Thesis for the Degree of Master of Science

The Mechanism of Non-thermal Plasma ion (NPi) Technology Sterilization Using *Escherichia coli*

by

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The Graduate School

Pukyong National University

February 2014

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저온 플라즈마 이온 (NPi) 을 이용한 *Escherichia coli* 의 살균 메카니즘

Advisor: Prof. Myung Suk Lee

by

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Contents

Abstract ····· 1

Introduction ····· 2

Materials and Methods ····· 4

1. Apparatus ····· 4
2. Preparation of microorganisms ····· 6
3. Plasma treatment ····· 6
4. Calculation of survival rate ····· 7
5. Cell LIVE/DEAD Kit ····· 7
6. Analysis of electron microscopy ····· 8
   6.1. FE-SEM (Field emission scanning electron microscope) ····· 8
   6.2. EF-TEM (Energy filtered transmission electron microscopy) ····· 8
7. Protein preparation ····· 9
8. Analysis of electrophoresis ····· 10
9. Identification of proteins (peptide mass fingerprinting) ····· 11

Results and discussion ····· 12

1. Calculation of survival rate ····· 13
2. Cell LIVE/DEAD Kit ····· 15
3. Analysis of electron microscopy ····· 17
4. Analysis of electrophoresis ····· 19
5. Identification of proteins (peptide mass fingerprinting) ····· 22

국문 초록 ····· 26

Acknowledgement ····· 27

References ····· 28

List of Tables

Table 1. Proteins differentially expressed in control and NPi irradiation 24

Table 2. Identified proteins showed changes in protein expression level 25

List of Figures

Fig. 1. The diagram of the test rig. ····· 5

Fig. 2. Effect of NPi treatment on the survival rate of bacterial cells in *Escherichia coli* cultures. The survival rate for each treatment was quantified by comparison with control cultures (100% survival). ····· 14

Fig. 3. Viability of *Escherichia coli* assessed using the LIVE/DEAD *Bac*Light Bacterial Viability Kit. (a) Control, (b) *E. coli* exposed to NPi for 60 min. ····· 16

Fig. 4. SEM and TEM images of *Escherichia coli*. (A) SEM photo of *E. coli* (a) control, (b) NPi-treated. (B) TEM photo of *E. coli* (a) control, (B) NPi-treated. ····· 18

Fig. 5. Analysis of the proteins expressed by using 12% SDS-PAGE. (a) Cytoplasmic proteins: lane M, standard protein molecular weight markers; lane 1, proteins (50 μg) from cell extracts (control); lane 2, proteins (50 μg) after 1 h of NPi exposure. (b) Membrane proteins: lane M, standard protein molecular weight markers; lane 1, proteins (30 μg) from membrane protein extracts (control); lanes 2, proteins (30 μg) following 1 hr (lane 2), 2 hr (lane 3), 3 hr (lane 4), 4 hr (lane 5), 6 hr (lane 6), 8 hr (lane 7) and 10 hr (lane 8) of NPi exposure. ····· 20

Fig. 6. Influence of NPi exposure on *Escherichia coli* cytoplasmic and membrane proteins, shown as a 2D PAGE profile. (A) Cytoplasmic proteins: (a) control, (b) 60 min of NPi exposure. (B) Membrane proteins: (a) control, (b) 60 min of NPi exposure. ····· 21

Fig. 7. Analysis of the variation in bacterial proteins by PMF. ····· 23

The Mechanism of Non-thermal Plasma ion (NPi) Technology Sterilization Using *Escherichia coli*

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Abstract

Indoor air quality is an important issue, since people spend considerable time indoors. Non-thermal plasma ion (NPi) technology is a new air-purification technique based on the production of positive and negative ions. In this study, denatured proteins from *Escherichia coli* were isolated and identified following NPi irradiation to investigate the mechanism by which bacteria are inactivated by an NPi generator. Based on the results, I propose that (i) positive and negative ions enter the bacterial cytoplasm; (ii) reactions between positive and negative cluster ions generate OH radicals; (iii) positive and negative cluster ions break down cytoplasmic proteins; (iv) bacteria cannot protect themselves from oxidative stress; (v) these processes also affect cell membrane proteins; thus, (vi) denatured bacterial membrane proteins cannot be repaired; and (vii) as a result, the osmotic pressure cannot be maintained, and the bacteria are killed.

Introduction

Indoor air quality has become progressively important, since people spend a large part of their time indoors. Many atmospheric pollutants found in indoor environments－including chemical agents, house dust, and microorganisms－access the body through inspiration (Anderson *et al*., 2004) and can cause building-related illnesses in humans (Brook and Frazier, 1998; Cayuela, 1995). Dales *et al*. (1998), have shown that approximately 10% of all colds are acquired in an indoor environment, with the high-risk group consisting of both young and old people. As a result, indoor air purification is now recognized as an important issue. Improved sterilization and disinfection technologies are constantly being investigated by the medical, food-processing, and air-conditioning industries. However, such technologies must conquer several problems, including high costs, thermal sensitivity, and the formation of toxic by-products. Recently, new air-purification technology has been developed, which is based on the production of positive and negative ions (Dales *et al*., 1991; Digel *et al*., 2005; Kayes *et al*., 2007; Lee *et al*., 2006; Venezia *et al*., 2006). Non-thermal plasma ion (NPi) technology uses an electronic device that produces positive and negative ions surrounded by a water shell (Fernandez *et al*., 1998; Grinshpun *et al*., 2007). The respective hydrated ions deposit on micro-particles (e.g., bacteria or odor-causing molecules), decompose, and chemically deactivate them (Kim *et al*., 2010; Rupf *et al*., 2010). The use of non-thermal plasma for

biological, chemical, and plasma decontamination, in medicine (Moisan *et al*., 2001), as well as electric discharges for plasma decontamination, have been studied and reported by various groups (Lebaron *et al*., 1998).

In the present study, I focused on the isolation and identification of denatured proteins from *Escherichia coli* following NPi irradiation. The resulting data provide insight into the antibacterial effects of air-borne plasma generated by cluster ions and may help to produce more efficient air-cleaning devices.

Materials and methods

1. Apparatus

The majority of experiments were carried out in an experimental chamber (0.005 m^3; 0.15 × 0.22 × 0.15) as shown in Fig. 1. Cluster air ions were generated with an NPi generator (LG Electronics). The device was located at the bottom of the chamber and attached to the chamber at half-height. Preliminary tests were run every 30 min; the chamber ion yield reached saturation.

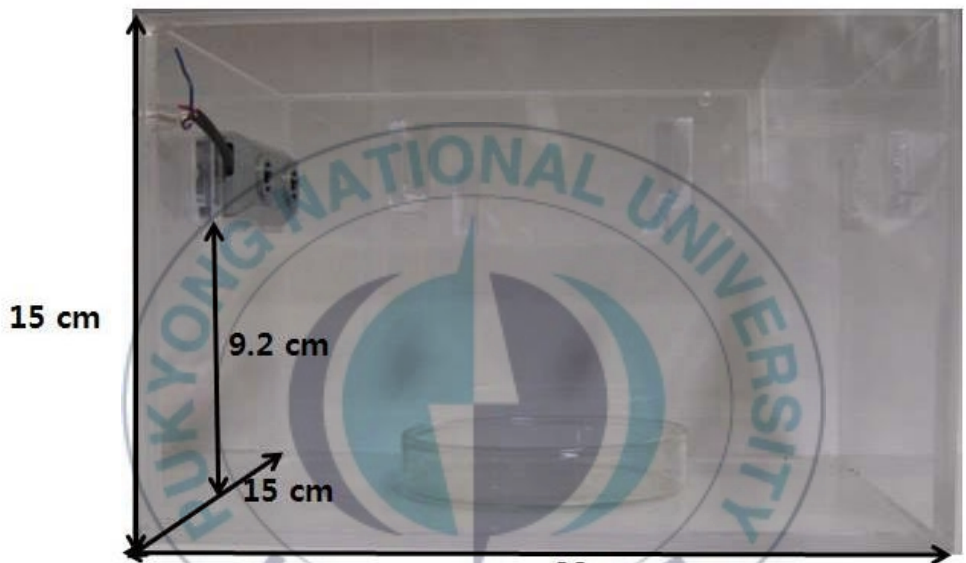

Fig. 1. The diagram of the test rig.

2. Preparation of microorganisms

Microorganisms used in the current study were Gram-negative bacteria (*Escherichia coli*), which were grown on brain heart infusion medium (BHI; Difco, USA) at 37°C. The overnight culture (500 µl) was transferred into 5 ml of fresh medium and incubated with shaking until the OD_{600} reached 0.5. Cells were then serially diluted in saline to concentrations of 10^2 to 10^6 colony forming units (CFU)/ml. One milliliter of each diluted sample was filtered through a 0.45 µm nitrocellulose membrane filter (25 mm diameter; Millipore, USA) by using a vacuum manifold (BioRad, USA), and the filtrate was air-dried.

3. Plasma treatment

Microorganisms on a filter were exposed to NPi for various time periods. During treatment, samples that had not been exposed to plasma were used as controls and kept at room temperature. After treatment, each filter was overlaid onto fresh BHI agar for *E. coli* and incubated at 37°C. The number of colonies that appeared on each filter was counted after an overnight incubation.

4. Calculation of survival rate

2

The survival rate (or death value) was defined as the time required achieving a 20% reduction of the initial numbers at the specific plasma treatment condition. The survival rate for *E. coli* was obtained by averaging the numbers obtained using various initial concentrations of cells.

5. Cell LIVE/DEAD Kit

Experiment was conducted in accordance with the manual of the cell LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, USA). Concentrate of plasma treatment *E. coli* by centrifugation at 10,000 × g for 15 min. Remove the supernatant and resuspend the pellet in 2 ml of 0.85% NaCl or appropriate buffer. Add 1 ml of this suspension to each of two 30-40 ml centrifuge tubes containing either 20 ml of 0.85% NaCl or appropriate buffer. Incubate both samples at room temperature for 1 hr, mixing every 15 min. Pellet both samples by centrifugation at 10,000 × g for 15 min. Resuspend the pellets in 20 ml of 0.85% NaCl buffer and pellet both samples by centrifugation at 10,000 × g for 15 min (Repeated 3 times). Add 3 μl of the dye mixture for each ml of the bacterial suspension. When used at the recommended dilutions, the reagent mixture will contribute 0.3% DMSO to the staining solution. Higher DMSO concentrations may adversely affect staining. Mix thoroughly and incubate at room temperature in the dark for 15 min after Observe in a fluorescence microscope.

6. Analysis of electron microscopy

6.1. FE-SEM (Field emission scanning electron microscope)

E. coli cells were fixed with a primary fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 mol/L Na-Cacodylate buffer, pH 7.2) for 30 min. The samples were then rinsed thrice with ultrapure water, followed by dehydration with a series of ethanol solutions (10%, 30%, 50%, 70%, 90% and 100%). The dehydrated samples were dried immediately by critical point dryer (Auto-Samdri 815 Automatic Critical Point Dryer; Tousimis, Rockville, MD, USA), followed by mounting onto SEM stubs and sputter-coating (BAL-TEC/SCD 005 Sputter Coater; Balzers Union AG, Balzers, Liechtenstein) with a thin layer of carbon. The coated samples were observed under FE-SEM (SUPRA 55VP FE-SEM; Carl Zeiss Ltd, Germany)

6.2. EF-TEM (Energy filtered transmission electron microscopy)

E. coli cell with ZnO NP were incubated overnight at 37°C and then placed into the primary fixative and microwaved (MW) under vacuum conditions in a Pelco Biowave (Ted Pella, Inc., Redding, CA, USA) at 120 W. The samples were rinsed several times with 0.1 mol/L cacodylate buffer and embedded in histogel, secondary MW fixation with a buffered (0.1 mol/L of cacodylate, 0.01 mol/L of 2-mercaptoethanol, 0.13 mol/L of sucrose) 1% osmium tetroxide. Then, the samples were quickly rinsed thrice with 0.1 mol/L of cacodylate, 0.01 mol/L of 2-mercaptoethanol, 0.13 mol/L

of sucrose and then rinsed thrice (5 min each) with ultrapure water, followed by dehydration with ethanol solutions (20%, 50%, 70%, 90% and 100%) and 100% acetone solution. The bacterial samples were infiltrated with Spurr's resin and polymerized at 60°C for 24 h. The sample blocks were processed into 85 nm thin sections with Microtome (HM 340E Microtome, MICROM Lab., Germany). The sections were placed onto 200 mesh thin bar grids and poststained for 20 min with 5% uranyl acetate and 10 min with Sato's triple lead stain. The stained samples were observed in EF-TEM (LIBRA 120 EF-TEM; Carl Zeiss, Germany) operating at 120 kV (Liu Y *et al*., 2009).

7. Protein preparation

Cultured bacterial cell pellets were washed twice with ice-cold PBS (pH 7.0) and sonicated for 10 s with a Sonopuls (BANDELIN electronic, Germany). The sample lysis solution consisted of 7 M urea; 2 M thiourea containing 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), and 2% (v/v) pharmalyte; and 1 mM benzamidine. The freeze-thaw step for yeasts or bacilli samples was repeated 5 times for 1 day. Occasionally, a bead beater was used in order to lyse rigid cells. Proteins were extracted for 1 h at room temperature with vortexing. After centrifugation at 15,000 rpm for 1 h at 15°C, insoluble material was discarded and the soluble fraction was used for two-dimensional gel electrophoresis. Protein concentration was assayed by the Bradford method.

8. Analysis of electrophoresis

IPG dry strips (4-10 NL IPG, 24 cm; Genomine, Korea) were equilibrated for 12-16 h in 7 M urea and 2 M thiourea containing 2% CHAPS, 1% DTT, and 1% pharmalyte, and subsequently loaded with 200 μg of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences) according to the manufacturer' instructions. For IEF, the voltage was linearly increased from 150 to 3,500 V during 3 h to allow sample entry, followed by a constant voltage of 3,500 V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 min in equilibration buffer (50 mM Tris-HCl (pH 6.8) containing 6 M urea, 2% SDS, and 30% glycerol), first with 1% DTT, and second with 2.5% iodoacetamide. Equilibrated strips were inserted into SDS-PAGE gels (20 × 24 cm; 10–6%), and SDS-PAGE was performed using the Hoefer DALT 2D system (Amersham Biosciences) as per the manufacturer' instructions. The 2D gels were run at 20°C for 1,700 Vh and subsequently stained with Coomassie G-250, as described by Anderson et al. Quantitative analysis of digitized images was carried out using the PDQuest (version 7.0; BioRad) software according to the protocols provided by the manufacturer. The quantity of each spot was normalized to the total valid spot intensity. Protein spots were selected for significant expression variation that deviated over 2-fold in expression level compared with the control or normal samples.

9. Identification of proteins (peptide mass fingerprinting)

For protein identification by peptide mass fingerprinting (PMF), protein spots were excised, digested with trypsin (Promega, USA), mixed with α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA, 114 and subjected to MALDI-TOF analysis (microflex LRF 20; Bruker Daltonics), as described Fernandez et al. Spectra were collected using 300 shots per spectrum over an m/z range of 600–3000 and calibrated by two-point internal calibration using trypsin auto digestion peaks (m/z 842.5099, 2211.1046). The peak list was generated using Flex Analysis 3.0. The threshold used for peak-picking was as follows: 500 for minimum resolution of monoisotopic mass and 5 for the signal:noise ratio (S/N). The search program MASCOT, developed by Matrix Science (http://www.matrixscience.com/), was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of 1 missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. PMF acceptance criteria is probability scoring.

Results and discussion

Non-thermal plasma produces negative and positive ions from water vapor in indoor air (Radetic M *et al.*, 2000). Since these ions form clusters around micro-particles, they may be detrimental to airborne microorganisms and allergens. In addition, due to chemical reactions that take place on the surface of cell membranes, negative and positive ions are transformed into OH radicals-more active and unstable radicals-which extract hydrogen atoms (H) contained in harmful substances (Shashurin A e*t al*., 2008). This results in damage to membrane proteins, thereby opening holes in the membrane and inactivating cells. The OH radicals instantly bond with the extracted hydrogen (H), form water vapor (H_2O), and return to the air (Stoffels EKI *et al*., 2006). Proteins are essential for carrying out important functions of cells and microorganisms. In particular, the cell membranes of bacteria are composed of protein (approximately 70% of the membrane weight). In addition, the structure of viruses consists of DNA surrounded by a protein envelope. Therefore, I aimed to analyze denatured proteins from *E. coli* following NPi treatment.

1. Calculation of survival rate

In total, 5 ml of the *E. coli* culture (with a cell count of 10^9-10^{10} CFU/ml in logarithmic growth phase) was exposed to NPi irradiation for different periods of time (20, 40, 60, 80, 100, and 120 min) at a distance of 10 cm. The relationship between the survival rate of the bacteria and NPi irradiation time is shown in Fig. 2, which reveals that the survival rate decreased with increasing NPi exposure time. The lowest survival rate of nearly 0% was obtained when the cells were exposed to NPi for 120 min.

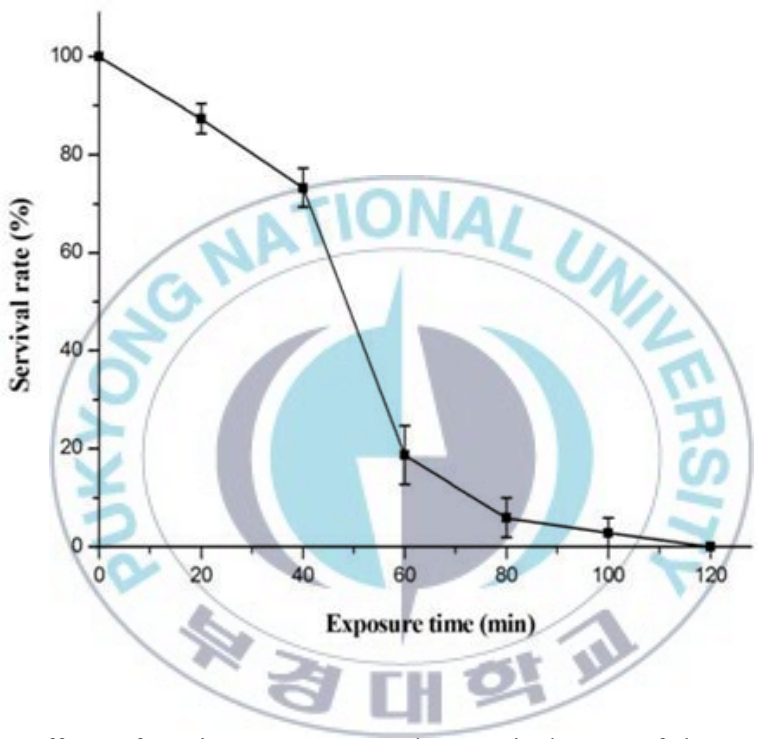

Fig. 2. Effect of NPi treatment on the survival rate of bacterial cells in *Escherichia coli* cultures. The survival rate for each treatment was quantified by comparison with control cultures (100 % survival).

2. Cell LIVE/DEAD kit

The novel two-color fluorescence assay, LIVE/DEAD BacLight Bacterial Viability Kit, has recently been successfully used to analyze a variety of bacteria (Thomas CW *et al.*, 2005). Thus, I used this kit to assess the viability of bacteria. *E. coli* cells were treated with NPi on the agar medium surface for up to 60 min. After irradiation, cells were extracted from the surface by using an inoculating loop, subsequently dissolved in liquid stain, and analyzed by fluorescence microscopy. In Fig. 3, green and red fluorescence indicate live and membrane- damaged bacteria, respectively. The results obtained show that the membranes of nearly 90% of bacteria are compromised after 60 min of plasma exposure.

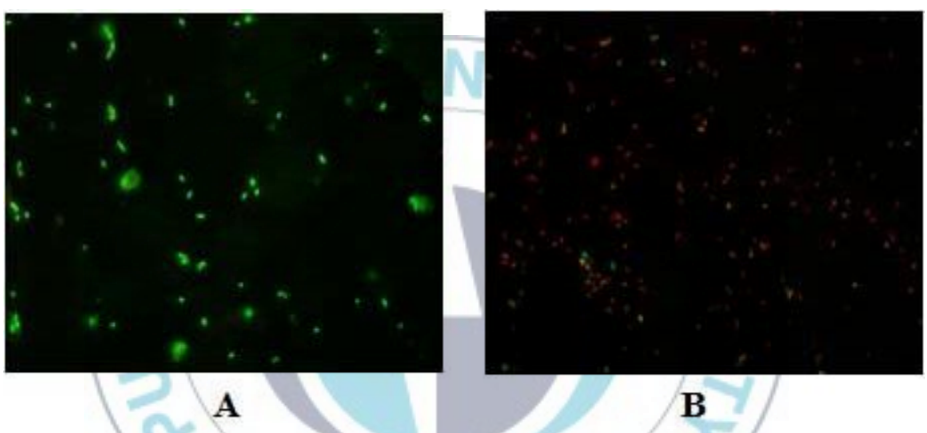

Fig. 3. Viability of *Escherichia coli* assessed using the LIVE/DEAD *Bac*Light Bacterial Viability Kit. (a) Control, (b) *E. coli* exposed to NPi for 60 min.

3. Analysis of electron microscopy

To determine bacterial cell death, electron microscopy analysis was used; scanning electron microscope (SEM) pictures of *E. coli* were taken before and after 60 min of NPi treatment (Fig. 4A). Figure 4B is a transmission electron microscope (TEM) image of *E. coli* after 60 min of NPi exposure. The electron microscopy images of *E. coli* showed the breakage of cell membranes and outflow of the internal cytoplasm. Therefore, it was concluded that NPi treatment inactivates bacteria.

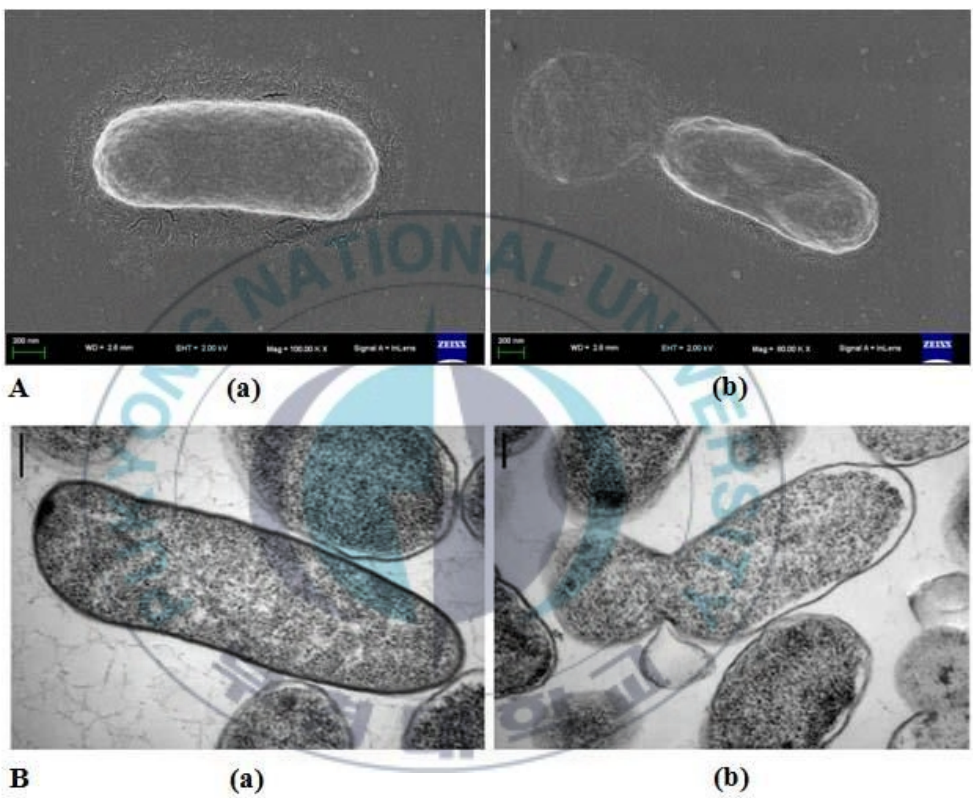

Fig. 4. SEM and TEM images of *Escherichia coli*. (A) SEM photo of *E. coli* (a) control, (b) NPi-treated. (B) TEM photo of *E. coli* (a) control, (B) NPi-treated.

4. Analysis of electrophoresis

In order to determine changes in proteins following NPi irradiation, SDS-PAGE and 2D-PAGE were used. After exposure of *E. coli* to NPi irradiation for 60 min, I extracted the cytoplasm and membrane proteins and used SDS-PAGE electrophoresis to measure their molecular weight distribution. The pattern of cytoplasm proteins is shown in Fig. 5a, which reveals that NPi irradiation caused silver staining to disappear for 58 kDa proteins and to appear for 23 kDa proteins. This change indicates that proteins with a molecular weight of 58 kDa were broken down into proteins with a molecular weight of 23 kDa. The pattern of membrane proteins is shown in Fig. 5b, which shows that NPi irradiation caused silver staining to disappear for 90 kDa proteins and to appear for 18 kDa and 25 kDa proteins. This result indicates that proteins with a molecular weight of approximately 90 kDa were broken down into proteins with molecular weights of 18 and 25 kDa under 4hr NPi irradiational exposure or more. At least, Figure 6 shows the results of 2D electrophoresis to map these cytoplasmic (Fig. 6A) and membrane (Fig. 6B) proteins. Some clear changes were definite by visual inspection. An important difference was observed between the control (Fig. 6A-a, Fig. 6B-a) and NPi-irradiated (Fig. 6A-b, 6B-b) samples. I have found that the expression level of the protein is reduced samples were investigated NPi is compared to control. This result shows that NPi irradiation denatures both cell membrane and cytoplasmic proteins.

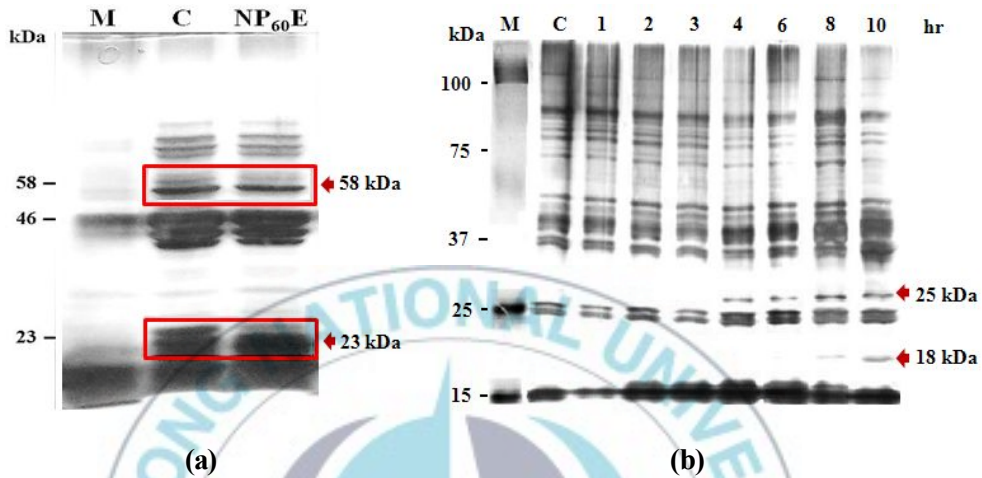

Fig. 5. Analysis of the proteins expressed by using 12% SDS-PAGE. (a) Cytoplasmic proteins: lane M, standard protein molecular weight markers; lane 1, proteins (50 μg) from cell extracts (control); lane 2, proteins (50 μg) after 1 hr of NPi exposure. (b) Membrane proteins: lane M, standard protein molecular weight markers; lane 1, proteins (30 μg) from membrane protein extracts (control); lanes 2, proteins (30 μg) following 1hr (lane 2), 2 hr (lane 3), 3 hr (lane 4), 4 hr (lane 5), 6 hr (lane 6), 8 hr (lane 7) and 10 hr (lane 8) of NPi exposure.

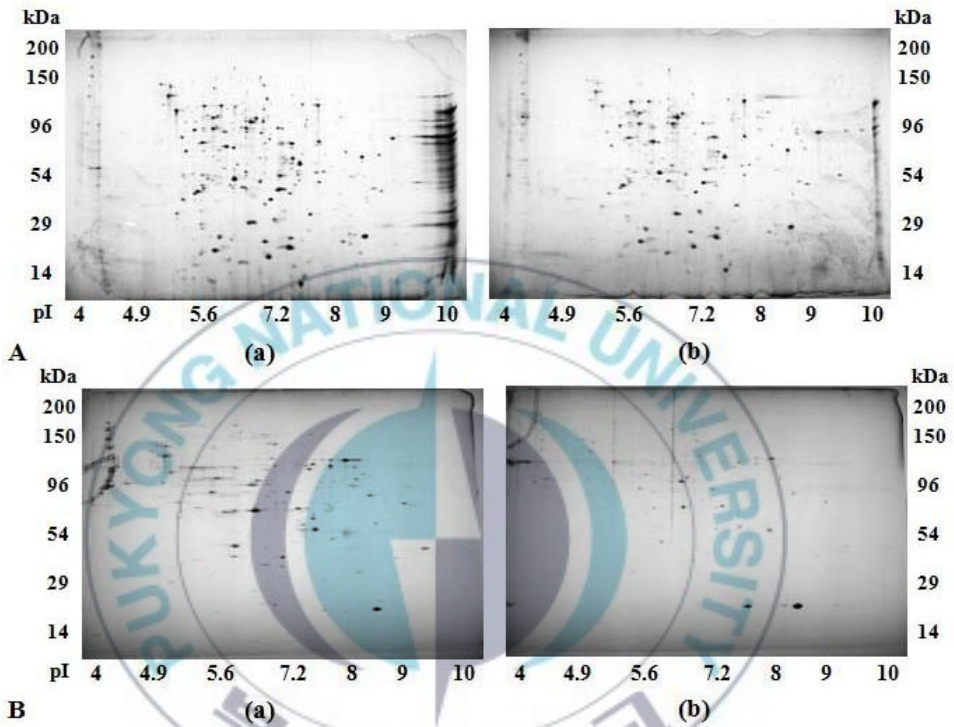

Fig. 6. Influence of NPi exposure on *Escherichia coli* cytoplasmic and membrane proteins, shown as a 2D PAGE profile. (A) Cytoplasmic proteins: (a) control, (b) 60 min of NPi exposure. (B) Membrane proteins: (a) control, (b) 60 min of NPi exposure.

5. Identification of proteins (peptide mass fingerprinting)

In order to identify the denatured proteins in *E. coli* following NPi irradiation, I used PMF. The variation in bacterial proteins shown by PMF analysis is presented in Fig. 7. A total of 32 protein spots showing a decrease in the intensity of about two times. These differentially expressed proteins are summarized in Table 1. Among them, 27 spots were identified (Table 2). They include DNA starvation/stationary phase protection protein Dps, Ion/manganese superoxide dismutase SOD, Outer membrane protein Tsx, and Lysine-arginine-ornithine-binding periplasmic protein. These results should contribute to our understanding of the mechanisms inherent to NPi-induced sterilization.

In conclusion, the result elucidates that positive and negative ions produced from NPi generator induce inactivation of bacteria by: (i) Positive and negative ions enter the bacterial cytoplasm; (ii) Reactions between positive and negative cluster ions generate OH radicals; (iii) Positive and negative cluster ions break down cytoplasmic proteins; (iv) Bacterial cells cannot protect themselves from oxidative stress. (v) These processes also affect the bacterial cell membrane proteins; (vi) As a result, the denatured bacterial membrane proteins cannot be repaired; (vii) The osmotic pressure cannot be maintained, and the bacteria are killed.

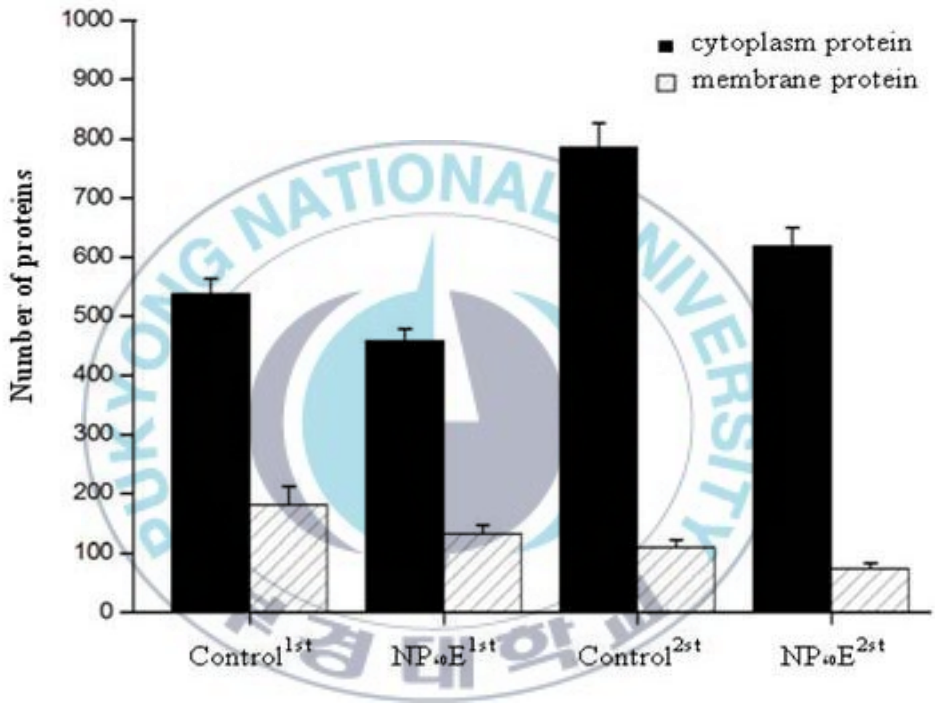

Fig. 7. Analysis of the variation in bacterial proteins by PMF.

Table 1. Proteins differentially expressed in control and NPi irradiation

Spot No.	MW/pI	Spot intensity	
		Control (avg)	$NP_{60}E$ (avg)
2101R	18.66 / 4.61	1265.98	1
2110R	22.84 / 4.98	1028.56	685.43
2203R	31.91 / 4.54	236.58	1
2302R	41.44 / 4.66	1299.45	573.64
3207R	35.23 / 5.21	1258.93	679.54
3209R	35.25 / 5.25	624.10	534.605
3301R	38.77 / 4.68	485.81	140.03
3310R	40.13 / 5.25	1019.47	684.3
3810R	144.26 / 5.25	2540.00	718.23
4102R	26.68 /5.12	2906.52	204.48
4106R	12.26 / 4.97	1221.57	161.165
4305R	38.99 / 5.12	1682.52	160.61
5101R	30.15 / 5.51	1717.27	624.785
5105R	16.41 / 5.28	812.66	1
5502R	54.15 / 5.47	2540.08	621.13
5810R	109.90 / 5.61	906.12	376.745
6110R	13.52 / 5.45	1665.14	290.45
6113R	23.24 / 5.51	662.45	103.895
6114R	26.38 / 5.52	2846.08	381.78
6202R	35.29 / 6.06	1194.65	409.305
6203R	36.63 / 6.16	1036.21	527.695
6306R	44.79 / 5.56	1831.96	589.57
6403R	47.94 / 5.98	1644.74	711.365
6603R	74.10 / 5.45	954.40	132.62
7108R	18.94 / 6.38	525.48	43.125
7205R	36.74 / 6.10	1625.33	425.86
7207R	33.90 / 6.15	4103.20	998.145
7301R	39.65 / 5.73	2338.60	1172.745
7307R	39.29 / 6.55	387.65	191.9
8111R	29.25 / 7.02	391.13	21.465
9103R	14.34 / 7.76	558.14	171.99
9104R	26.03 / 8.17	2769.05	1128.78

Table 2. Identified proteins showed changes in protein expression level

Number	Protein name	Function
2101R	Thiol peroxidase	Oxidative stress and defense mechanisms
4106R	Universal stress protein A	
6110R	Osmotically inducible Protein C	
6603R	Trasnscriptional regulator Bol A	
7108R	DNA starvation/stationary phase protection protein Dps	
9104R	Ion/manganese superoxide dismutase SOD	
2203R	Outer membrane protein Tsx	Osmoregulation and transportation
3207R	Lysine-arginine-ornithine-binding periplasmic protein	
3310R	Aerobic respiration contral protein arc A	
7205R	Galactofuranose binding protein	
8111R	Glutamine ABC transporter periplasmic protein	
3301R	Ribokinase	Metabolic pathway
3810R	2-methylisocitrate	
5101R	Acetyl-CoA synthase	
5810R	NADH; ubiquinone oxidoreductase, Chaine E	
6114R	KDPG and KHG aldolase	
6306R	Transcriptional regulator BolA	
6403R	2-deoxy-D-gluconate 3-dehydrogenase	
7207R	4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase	
7301R	Malate dehydrogenase	
5102R	Glycine cleavage system T protein	Cells essential substances and vitamin synthase
6202R	Riboflavin (vitamin B2) synthase alpha subunit	
6203R	Peptidase E	
7307R	Cystein synthase A	
9103R	*rplI*geneproduct	
5105R	Predicted hydrolase, isochorismatase family	Unconfirmed protein
4102R	Hypothetical proteion ECS 5299	

국문 초록

최근 사람들의 실내에서 활동하는 시간이 증가함에 따라 실내 공기 정화는 중요한 문제로 인식 되고 있다. 공기정화와 오염원을 제거하기 위해 다양한 방법들이 개발되고 있으며 특히 인체에 무해한 저온 플라즈마 이온 (NPi)을 이용한 공기 정화법이 활발히 연구 되고 있다. 저온 플라즈마 방식에 의해 생성되는 다양한 양이온과 음이온 중 특히 수산화 이온이 공기 중 미생물에 대하여 높은 살균력을 나타낸다고 알려져 있다.

본 연구에서는 저온 플라즈마 이온이 대장균에 미치는 살균 메카니즘을 규명하고자 하였다. 먼저 이온 조사 시간에 따른 대장균의 살균력을 측정하여 생존율을 정량적으로 나타내었고, 형광 현미경 (LIVE/DEAD Kit)과 전자현미경 (SEM, TEM)으로 사멸된 대장균을 직접 관찰 하였다. 그리고 세포막, 세포질 단백질을 분리하여 SDS-PAGE, 2D-PAGE, PMF 단백질 동정 과정을 거쳐 변성이 일어난 단백질의 기능을 분석 하였다.

그 결과, 60분간 이온 조사한 대장균을 전자현미경으로 관찰하였을 때 세포막에서 세포질이 흘러나오는 형태로 관찰 되었다. 이는 저온 플라즈마 이온이 세포 내에 침투하여 바이오 라디칼을 생성하게 되면 세포질 단백질에 변성작용을 일으켜 산화적 스트레스로부터 자신을 보호할 수 있는 물질 분비가 억제 되고, 대장균 세포의 자가 회복 능력을 상실하여 사멸된 것으로 추정된다.

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2009년부터 약 5년간 생활하면서 제가 부족함에도 도움 많이 주시고 지금 이 자리에 있게 해준 일반 미생물 실험실의 많은 선배님 후배님들, 특히 실험하는데 많이 도움 주신 서용배 박사님, 2년동안 고생 많이 한 선영이, 앞으로 고생할 승용이, 앞으로 더 고생할 예지, 약 1년 동안 프리폴에 일하면서 많은 업무 노하우 전수해주신 정희록 이사님, 유일하게 05학번 동기가 많았던 생태방 친구들 영삼이, 동현이, 은수, 프리폴에 일할 때 일손 부족하면 많은 도움 줬던 생화학방 선후배님들, 그 외 바이러스방, 세포방, 유전방 선후배님들, 대학교 입학 05년부터 아직까지 우정 이어져가고 있는 우리 동기들 대규, 창배, 병하, 광모, 경영이, 성우, 현석이, 은수 그 외 기타 많은 분들 힘이 되어 줘서 고맙다고 전하고 싶습니다.

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