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**Full Paper**

# Effects of ozone treatment on cell growth and ultrastructural changes in bacteria

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**Ozone appeared to inhibit growth and caused the death of gram negative and gram positive tested bacteria: *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus* and *Bacillus subtilis*. Bacterial cultures at $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ cfu/ml dilution were exposed to 0.167/mg/min/L of ozone at different time intervals (0, 5, 10, 15, 30, 60, 90, 120, and 150 min). Cell viability was observed in all types of tested bacteria at $10^3$, $10^4$, $10^5$ cfu/ml within 30 min after ozone exposure. However, cell inactivation was not significantly observed at concentrations of $10^6$, $10^7$ cfu/ml even after an exposure of 150 min. Ultrastructural changes of treated bacteria showed deformation, rough damage and surface destruction revealed by scanning electron microscopy. Some bacterial cells showed collapsed and shrunken patterns within 60 min and severe rupture and cellular lysis after 90 min of ozone treatment. This study supports the proposed mechanism of the bacteria inactivation by ozone that caused cell membrane destruction and finally lysis reaction. Thus, the precaution of using ozone as a biocide should be used to address appropriate concentrations of bacterial contamination in water.**

**Key Words**——bacteria; biocide; cell growth; ozone; scanning electron microscope; ultrastructure

## Introduction

For many years, the role of ozone ($O_3$) against pathogens has been studied and it has been revealed that ozone can display biological effects and therapeutic index (Berson et al., 1996; Bocci, 1996; Hernandez et al., 1995; Turcic et al., 1995; Verrazzo et al., 1995; Wolfe et al., 1989). Ozone is a natural gas that is distributed at approximately 0.01–0.04 ppm (part per million) of the world's atmosphere. It is an unstable gas which can be broken down to oxygen gas ($O_2$) and oxygen atoms (O). The oxygen atom, being a strong oxidizer, appears to be a good agent for sterilization by the oxidation process. This property, therefore, becomes very useful for antioxidant protection system in arterial diseases and the immune system (Jakab et al., 1995; Peden and Dailey, 1995; Verrazzo et al., 1995) and in the inactivation of microorganisms (Broadwater et al., 1973; De Mik and De Droot, 1977; Foegeding, 1985; Ishizaki et al., 1986; Komanapalli and Lau, 1998). The use of ozone is applied in decontamination of microorganisms in biocleaned rooms (Masaoka et al., 1982), food preservation (Chen et al., 1992; Rice et al., 1982; Sheldon and Brown, 1986) and drinking water (Boyce et al., 1981; Glaze, 1987; Katzenelson and Biedermann, 1976).

The ozone treatment was effective against both

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gram positive and gram negative bacteria (Greene et al., 1993) and caused sensorial and microbial effects on fish bacteria (da Silva et al., 1998). Moreover, damage to cell membranes and cytoplasmic components was proposed to be responsible for inactivation of bacteria and fungi (Hinze et al., 1987; Mudd et al., 1969; Pryor et al., 1991; Scott and Lesher, 1963). However, no reports on the ultrastructural changes to these cells after ozone exposure are available. Therefore, in this study, the effect of ozone exposure in a time-dependent manner to various concentrations of gram positive (*Staphylococcus aureus*, *Bacillus subtilis*) and gram negative (*Escherichia coli*, *Salmonella* sp.) bacteria cultures on cell viability and cell structure at the ultramicroscopic level using a scanning electron microscope was investigated.

## Materials and Methods

*Microorganisms and media.* *Escherichia coli*, *Salmonella* sp., and *Staphylococcus aureus* were isolated from patients and kindly provided by the Microbiological Laboratory, Chulalongkorn Hospital. *Bacillus subtilis* was isolated from soil and an interval subculture was kept as a culture stock in the laboratory. Nutrient broth, McConkey and nutrient agar media were purchased from Difco Laboratories, Detroit, MI, USA.

*Ozone.* Ozone was generated with a commercial ozone generator (Brightzone) model OZ100 (Brightgreen Technology Co. Ltd., Hsin-Tien City, Taiwan) using atmospheric air as the oxygen source. The ozonated air produced at a constant flow rate by the apparatus was passed by a silicone tube to a diffuser. The concentration of ozone used was 20 mg/h with the fixed volume of the tested suspension of 2 L or equivalent to 0.167 mg/min/L. Bacterial suspensions (2 L) were exposed to $O_3$ directly through the diffuser at room temperature (28°C).

*Growth condition and cell viability test.* Each of the four bacteria species was cultivated in 1 L of nutrient broth for 18–20 h at 37°C except for *Bacillus subtilis* which was grown at 30°C. Without washing, the cultures were diluted with sterile water up to 2 L to the dilution of $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ cfu/ml. Both non-treated control (without ozone) and ozone-treated samples, of 0.1 ml each, were collected at different time intervals (0, 5, 10, 15, 30, 60, 90, 120, and 150 min). Then, the samples were spread onto McConkey agar (for *E. coli* and *Salmonella* sp.) or nutrient agar (for *S. aureus* and *B. subtilis*) and incubated at 37°C. The colonies appearing on the plates were counted with a colony counter (Branson, Quebec, Buffalo, NY, USA) and calculated as cfu/ml.

*Scanning electron microscopic study.* The bacteria cultures at a dilution of $10^7$ cfu/ml were collected at intervals of 0, 5, 10, 15, 30, 60, 90, and 120 min, concentrated by centrifuging at 1,000 rpm for 5 min, and prepared immediately for scanning electron microscopy. All specimens were fixed sequentially in two fixatives; firstly, in ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, for 2 h and followed by three washes with the same buffer. Secondly, the specimens were fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate for 2 h at 4°C, and washed three times with distilled water. They were then dehydrated in increasing concentrations of ethanol (from 50%, 70%, 80%, 90%, 95% to absolute alcohol) and dried in a Denton vacuum critical point drying machine using liquid $CO_2$ as a transitional medium. The specimens were mounted on aluminum stubs and coated with gold in an ion-sputtering apparatus, SPI-module, with a setting of 1.4 kV and 12 mA for 4 min. The specimens were then examined by the JEOL, JSM-5400 scanning electron microscope (SEM) operating between 0.5 and 30 kV. The ultrastructural changes of bacteria at various times of ozone exposure were studied and photographed as SEM micrographs.

## Results and Discussion

### *Cell viability test*

The bacteria used in this study are the microorganisms that are usually found in natural water. Some species, i.e. *S. aureus* and *Salmonella* sp., can cause symptoms in humans. They were selected to provide models for the study of bacteria structure, i.e. gram positive vs. gram negative and cocci vs. bacilli forms. The hypothesis is that ozone treatment is capable of causing changes to and destruction of bacterial architectures, especially at the membrane portions, which can be observed at ultrastructural level. The inactivation effect of ozone was observed in both gram positive and gram negative bacteria. After ozone exposure, the numbers of bacteria in cultures at $10^3$, $10^4$, and $10^5$ cfu/ml decreased in a time-dependent manner and bacterial growth was no longer detectable within 30 min of treatment (Fig. 1). However, at high bacterial

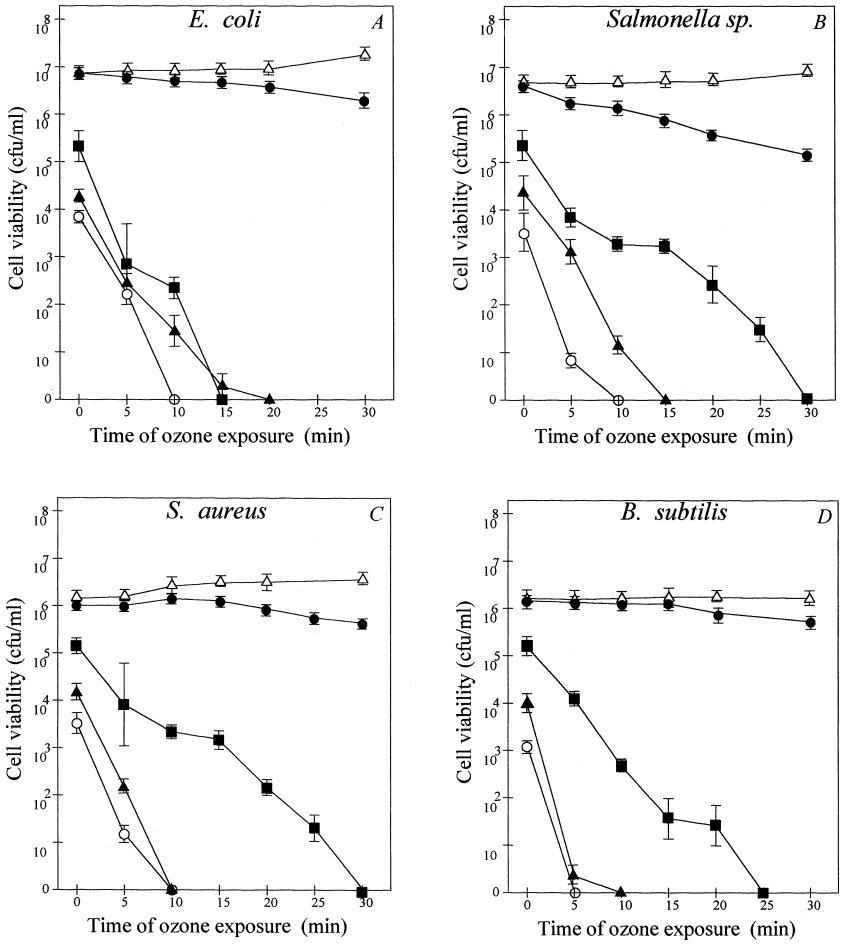


Fig. 1. Comparison of the effects of ozone exposure on cell viability.

Four types of bacteria groups were exposed to ozone at 0.167 mg/min/L for 0–30 min. Data represent means±standard errors. A: *Escherichia coli*, B: *Salmonella* sp., C: *Staphylococcus aureus*, D: *Bacillus subtilis*. Concentrations: (○) $10^3$, (▲) $10^4$, (■) $10^5$, and (●) $10^6$ cfu/ml. Non-treated control bacterial sample was demonstrated as (△).

concentrations, i.e. at $10^6$ and $10^7$ cfu/ml, a gradual decline in cell survival was demonstrated but ozone did not show effective activity against all bacterial cells even after a prolonged period of treatment (150 min). This finding correlates well with previous reports (da Silva et al., 1998; Komanapalli and Lau, 1996, 1998). On the other hand, without ozone treatment, all kinds of control bacterial cultures grew normally after being left at room temperature (28°C) as shown by the increased numbers of bacterial cells (Fig. 1).

*Ultrastructural changes of bacterial surface structure*

Only bacterial cells at $10^7$ cfu/ml were used for ultrastructural study because the density of cells was sufficient. Ozone treatment of two groups of bacteria; i.e. gram positive and gram negative bacteria, resulted in ultrastructural changes of surface structure. The severity of destruction increased in correspondence with the period of ozone exposure (Figs. 2–5). The degree of ultrastructural change can be divided into three steps according to the SEM morphological patterns. The first step was defined at the period of 30 min after the ozone exposure. The damage and deformity of the surface structure of ozone-treated bacteria of both gram positive and gram negative groups were clearly observed, whereas the normal surface structures of the control groups were still intact (Figs. 2A–B, 3A–B, 4A–B, 5A–B). The damage by ozone greatly affected the integrity of the cells under osmotic pressure so the bacterial cells could not survive at lower concentrations of $10^3$, $10^4$, and $10^5$ cfu/ml in which bacterial growth was not detected (Fig. 1). The second step was at the period of 60 min after the ozone exposure. This was the critical point of bacterial viability because most

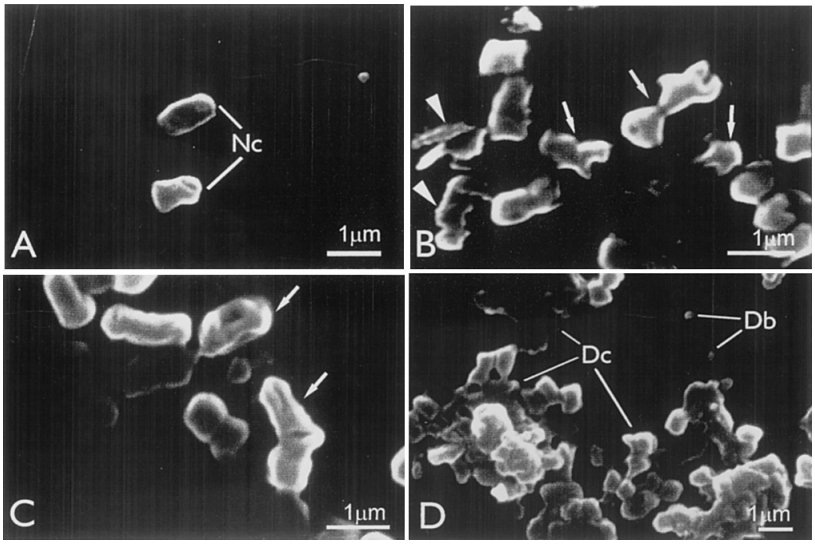


Fig. 2. Scanning electron micrograph of *Escherichia coli* treated with ozone.

After ozone exposure of 30–90 min, ultrastructural changes and damage to surface morphology were observed. A: Normal cell (Nc) of control group, B, C: Bacteria treated with ozone for 30 and 60 min, respectively, showed deformity (arrows) and destruction (arrowheads) of cells. D: Bacteria treated with ozone for 90 min showed destroyed cell (Dc) characteristics and some exploded debris (Db).

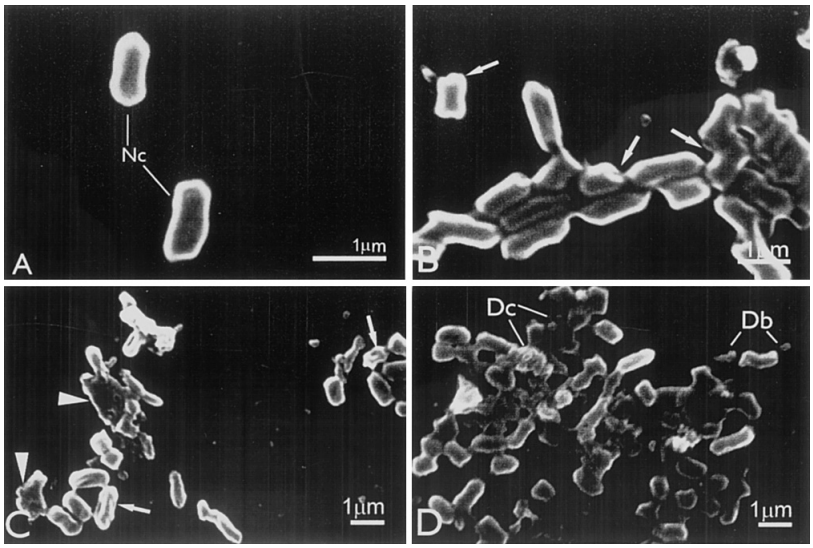


Fig. 3. Scanning electron micrograph of *Salmonella* sp. treated with ozone.

After ozone exposure of 30–90 min, ultrastructural changes and damage to surface morphology were observed. A: Normal cell (Nc) of control group, B, C: Bacteria treated with ozone for 30 and 60 min, respectively, showed homogeneous patterns of cell deformity (arrows) and cell destruction (arrowheads). D: Bacteria treated with ozone for 90 min showed destroyed cell (Dc) characteristics and some exploded debris (Db).

bacterial cells showed homogeneously irregular patterns of damage and deformity (Figs. 2C, 3C, 4C, 5C). In the case of *Bacillus* sp. (Fig. 5C), cell destruction and cell debris could be observed particularly clearly. The cellular cytoplasm exploded and was released into the media, defined by numerous rough particles around the bacterial bodies and throughout the media. The whole bodies of ozone-treated bacteria collapsed and shrank. These characteristics should be traced to the cause of bacterial cell lysis and bacterial death. The third step was at the period of 90–120 min after the ozone treatment. The destruction demonstrates

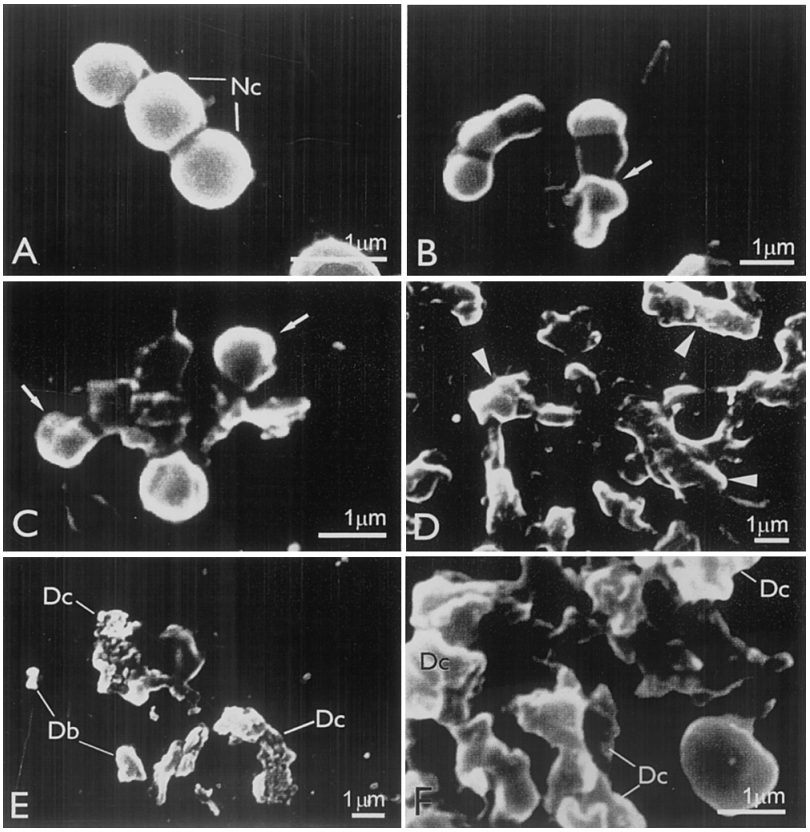


Fig. 4. Scanning electron micrograph of *Staphylococcus aureus* treated with ozone.

After ozone exposure of 30–120 min, ultrastructural changes and damage to surface morphology were observed. A: Normal cell (Nc) of control group, B–D: Bacteria treated with ozone for 30, 60, and 90 min, respectively, showed characteristics of cell deformity (arrows) and cell destruction (arrowheads). E, F: Bacteria treated with ozone for 120 min showed severe destruction (Dc) and copious debris (Db).

that severe rupture of the bacterial surface occurred. Almost all bacterial bodies of both gram positive and gram negative bacteria were broken and the debris of bacterial cells were clumped and distributed throughout the media (Figs. 2D, 3D, 4D–F, 5D–E).

Interestingly, when a cell viability test was carried out at a bacteria concentration of $10^6$ and $10^7$ cfu/ml, many bacterial cells still survived after 150 min of exposure. This may be due to copious cell debris or intracellular component leakage quenching the ozone and decreasing the inactivation efficiency (Finch et al., 1988; Komanapalli and Lau, 1996). The ultrastructural damage and destruction results support the previous reports that the primary target of ozone exposure was the physical effect on bacterial membrane whereas prolonged exposure would affect intracellular components (Komanapalli and Lau, 1996; Pryor,1992). The progressive degradation involved the changes of membrane permeability and cell integrity and was followed by the lysis reaction which corresponded to the biological effect on cell viability.

A study on the comparative responses between cocci vs. bacilli forms and between gram positive vs. gram negative bacteria showed that the deformation and cell viability of the two groups were similar at the first step within 30 min of the ozone exposure. However, a difference in severity and damage could be demonstrated. Gram positive bacteria samples, particularly that of *B. subtilis* which has a rapid cell wall turnover rate, showed much more destruction than the others (Fig. 5D–E). Moreover, in non-treated *B. subtilis* after a 90-min interval, most of the bacterial cells had a normal appearance whereas very few cells were damaged (Fig. 5F–G). Thus, it should be suggested that ozone would exert a stronger damaging effect on the bacteria than would ordinary autolysis.

In general, the related explanation might account for the difference in cell wall structure between gram positive and gram negative bacteria. Cell walls of gram positive bacteria consist of many layers of peptidogly-

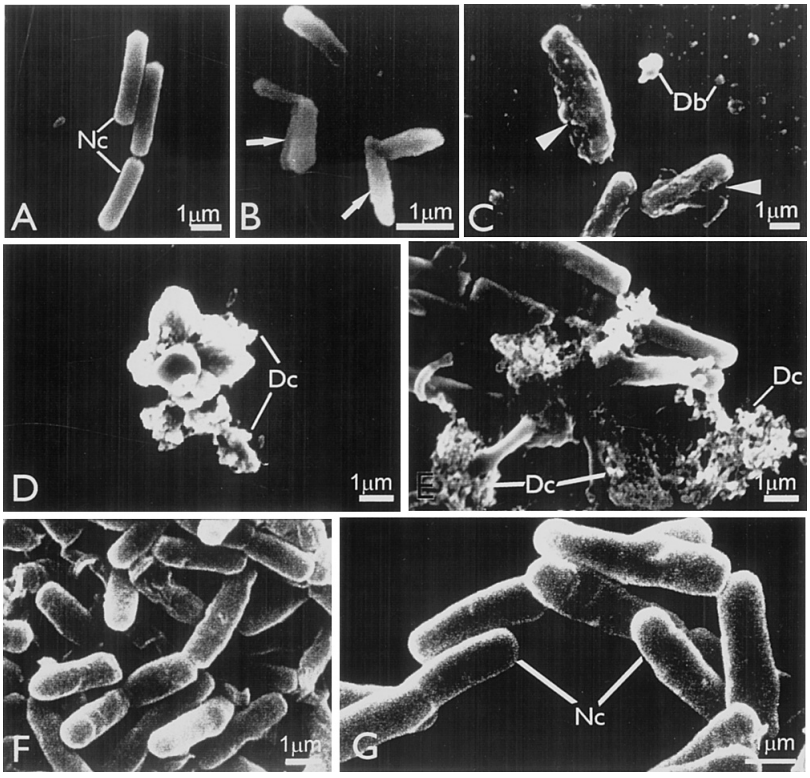


Fig. 5. Scanning electron micrograph of *Bacillus subtilis* treated with ozone.

After ozone exposure of 30–120 min, ultrastructural changes and damage to surface morphology were observed. A: Normal cell (Nc) of control group. B, C: Bacteria treated with ozone for 30 and 60 min, respectively, showed collapsed and shrunken patterns of cell deformity (arrows), cell destruction (arrowheads) and cell debris (Db). D, E: Bacteria treated with ozone for 120 min showed severe cell rupture and destruction (Dc). F, G: Most non-treated control bacteria at 90 min demonstrated normal cellular appearance (Nc).

cans forming a thick rigid structure whereas cell walls of gram negative bacteria consist of an outer membrane, which contains lipoprotein, lipopolysaccharides and a few layers of peptidoglycans beneath. This result support the study of Komanapalli and Lau (1998) which suggested that ozone reacts more readily with proteins than lipids.

Generally, most data revealed the antimicrobial effects of ozone against bacteria and virus (da Silva et al., 1998; Herbold et al., 1989; Komanapalli and Lau, 1998; Mackey, 1984). However, the sensitivity of bacteria to ozone varied enormously, depending on many factors. These factors include environmental factors or suspension media, laboratory conditions, the types of organisms, the stages of cellular growth, time period, the ozone concentrations, etc. However from this study, it can be concluded that ozone at 0.167 mg/min/L can be used to sterilize water which is contaminated with up to $10^5$ cfu/ml bacteria within 30 min. Nevertheless, this ozone concentration will not have significant efficiency on the cell viability of bacterial cultures at higher concentrations of $10^6$ and $10^7$ cfu/ml. Therefore, we can conclude that the appropriate amount of ozone can exert effective microbicidal activity by destroying the bacterial cell membrane, subsequently producing intracellular leakage and eventually causing cell lysis.

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