBC concentration (M)	MIC/MBC ratio
Effect on spore	AgO	0/1	0/1	0/1
	CuO	1	-	-
Effect on bacteria	AgO	0/001	0/005	0/2

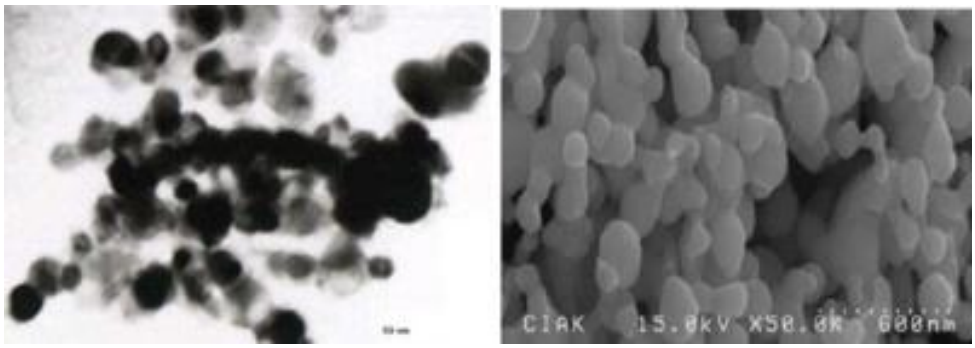

Figure 3. SEM and TEM images of nanoparticles (right, silver oxide and left copper oxide).

silver and copper can be used to prevent building damage, casualties and a suitable replacement for many disinfectants. This alternative is affordable and also economical (Lin and Xing, 2007).

Many results reported reactions between particles with biological macromolecular. The contrast between the negative charges of microorganisms with a positive charge of nano-particle created an electromagnetic attraction between the microbe and effective levels of active nano-particle. Finally, a large number of contacts led to oxidation of surface molecules of microbes and the immediate cause of death. Metallic silver and copper oxides show biodestructive effects, such as degradation of DNA (Rezaei-Zarchi et al., 2010; Khani et al., 2010). All toxicity tests of AgO and CuO nanoparticles on Gram-positive bacteria, immune cells and bacteria in human toxicity of nanoparticles of Ag and Cu suggest a variety of biological systems (Ghosh, 2007).

On the release of nonmaterial, they react with thiol groups (-SH) cell surface proteins in bacterial cell and perhaps in spores. Nano-materials cause inactivation and impermeability in the membranes of the protein of bacteria (Kawata and Okabe, 2009; Gant et al., 2007).

Carbonic acid or phenol is used as a standard to determine the germicidal effects. Phenol coefficient of disinfectants and disinfection with phenol show how much power is dramatic. When phenol coefficient is much, the sanitizer is stronger and microorganisms are destroyed in 10 min. In fact, the matter diluted is more antiseptic than phenol.

MIC/MBC test results confirm that silver nanoparticles have sporicidal property stronger than copper nanoparticles. Perhaps, powerful sporicidal silver has effect on the metabolism of bacteria, and stimulates hydrogen peroxide. Nanoparticles have a relatively large surface area and have more contact with outdoors, resulting in high sporicidal or sporestatic property.

Conclusion

These results show that copper and silver nanoparticles have high capability to inhibit the growth of spores. It is hoped that in future nanoparticles will be prevented in the treatment of diseases caused by pathogenic bacteria.

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