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EFFECT OF L-ASCORBATE/Cu(II) ON GROWTH OF *BACILLUS SUBTILIS* AND *ESCHERICHIA COLI*

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ABSTRACT

Antiviral and antitumoral effect of L-ascorbate (L-AsA) / Cu (II) have been established. We studied the antibacterial effect of L-ascorbate / Cu (II) and its action on the bacterial cell membrane. Oxidation products of ascorbate (AsA) produced in the presence of Cu (II). It has been known that these oxidation products cause breaks in protein and nucleic acids. They also lead lipid peroxidation. Effects of different concentrations of AsA + $CuCl_2$ on growth of *Escherichia coli* and *Bacillus subtilis* were investigated. Also subtoxic concentration of AsA / Cu (II) that affect bacterial growth were established. It was found that the inhibitory concentration for *B. subtilis* was 4 mM AsA + 100 μM $CuCl_2$ and *E. coli* was 8mM AsA+100 μM $CuCl_2$. The growth of bacterial cells were followed spectrophotometrically. Also viable cell counts were made.

Bacterial membranes isolated by Lysozyme-EDTA method to show changes caused in membranes.

Also membranes isolated from control cells and bacteria grown in AsA+$CuCl_2$ medium were applied on SDS-PAGE to show the changes occured in the membranes.

INTRODUCTION

Ascorbate (AsA) is a strongly reducing agent. Antiviral (Murata and Kitagawa, 1973; Wong et al., 1974; Morgan et al., 1976; Shamberger, 1984), antitumoral and antimutagenic (Pavellic et al., 1989) effects of AsA has been established.

AsA shows its chemical effects by its free radicals and oxidation products. Free radicals of AsA cause breaks in proteins and nucleic acids of the cell (Chiou, 1983; Gutteridge and Wilkins, 1983) and also leads lipid peroxidation (Düzgün ve ark., 1997; Feher, 1987; Chattopadhyay, 1992). It has been known that various and effective oxidation products of AsA occur in the presence of Cu (II).

Antiviral and antitumoral effects of AsA has been studied. So, we study the antibacterial effects of AsA/Cu (II), on *B. subtilis* and *E. coli.*

MATERIAL AND METHODS

Organisms:

B. subtilis (I. P. Eh. 1. 1953) was supplied from Institute of Hıfzısıhha (Ankara, Türkiye) and *E. coli* (ATCC 25822) was supplied from Hacettepe Univ. Medical School, Department of Microbiology.
Bacterial strains were grown on Nutrient Agar plates. They were stored at 4°C and subcultured monthly.

Medium:

Nutrient Broth (NB) was used for overnight culture. It was autoclaved at 121°C for 2 hours. Bacterial growth followed by spectrophotometrically at 400 nm.

Culture Condition:

Overnight culture was prepared by growing bacterial cells in NB at 37°C by shaking at 100 rpm. 4 mM AsA + 100 μM $CuCl_2$ for *B.subtilis* and 8 mM AsA+100 μM $CuCl_2$ for *E. coli* were added in the growth media (NB) to show their antibacterial effects.

Chemicals:

AsA (Merck) was dissolved in distilled water and sterilized by passing millipore filter. $CuCl_2$ (Merck) stock solution autoclaved at 121°C for 2 hours.

Preparation of Bacterial Membranes:

Osmotically sensitive cells (protoplast and spheroplast) were prepared. Spheroplasting of cells by Lysozyme-EDTA (Graham and Higgins, 1997; Copeland et al., 1982) was performed as follows. Cells were harvested at late logaritmic growth phase (2900xg, 10 min, 4°C) and washed once in 10 ml of 30 mM Tris- HCl buffer, pH 8.0. The pellet was resuspended in 10 ml of 30 mM Tris-HCl buffer, containig 20 %(W/V) sucrose, pH 8.0 warmed to 37°C. A freshly prepared solution of 60 μg/ml of lysozyme in 100 mM EDTA was then diluted 10 fold into the suspension of cells at 37°C.

The extend of spheroplasting as a function of time was followed by phase-contrast microscopy and by monitoring susceptibility to cell lysis as indicated by absorbance at 400 nm for 1:30 dilutions of cell suspensions into distilled water.

After 10 minute of incubation at 37°C, less than 5% of cells were rods. After an additional 10 minute of incubation the spheroplasting was judged complete. Spheroplasts and protoplasts were centrifuged (2900xg, 10 min.). Cold 10 mM Tris-HCl (pH 8.0), 1 mM EDTA were added on cell pellet and vortexed. This suspension was added slowly in a 4 fold distilled water in order to lyse the spheroplasts and protoplasts. After lysis, stirred magnetically at 0 - 2°C for 10 minute and microfuged (2400xg, 0 - 2°C, 10 min.). Supernatant centrifuged at 50.000xg for 2 hours to get membranes.

The bacterial membranes stored in 30 mM Tris-HCl buffer (pH 8.0) at -20°C.

SDS-PAGE:

Membranes isolated from *E. coli* and *B. subtilis* (control and grown on AsA + $CuCl_2$ media) were applied on SDS-PAGE 10 % to show the changes occured in membrane proteins (Graham and Higgins, 1997; Bollag and Edelstein, 1991; Laemmli, 1970).

60 μl membrane sample was put in each well. Samples were boiled for 3 minutes before put on the gel. Running buffer was composed of Tris-Glisin (pH 8.0). Electrophoresis were run at 220 V for 2 hours. Silver staining method was used to stain protein bands on the gel (Trpis et al., 1981; Merril et al., 1984).

RESULTS

Various concentrations of AsA(1 - 10 mM) and $CuCl_2$(50 - 100 μM) were tried to find subtoxic concentration that affect bacterial growth. The effective concentrations were determined for *B. subtilis* as 4 mM AsA+100 μM $CuCl_2$ and 8 mM AsA+100 μM $CuCl_2$ for *E. coli* (Figure 1, Figure 2). Viable cell counts were also made (Table1, Table2).

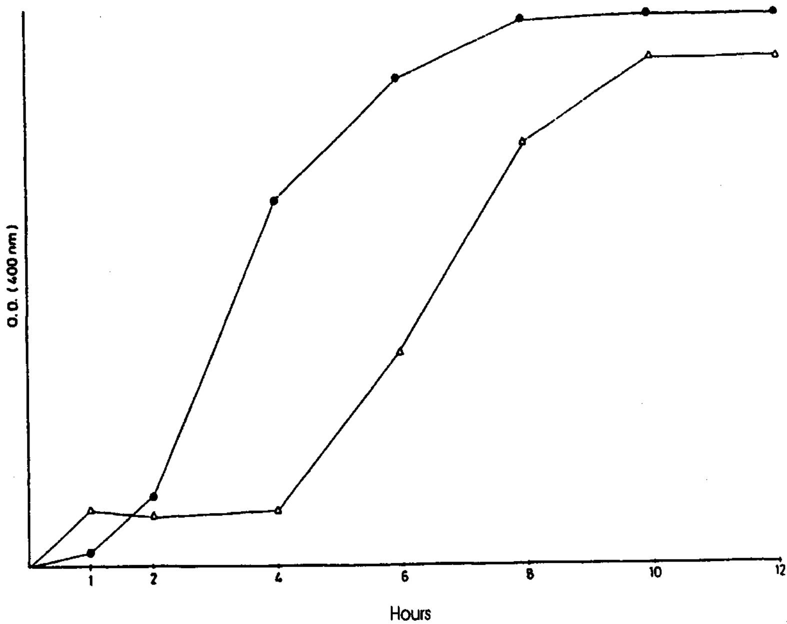

Figure 1. • Growth curve of *B. subtilis* (Control)
Δ Growth curve of *B. subtilis* in the presence of
100 µM $CuCl_2$ + 4 mM AsA
(Values are the average of three experiments)

Table 1. Viable cell counts of *B. subtilis* *(x10^5)*

Hours	Control	100 µM $CuCl_2$ + 4 mM AsA
1. Hour	13	8
2. "	77	20
4. "	895	70
6. "	3100	157
8. "	4715	639
10. "	7300	700
12. "	7500	750
	SD = 0.0085 $p< 0.05$	SD = 0.0071 $p< 0.05$

SD : Standart Deviation

13–(3371.42 ±0.0085)–7500 8–(334.85±0.0071)-750

(Values are the average of three experiments)

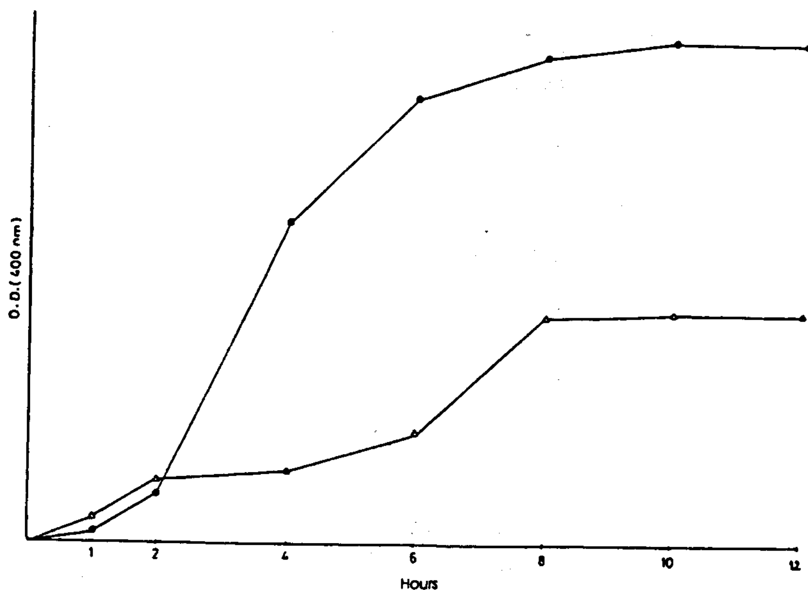

Figure 2. • Growth curve of *E. coli* (Control)
Δ Growth curve of *E. coli* in the presence of
100 μM $CuCl_2$ + 8 mM AsA

(Values are the average of three experiments)

Table 2. Viable cell counts of *E. coli* ($x10^5$)

Hours	Control	100 μM $CuCl_2$ + 8 mM AsA
1. Hour	51	10
2. "	154	20
4. "	5459	30
6. "	6944	137
8. "	9754	1712
10. "	13.491	1800
12. "	20.076	2100
	SD = 0.0071 $p< 0.05$	SD = 0.0071 $p< 0.05$

SD : Standard Deviation

51-(7989.85±0.0071)-20076 10-(829.85±0.0071)-2100

(Values are the average of three experiments)

Bacterial membranes were isolated by Lysozyme-EDTA method to show the effects of AsA/Cu (II) on membranes (Graham and Higgins, 1997; Copeland et al., 1982). These membranes were applied on SDS-PAGE (slab gel) to observe the changes on membrane proteins (Laemmli, 1970; Bollag and Edelstein, 1991; Graham and Higgins, 1997). Silver staining method was used. We found that some of the soluble protein bands lost in bacterial membranes isolated from bacteria grown in AsA/Cu (II) containing media (Figure 3).

Figure 3 . Gel profile of membrane proteins on 10 % SDS-PAGE gel.

1. Marker proteins
   Bovin serum albumin (M.W. 68.000), Lipase (M.W. 54.000)
2. *E. coli* (Control)
3. *E. coli* (Medium with AsA + $CuCl_2$)
4. Marker proteins
   Bovin serum albumin (M.W. 68.000), Lipase (M.W. 54.000)
5. *B. subtilis* (Control)
6. *B. subtilis* (Medium with AsA + $CuCl_2$)

DISCUSSION AND CONCLUSION

We investigated the antibacterial effect of AsA/Cu(II) in this study. It has been known that various and effective oxidation products of AsA occur in the presence of Cu (II) (Chiou, 1984). Therefore we chose $CuCl_2$ in our study.
The subtoxic concentration that affect bacterial growth 4mM AsA + 100 μM $CuCl_2$ for *B.subtilis* and 8 mM AsA + 100 μM $CuCl_2$ for *E. coli* were established. It seemed that *B.subtilis* was more susceptible to AsA/Cu (II) than *E. coli.*
We established that ascorbate and $CuCl_2$ has no effect by itself on bacteria.
It was determined that above 4 mM ascorbate was toxic for *B.subtilis* and above 8 mM ascorbate was toxic for *E.coli* in the presence of Cu (II).

The cell wall of Gram-positive and Gram-negative bacteria are structurally different. Cell wall of Gram-negative bacterium is thinner than Gram-positive one, but it has more complex structure. Therefore subtoxic dose of L-ascorbate on microorganisms is higher in Gram-negative bacteria than Gram-positives.

B. subtilis is a Gram-positive bacterium. Gram-positive bacterial cell wall contains teichoic acid. Teichoic acid has glucose units in its R group. AsA can take place glucose in teichoic acid because of structural similarity (Ngkeekwong, 1997). Therefore oxidation products of AsA concentrates locally in the cell wall. So, this can explain why *B. subtilis* is more susceptible to AsA/Cu (II) than *E. coli.*

We can say that oxidation products of L-ascorbate enter from porines in the outer membrane of Gram-negative bacterium. So, they cause damage in protein structure of porines. This cause disturbances in membrane transport. Therefore bacterial growth decreases. When the outer membrane is damaged, it can not protect inner membrane. So, cytoplasmic membrane is directly affected by oxidation products of ascorbate.

Loosening on outer membrane structure of Gram-negative bacterium formed by Ascorbate/Cu (II) might cause lost of binding proteins and detoxifying enzymes in periplasmic space. Nutrition transport decreased by losing binding proteins. Lost of detoxifying enzymes cause decrease in antibiotic resistance.
Some of the soluble protein bands of bacterial membranes lost that prepared from bacteria grown in AsA/Cu (II) containing media. It was showed that AsA/Cu (II) cause breaks in protein structure. So, we can attribute the loss of some protein bands to this effect of AsA/Cu (II).

It can be concluded that AsA/Cu (II) showed its antibacterial effect by causing damage in bacterial membranes.

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