보건학석사 학위논문

Cyto-/genotoxicity of N-nitrosodimethylamine in human lymphocytes

사람 림프구에서 N-nitrosodimethylamine 의 세포 유전독성 평가

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보건학과 보건학전공

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지도교수 정해원

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김 계 은

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위 원 장 정 효 지 (인)

부 위 원 장 조 성 일 (인)

위 원 정 해 원 (인)

Abstract

Cyto-/genotoxicity of N-nitrosodimethylamine in human lymphocytes

Kim, Gye Eun
Department of Molecular Epidemiology
Graduate School of Public Health
Seoul National University, Korea

Directed by professor Hai Won Chung, Ph.D.

N-nitrosodimethylamine (NDMA), present in many foodstuffs such as cured meat, cooked ham, smoked fish, and alcoholic beverages. It is known to be a potent carcinogen and to cause acute hepatotoxicity.

NDMA requires metabolic activation to exert its mutagenicity and carcinogenicity. Cytochrome P450 2E1 (CYP2E1) plays a major role in this activation. CYP2E1 is mainly included in hepatocytes, in which it has been commonly

studied regarding NDMA-induced toxicity. CYP2E1 was also found in human lymphocytes, but little is known about whether NDMA has harmful effects in this type of cells. In the present study, the cyto-/genotoxicity of NDMA on human lymphocytes was found by trypan-blue assay, single-cell gel electrophoresis (comet) assay, cytokinesis-block micronucleus (CBMN) assay and flow cytometry.

Exposure of cells to various concentrations of NDMA (10, 25, 50, 100, and 150mM) for 48 hr significantly decreased cell viability and induced DNA damage resulting from DNA strand breaks in a dose dependent manner, but the frequency of MN is not increased in human lymphocytes.

Moreover, I found that Hypodiploid sub-G1 apoptosis peak showed no change, suggesting that NDMA is not related to apoptotic cell death. However, NDMA caused cell-cycle arrest at Go/G1 phase, and cytokinesis-block proliferation index (CBPI) decreased 20% in 50mM NDMA-treated cells and 40% in 150mM NDMA-treated cells compared to the control, respectively. In addition, intracellular ROS levels were increased with concentrations of NDMA. Pre-treatment of human lymphocytes with N-acetylcysteine (NAC), direct ROS

scavenger, reduced NDMA-induced ROS release and DNA damage, and it also restored cell proliferation and decreased G1 phase cell arrest. Through these result, I found the protective effect of NAC against NDMA-induced toxic effects in human lymphocytes.

In conclusion, NDMA induces DNA damage via ROS generation, which triggers cell-cycle arrest in human lymphocytes. Taken together, the results of this study suggest that NDMA-mediated cyto-/genotoxicity in human lymphocytes through generation of ROS.

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Keywords : N-nitrosodimethylamine (NDMA); Lymphocytes;
DNA damage; Reactive oxygen species (ROS);
Cell-cycle arrest

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I. Introduction

Occupational and environmental exposure to pre-formed N-nitroso compounds (NNC), as well as endogenous exposure is considered to be a potential health hazard to humans (Arranz, Haza et al., 2008; Mirvish, 1995). N-nitrosamines, known N-nitroso compounds, are one of the potent food-derived genotoxins. In particular, N-nitrosodimethylamine (NDMA), which is the simplest stable and the most extensively occurring nitrosamine, is known to be an acute hepatotoxin and potent carcinogen in many animal species as well as humans (Lin, Parsels et al., 1999; Sugimura, 2000). It can be formed endogenously from the interaction of ingested nitrate and nitrite with secondary amine (dimethylamine) in the stomach and/or other body sections (C.D. leaf, 1989; Marletta, 1988). Also, it can be generated in food products through the process of heating and/or drying of foods using combustion gas (Kyrtopoulos, 1998; Lijinsky, 1999; Marletta, 1988; Tricker and Preussmann, 1991).

NDMA is present in many foodstuffs such as cured meat, cooked ham, smoked fish, alcoholic beverages, smoking and

cosmetics. Human couild be exposed to NDMA through ingestion, inhalation, and dermal contact. Also, workers in rubber industry are exposed to NDMA due to the extensive use of vulcanization agents (De Stefani, 1996; Dich, Jarvinen et al., 1996; Oury, Limasset et al., 1997; Tricker and Preussmann, 1991; Wainwright, 1986).

The chronic exposure to low doses of NDMA induced tumors of liver and esophagus in rat and mouse (Magee and Barnes, 1956; Peto, Gray et al., 1991; Peto, Gray et al., 1991).

A number of epidemiological studies have yielded results compatible with a carcinogenic role of NDMA in humans. Knekt et al (1999) found positive correlation between intake of NDMA and occurrence of colorectal cancer in population through a long term follow-up study, and consumption of processed meat, smoke and beer was associated with a higher risk of lung cancer. Salted meat increased the risk of oropharyngeal, laryngeal cancer in humans (De Stefani, Correa et al., 1993; De Stefani, Oreggia et al., 1995; De Stefani, Oreggia et al., 1994; Goodman, Hankin et al., 1992; Potter, 1990). Also, it has been reported that exposure to high concentrations of NDMA was linked with increased mortality from cancers of the oesophagus, oral cavity

and pharynx through cohort studies of rubber workers (Oury, Limasset et al., 1997; Straif, Weiland et al., 2000; Tsutsumi, Matsuda et al., 1993; Vocht, Burstyn et al., 2007).

In order to exert its mutagenicity and carcinogenicity in cells, NDMA requires metabolic activations, and cytochrome P450 2E1 (CYP2E1) plays a major role in this activation (Anundi and Lindros, 1992; Schulze, 1999). It has been suggested that NDMA metabolized by CYP2E1 can cause oxidative stress and cellular injury by the generation of reactive oxygen species (ROS). These oxidative attack leads to the generation of DNA strand breaks and formation of DNA base adducts such as O^6-meG, N^7-meG, and it causes cell damage both in vivo and in vitro (Bartsch, Hietanen et al., 1989; Korr, Botzem et al., 2001; Tunc and Tremellen, 2009).

It has been known that hepatocytes, cells of the main tissue of the liver, are a major source of CYP2E1 (Lin and Lu, 1998; Nagai, Hiyoshi et al., 2002). Precedent studies on toxicity of NDMA were carried out using liver cell become it could activate NDMA through expression of CYP2E1. Valentin-Severin et al (2003) and Helma et al (2000) reported genotoxic effect of NDMA in HepG2 cell, human derived hepatoma cell line. Cytotoxicity

and mutagenicity of NDMA were also reported in V79 cells (Kuroki, 1977; Uhl, Helma et al., 2000; Valentin-Severin, Le Hegarat et al., 2003).

Recent study reported that CYP2E1 is also found in human lymphocytes (Dey, Parmar et al., 2002; Nagai, Hiyoshi et al., 2002; SONG, VEECH et al., 1990). And toxic effect of NDMA could be also expected, but little known about the toxicity of NDMA in blood cells.

The objection of the study was to find whether NDMA could induce cyto-/genotoxic effect in human lymphocytes. In this study, I assessed the cytotoxic effect of NDMA by flow cytometry assay and the genotoxic effect of them by using alkaline single cell gel electrophoresis (Comet) assay and cytokinesis-block micronucleus (CBMN) assay in human lymphocytes.

II. Materials and Methods

2.1. Isolation and Culture of Lymphocytes from Human Blood

Human Blood was obtained from healthy, non-smoking female adult donor (26 years old) and collected in heparinized tubes. Peripheral Blood Lymphocytes were separated by a modified method of Boyum (Boyum, 1976). In brief, lymphocytes were isolated by buoyancy density centrifugation at 400 × g for 30 min at room temperature, using Ficoll-Paque™ (Amersham Biosciences, Uppsala, Sweden) in the dark, removed from the interface, washed twice with phosphate buffered saline (PBS) and resuspended in RPMI-1640 (Gibco, Paisley, UK) containing 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) and 100 U/ml penicillin and streptomycin (all from Sigma, St. Louis, MO, USA). The cultures were stimulated with 1% phytochemagglutinin (PHA) (Gibco, Paisley, UK) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.2. Chemicals

N-nitrosodimethylamine (NDMA) was purchased from Wako Pure Chemicals Industries Ltd (Japan). This chemicals was dissolved in distilled water at the concentration of 10M as stock solution. The stock solutions were stored at -80°C. All other chemicals and solvents used were the highest purity available from commercial sources.

2.3. Cell Viability assay

Cell viability was determined by trypan blue dye exclusion. Lymphocytes were exposed to various concentrations (0, 10, 25, 50, 100, 150mM) of NDMA for 48hr at 37°C. An equal volum of 0.4% trypan blue reagent was added to a cell suspension and the percentage of viable cells evaluated under a field microscope.

2.4. Single–cell gel electrophoresis (Comet) assay

The comet assay was performed under alkaline conditions according to the procedure of Singh et al (Singh, McCoy et al., 1988) with modifications. Lymphocytes were exposured to different concentration of NDMA for 12hr at 37°C and harvested. To prevent DNA repair, the process was maintained at 4°C. The cells were mixed with 85 µl 0.6% low melting point agarose dissolved in PBS and dropped onto fully frosted microscope slides precoated with 0.7% normal melting agarose. Following harden in the ice, another 85 µl of 0.6% low melting agarose was dropped on top layer. The cells were then lysed for 1 hr at 4°C in a buffer consisting of 2.5 M NaCI, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100, pH 10. After the lysis the slides were placed in an electrophoresis unit, allowing DNA to unwind for 20 min in the electrophoretic buffer consisting of 1 mM Na_2–EDTA, 300 mM NaOH, pH > 13. Eelectrophoresis was conducted at an electric field strength 22 V / 295 mA for 25 min. The slides were then neutralized with 0.4 M Tris, pH 7.5, three times and fixed by absolute ethanol.

To prevent additional damage, all the steps described above were conducted under a dimmed light or in the dark. The 60 images of cells from each slide were randomly selected and measured Olive tail moment (OTM) as an index of DNA damage using a fluorescence microscope (Nikon, Japan) equipped with an excitation filer of 515 to 560 nm and a barrier filter of 590 nm, analyzed using the KOMET 5.5 imaging program.

To determine the protective effect of antioxidants, lymphocytes were pretreated with 1 mM N-acetylcysteine (NAC; Sigma-Aldrich, St. Louis, MO, USA) for 1hr prior to 150 mM NDMA treatment. The concentration and treatment time were determined by three preliminary experiments.

2.5. Cytokinesis-block Micronucleus (CBMN) assay and Cytokinesis-block proliferation index (CBPI)

The cytokinesis-block micronucleus (CBMN) assay was carried out as described by Fenech et al (1993). Peripheral whole blood (1 ml) were cultured in 9 ml of RPMI 1640 medium containing 10% FBS. After 24 hr of culture, NDMA (0, 10, 25, 50,

100, 150 mM) was treated into the culture and after 20 hr of incubation, cytochalasin-B (4 μg/mL, Sigma-Aldrich) was treated into the culture, and the cells were incubated for another 28 hr. The cells were collected, added 0.075 M KCl hypotonic solution, fixed in methanol: acetic acid (3 : 1). The cells were dropped on the slide glass and stained with 5% Giemsa solution, examined using a microscope of 1000 magnifications. The score of binucleated cells and detection of micronuclei were followed as Fenech et al (Fenech, 2000). Cytokinesis block proliferation index (CBPI) was scored 1000 cells in between bi-to multi nuclei to measure the extent and progression of nuclear division in a dividing cell population. The CBPI value was determined by the standard calculation (Fenech, 1997).

2.6. Measurement of Reactive Oxygen Species (ROS)

Measurements of intracellular ROS were determined by using 5-carboxy-2', 7'-dichlorofluorescein diacetate (DCFDA, Molecular Probes, Eugene, OR, USA) as described by Wang et

al.(1999) (Wang and Joseph, 1999). Freshly prepared lymphocytes were seeded into 24 well-plates at a density of 1×10^5 cells per ml. The cells were treated with various NDMA concentrations (0, 10, 25, 50, 100, 150 mM) for 3 hr. After removing the medium, remained cells were added new culture medium containing 30μM DCFDA under a dimmed light condition and incubated for 25 min at 37°C. After DCFDA was removed, the cells were filled with PBS and measured for fluorescence distribution using flow cytometry (FACSCalibur, Becton-Dickinson, CA, USA). To confirm the protective effect of antioxidant, lymphocytes were pre-treated with 1 mM NAC for 1 hr before NDMA treatment.

2.7. Analysis of Apoptotic Cell Death and Cell Arrest

The Apoptotic cell death was investigated using propidium iodide (PI) staining as reported by Nicoletti et al. (1991) (Nicoletti, Migliorati et al., 1991). Freshly prepared lymphocytes were seeded into 24 well-plates at a density of 1×10^6 cells per ml. The

cells were treated with various NDMA concentrations (0, 10, 25, 50, 100, 150 mM) and/or NAC (to confirm the protective effect of antioxidant) for 48 hr. The cells were fixed in 70% ethanol, stored at 4°C for at least 24 hr. After removing the ethanol, cells were washed in PBS and then stained with PI staining solution containing RNase A (10 mg/ml), PI (10 mg/ml) in 10 ml of PBS in the dark at room temperature for 30 min. Cells were washed in PBS again, and analyzed by a flow cytometry (FACSCalibur, Becton-Dickinson, CA, USA). The cell arrest is also determined to confirm the identification of the cell distribution during the various phases of the cell cycle (G1, S, G2/M-phase) using flow cytometry.

2.8. Statistical Analysis

Statistical analysis was performed using the SPSS for Windows 9.05 package program. Data were compared by Mann-Whitney's *U* test. Multiple comparisons were performed by least significant difference (LSD) test. $P < 0.05$ was considered as level of significance.

III. Results

3.1. Cell Viability

Cytotoxicity of NDMA in cultured human lymphocytes was measured by the trypan blue assay. The cell viability of lymphocytes with various concentrations (10, 25, 50, 100, 150 mM) of NDMA during the 48 hr culture period is explained by Fig.1. The concentration has been related to previous experiment in NDMA cytotoxicity (Zhang, Lipsky et al., 1990). The result shows that cell viability decreased in a dose-dependent manner in lymphocytes exposed to NDMA ($P<0.05$, Mann-Whitney's U test). The experiments were repeated three times.

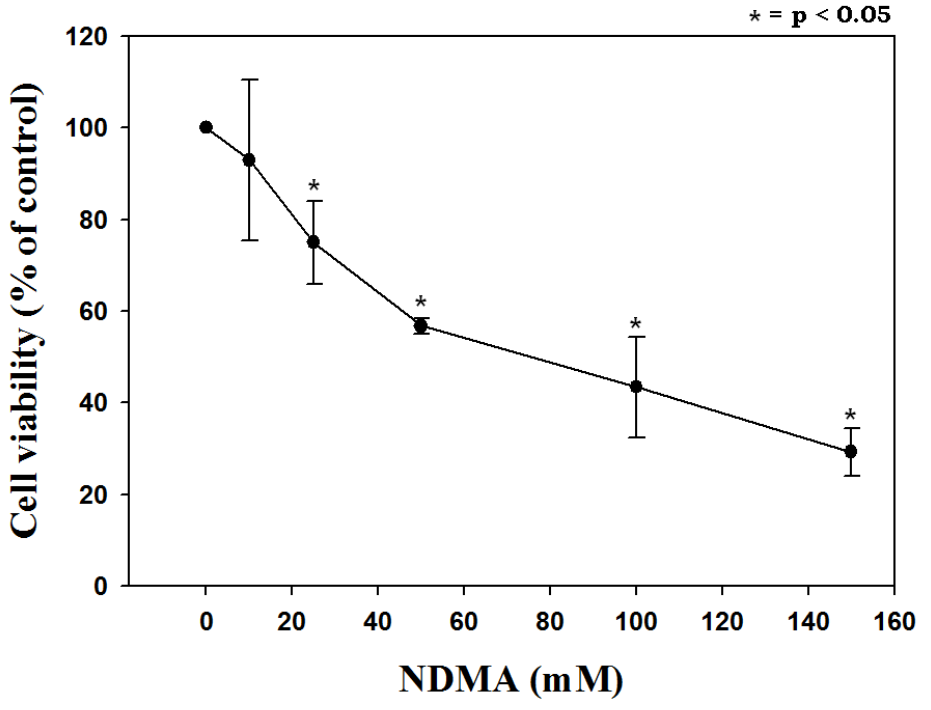

Fig. 1. Value of viable cells determined by counting the trypan blue-excluding cells was significantly decreased in the NDMA-treated peripheral blood lymphocytes for 48 hr exposure. The results indicate the means ± S.D. for Three independent experiments. * = $p < 0.05$ compared to control.

3.2. Analysis of Apoptotic Cell Death and Cell Arrest

To investigate the apoptotic cell death and cell arrest in NDMA-induced lymphocyte, the cells were treated with various concentrations of NDMA for 48 hr and measured by using flow cytometry. As shown in Fig. 2, NDMA didn't induced the apoptotic cell death in any concentrations, whereas Fig. 3 clearly indicates that treatment of NDMA in lymphocytes induces a significant increase in the percentage of G1 phase cells and significant decrease in the percentage of S phase cells in dose-dependent manner, but the percentage of G2/M phase cells is significantly decreased at the highest dose only. Consequently, NDMA-induced G1 arrest was manifested in lymphocytes in dose-dependent manner.

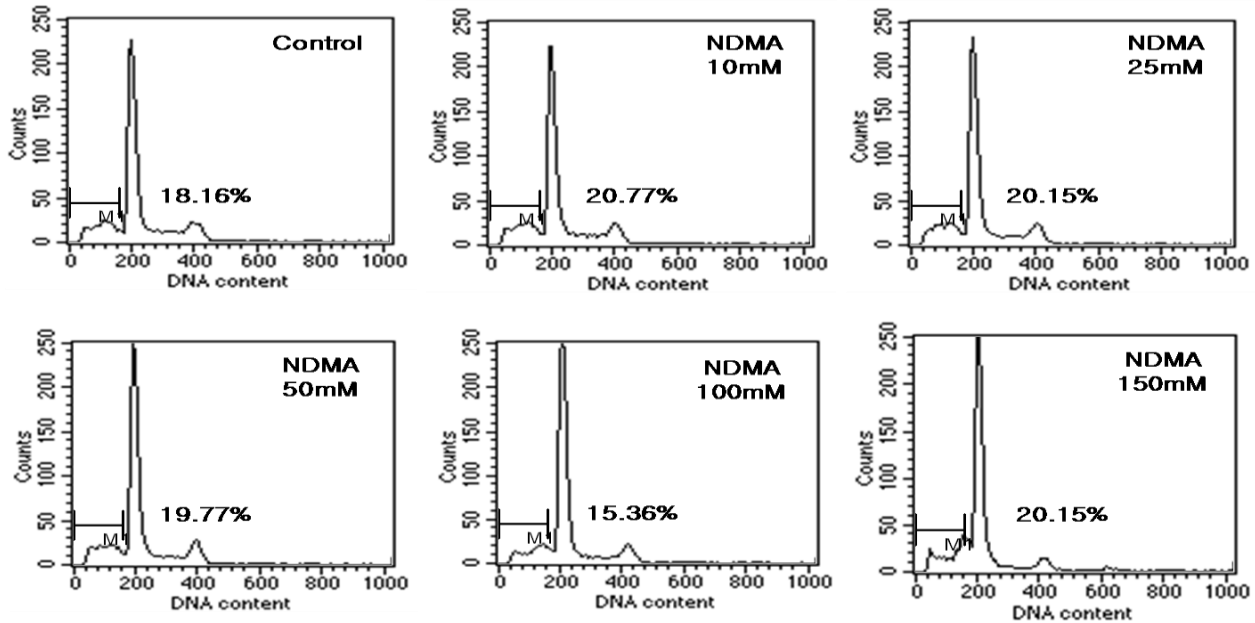

Fig. 2. Apoptotic cell death in NDMA-induced lymphocytes for 48 hr at 37°C. Results of one experiment are shown; similar patterns were revealed in three separate experiments.

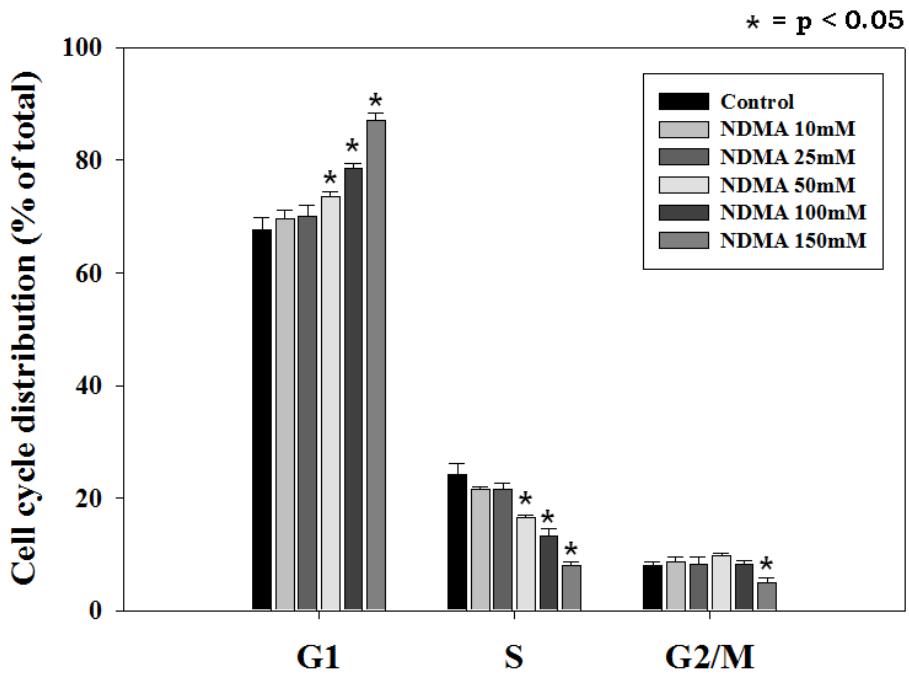

Fig. 3. Induction of cell growth arrest in peripheral blood lymphocytes treated with various NDMA for 48 hr. Cells stained with PI were subjected to flow cytometric analysis for cell distributions at each phase of cell cycle. The results represent the means ± S.D. for three separate experiments. * = $p < 0.05$, compared to control.

3.3. Single – Cell Gel Electrophoresis (Comet) Assay

Genotoxic effect of NDMA in human lymphocytes was determined using an alkaline single-cell gel electrophoresis (Comet) assay. Fig. 4 shows a change of DNA damage induced by NDMA in lymphocytes for 12 hr exposure. The result shows that DNA damage increased in a dose-dependent manner in lymphocytes exposed to NDMA. OTM (olive tail moment) indicated a level of DNA single strand breakage. Especially, the level of OTM at the highest dose has significantly greater than control (control vs. NDMA 150 mM ; 100% vs. 752%, $P<0.05$, Mann-Whitney's U test).

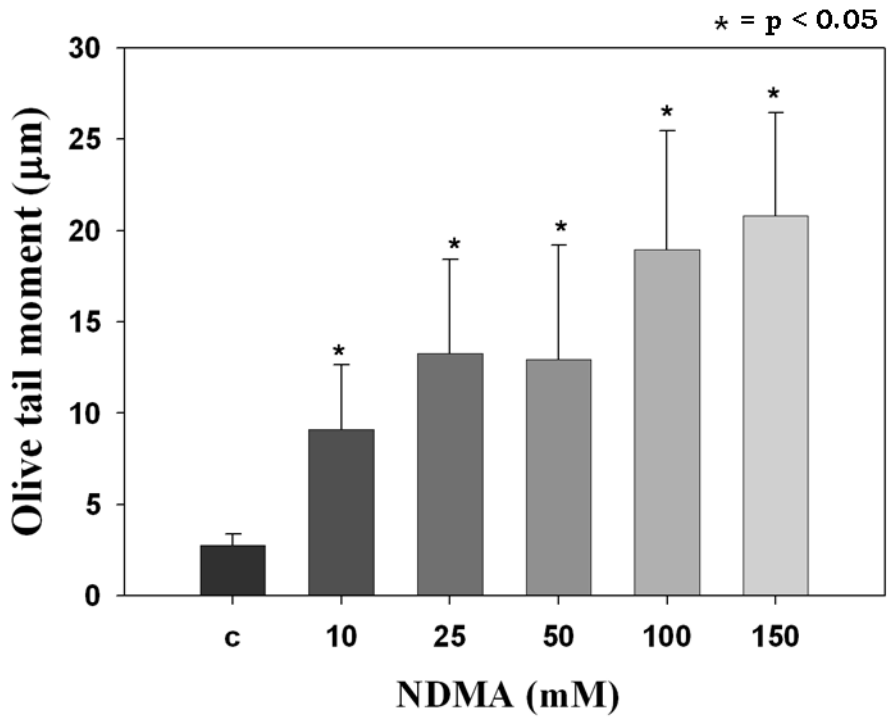

Fig. 4. NDMA treatment induces does-dependent DNA damage in peripheral blood lymphocytes as estimated by the single-cell gel electrophoresis (comet) assay. The alkaline comet assay was used to determine DNA single-strand breaks, and the Olive tail moment was used as an index of damage [(tail mean-head mean) × Tail%DNA/100]. Each bar represents the mean ± S.D. for Three independent experiments. * = $p < 0.05$ compared to control.

3.4. Cytokinesis-block Micronucleus (CBMN) Assay

To assess the genotoxic effect of NDMA, I performed the CBMN assay in human lymphocytes exposed to NDMA for 48hr. Micronuclei(MN) in lymphocytes treated with NDMA was slightly increased and not significant (data not shown).

To confirm that the cell proliferation is suppressed by NDMA, Cytokinesis block proliferation index (CBPI) was scored 1000 cells in between bi-to multi nuclei to measure the extent and progression of nuclear division in a dividing cell population. The CBPI value was determined by the standard calculation (Fenech, 1997). Fig. 5 shows that value of CBPI significantly decreased in a dose-dependent manner in lymphocytes exposed to NDMA. The mean CBPI represented 1.54±0.005, 1.40±0.021, 1.38±0.012, 1.35±0.002, 1.30±0.011, 1.16±0.014 in lymphocytes exposed to 0, 10, 25, 50, 100, 150mM of NDMA.

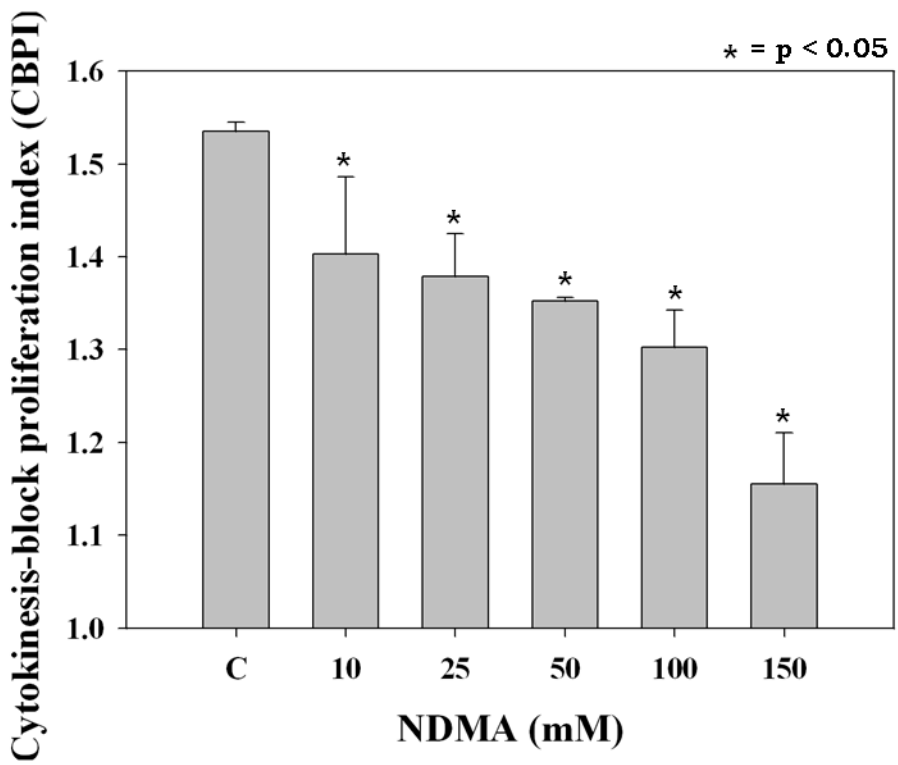

Fig. 5. Induction of CBPI values in peripheral blood lymphocytes treated with NDMA for 48 h. Data are the means ± S.D. for Three independent experiments. * = $p < 0.05$ compared to control.

3.5. Measurement of Reactive Oxygen Species

To analyze the level of ROS in NDMA-induced lymphocytes, various NDMA concentrations were treated in cells for 3 hr. ROS production was measured by using 5-carboxy-2', 7'-dicholrofluorescein diacetate (DCFDA, Molecular Probe, OR). Levels of ROS were significantly increased with NDMA concentrations and expressed as the relative fluorescence intensity (%) with respect to control (50 mM; 131.55%, 100 mM; 141.68%, 150 mM; 169.65% compared to control; 100%) (Fig. 6).

To determine the protective effect of N-acetylcysteine (NAC) in lymphocytes exposed to NDMA, NAC (1mM) was pre-treated for 1 hr before NDMA (150 mM) exposure. Fig. 7 shows value of ROS in NDMA-induced lymphocytes significantly decreased by NAC (NDMA 150mM; 162.3% vs. NDMA 150mM + NAC; 121.5%). All experiments were repeated three times.

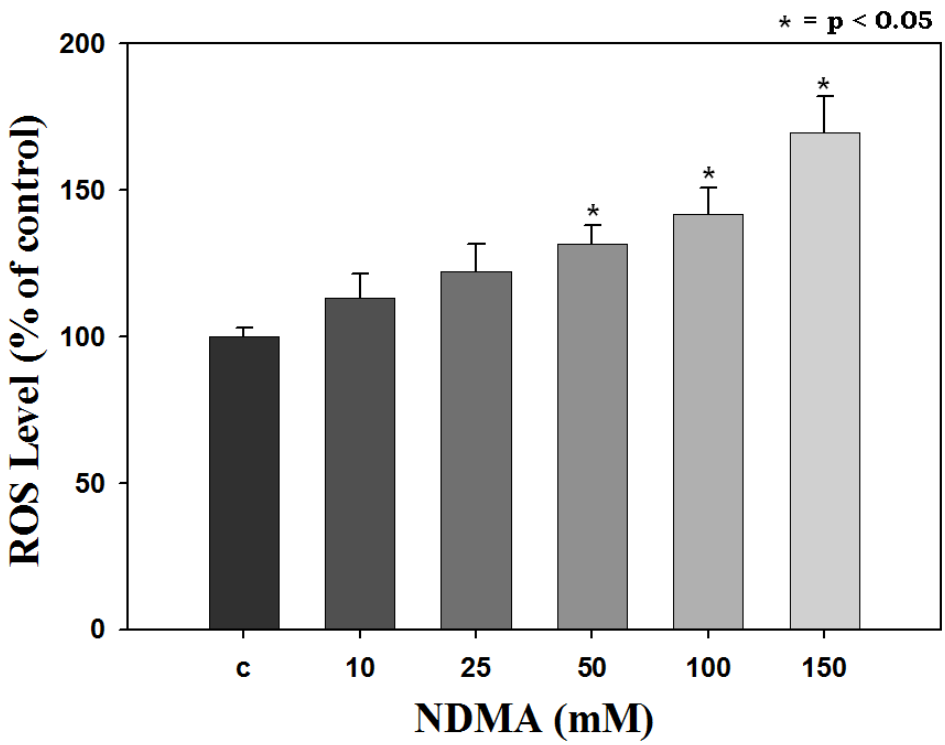

Fig. 6. ROS production in peripheral blood lymphocyte treated with NDMA for 3hr. Intracellular ROS levels were assayed using the fluorescent Probe DCF-DA with a FACS Calibur flow cytometer. Data are the means ± S.D. for Three independent experiments. * = $p < 0.05$ compared to control.

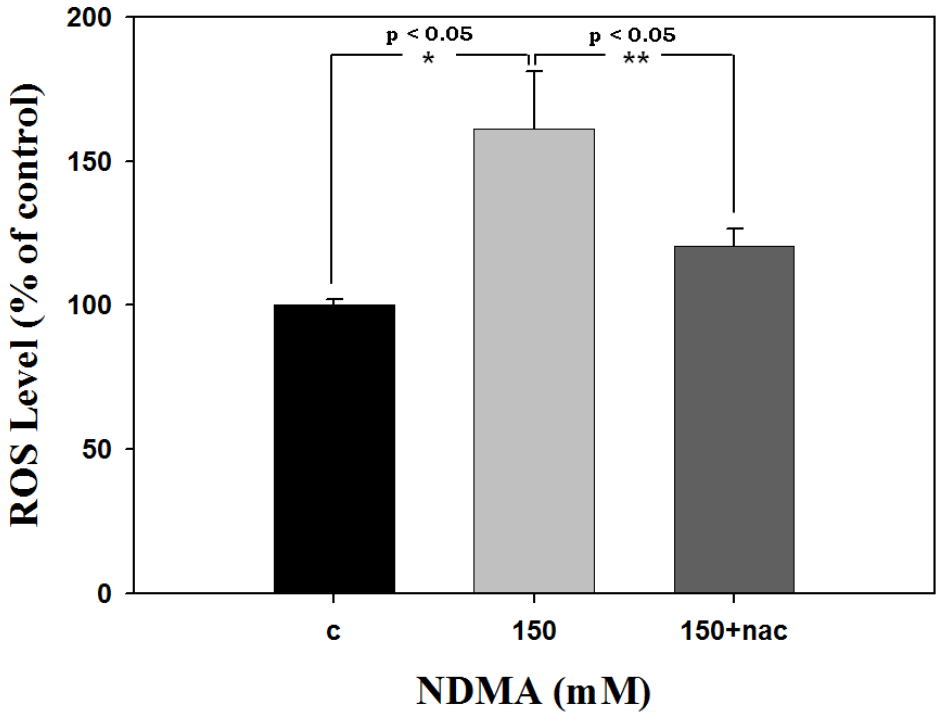

Fig. 7. The effect of NDMA on ROS production in the absence or presence of NAC pre-treatment (1mM) before 1 hr. Intracellular ROS production was assayed using the fluorescent probe DCF-DA and revealed that ROS revel reduced by NAC. The results are the means ± S.D. for Three experiments. * = $p < 0.05$ for control vs. NDMA; ** = $p < 0.05$ for NDMA vs. NDMA + NAC.

3.6. Protective effect of N-acetylcysteine against NDMA-induced DNA damage in human lymphocytes

To confirm that NDMA-induced DNA damage is related to production of ROS, NAC (1mM) and NDMA (150 mM) were treated with the cells for 12 hr and olive tail moment of this sample was measured using the comet assay. The result indicated that DNA damage in NDMA-induced lymphocytes significantly decreased by NAC ($P<0.05$, Mann-Whitney's U test) (Fig. 8).

To evaluate that decreased cell proliferation and cell cycle arrest could be restored through the protective effect of antioxidant in lymphocytes exposed to NDMA, NAC (1mM) was simultaneously treated with NDMA (150 mM) in lymphocytes for 48 hr. The result demonstrated that the value of CBPI was significantly higher in lymphocyte exposed to NDMA with NAC than lymphocytes treated with NDMA-only (Fig. 9). Also, the proportion of G1 phase cells was significantly lower and that of S phase cells was significantly higher in lymphocyte exposed to NDMA with NAC than lymphocytes treated with NDMA alone (Fig. 10).

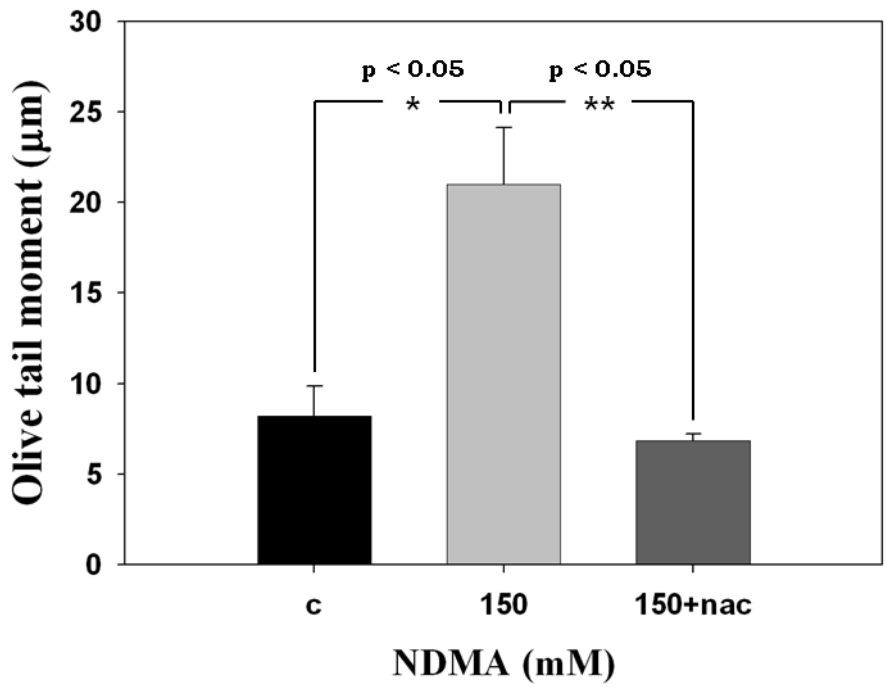

Fig. 8. The effect of NDMA-induced DNA damage in the absence or presence of NAC treatment (1mM). The addition of NAC significantly decreased NDMA-induced DNA breakage. Each bar represents the mean ± S.D. for Three independent experiments. NAC, N-acetylcysteine. * = $p < 0.05$ for control vs. NDMA; * * = $p < 0.05$ for NDMA vs. NDMA + NAC.

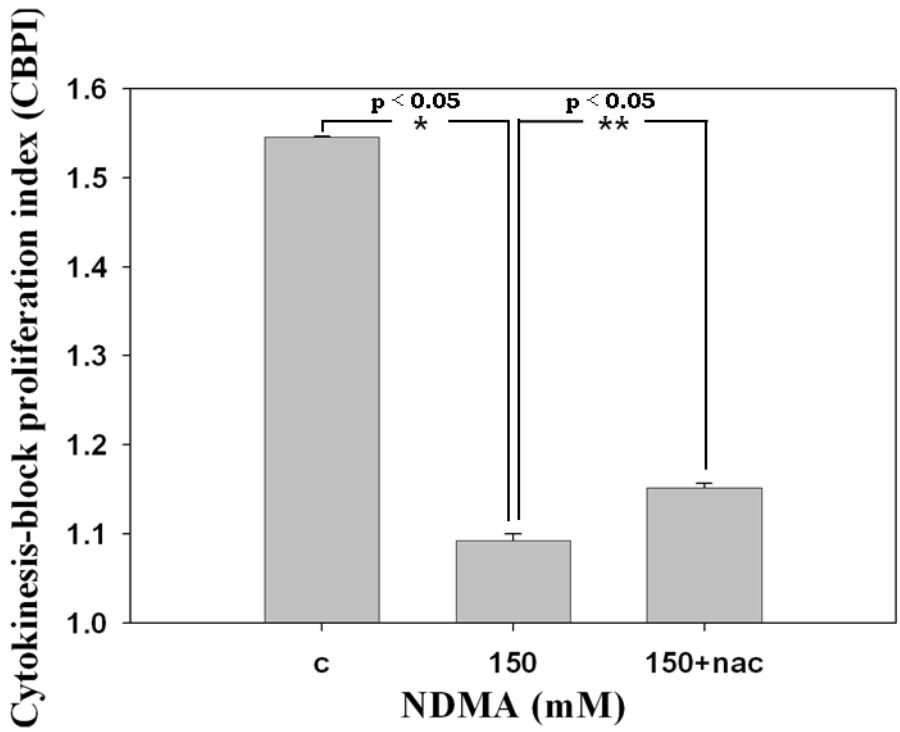

Fig. 9. The effect of NDMA on the cell proliferation in the absence or presence of NAC treatment (1mM) for 48 hr exposure. The CBPI value was increased by NAC compared to the cell treated with NDMA 150mM. The results are the means ± S.D. for Three experiments. * = $p < 0.05$ for control vs. NDMA; ** = $p < 0.05$ for NDMA vs. NDMA + NAC.

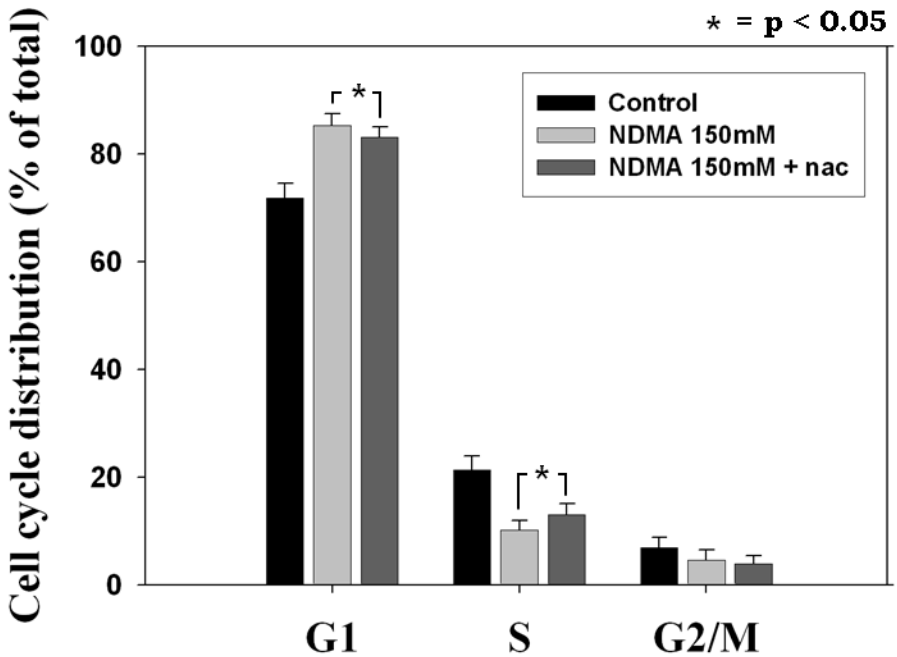

Fig. 10. The effect of NDMA on the cell cycle distribution in the absence or presence of NAC treatment (1mM) for 48 hr exposure. The results represent the means ± S.D. for Three separate experiments. * = $p < 0.05$ for NDMA vs. NDMA + NAC.

IV. Discussion

N-nitrosodimethylamine (NDMA), which is one of the most toxic representatives of nitrosamines, has been shown to be an acute hepatotoxin and potent food-derived genotoxin, carcinogen in many animal species as well as humans (Lin, Parsels et al., 1999; Sugimura, 2000). It is known that NDMA is present in tobacco smoke, alcoholic beverages, nitrate- or nitrite-treated foods and certain occupational setting e.g. rubber industry. Also, appreciable amounts of NDMA are formed endogenously (Kyrtopoulos, 1998; Oury, Limasset et al., 1997). The carcinogenic action of NDMA is mediated via CYP2E1, which is known to be mainly included in hepatocytes, cells of the main tissue of the liver (Anundi and Lindros, 1992; Nagai, Hiyoshi et al., 2002; Schulze, 1999).

Several studies have demonstrated that CYP2E1 is also found in human lymphocytes as well as hepatocytes (Nagai, Hiyoshi et al., 2002; Raucy, Schultz et al., 1997). Ingested NDMA is transferred from the gastrointestinal tract to the systematic circulation, and is transported mainly to the liver through blood (Tricker and Preussmann, 1991). NDMA may be able to produce

toxicity by its metabolizing enzymes in CYP2E1 expressing lymphocytes through those processes. Although many reports have demonstrated that NDMA exposure leads to cytotoxicity and genotoxicity in various cells including hepatocytes, the toxic effects of NDMA in human lymphocytes have not yet been fully understood. In this study, cytotoxic effect and genotoxic effect of NDMA on human lymphocytes was investigated.

In the present study, I used the concentration of NDMA of 10-150mM, since the cytotoxicity was previously examined at these concentrations (Zhang, Lipsky et al., 1990). As shown in Fig.1, NDMA induced a significant decrease of cell viability in a dose-dependent manner in human lymphocytes.

Several precedent studies have demonstrated that NDMA induced cell cycle arrest in G2/M phase as well as apoptosis in human hepatoma (HepG2), lymphoblastoid, embryonic kidney (HEK293T), leukemia (HL-60) cells and rat neutrophils (Dobo, Eastmond et al., 1997; Jablonski, Jablonska et al., 2001; Kim, Kim et al., 2008; Lin, Parsels et al., 1999). To identify that NDMA triggers cell cycle arrest and/or apoptosis in human lymphocytes, I examined cell cycle distribution and proportion of subG1 phase in cells exposed to NDMA by using flow cytometry.

It was different from the results of precedent studies in that NDMA-induced G1 phase arrest occurred in a dose-dependent manner but not induced the apoptotic cell death in any concentrations in human lymphocytes (Fig.2, Fig.3). This result can be suggested that NDMA exert cytotoxic effects on human lymphocytes by causing G1 arrest through different mechanism compare with the existing studied cell types.

To evaluate genotoxic effect of NDMA caused by DNA strand breaks in human lymphocytes, I measured Olive Tail Moment (OTM), which indicates DNA damage, as using the comet assay (Fairbairn, Olive et al., 1995). In the present study, the result showed that NDMA dose-dependently induces significant increase of OTM in human lymphocytes (Fig.4). It is consistent with several studies which demonstrated that NDMA induced cell damage by DNA strand breaks in rat, cow, human (Bowen, Whitwell et al., 2011; Sivikova and Dianovsky, 1993; Uhl, Helma et al., 2000; Wilkening, Stahl et al., 2003). In addition, Lin et al. (1999) has shown that NDMA induces 8-Hydroxydeoxyguanosine (8-OHdG), a major product of oxidative DNA damage, resulting from the reaction of the hydroxyl radical (HO) with guanine residue in DNA in CYP2E1 expressing cells (Lin, Parsels et al., 1999). Lin et al.

(2001) and Souliotis et al. (2002) indicated that NDMA causes DNA damage due to generation of DNA adducts formed by methylating agents, such as O^6-methylguanine (O^6-meG), N^7-methylguanine (N^7-meG) in rat, human hepatocytes (Lin and Hollenberg, 2001; Souliotis, Henneman et al., 2002).

I also confirmed the frequencies of micronuclei (MN) in binucleated cells and the cytokinesis block proliferation index (CBPI) to determine to percentage of cells with bi- to multi nuclei after treatment with NDMA by using the CBMN assay, which enables other kinds of damage to be detected, such as chromosome breaks and aneuploidy (Decordier and Kirsch-Volders, 2006). NDMA-induced MN was slightly increased but not dose-dependent manner and not significant (data not shown). The level of CBPI in treated groups was lower than that of control (Fig. 5). These data can be evidence that cell proliferation is slowed down by NDMA, which is related to induction of G1 arrest in human lymphocytes.

Several studies have reported that NDMA can be the cause of the intracellular ROS production and various antioxidants, such as ascorbic acid (vitamin C) and vitamin B_{12} have a beneficial role in protecting against NDMA-induced toxic effects in human

hepatoma and leukemia cells (Arranz, Haza et al., 2008; Erkekoglu and Baydar, 2010; Isoda, Kagaya et al., 2008). I found that NDMA-induced toxic effect is linked to an increase of ROS in human lymphocytes (Fig. 6). It was reported that N-acetylcysteine (NAC), a precursor of reduced glutathione (GSH) and direct ROS scavenger, has a protective effect on DNA damage in peripheral blood lymphocytes (Sudheer, Muthukumaran et al., 2007). Pretreatment of human lymphocytes with NAC significantly inhibited NDMA-induced generation of ROS (Fig. 7).

It is known that the production of ROS leads to lasting oxidative stress, which can trigger cell damage and ultimately cause cell cycle arrest (Jacobson, 1996; Verbon, Post et al., 2012; Zhao, Tsuchida et al., 2002). In the present study, I assessed the protective effect of NAC against NDMA-induced DNA damage in human lymphocytes. The result indicated that NAC reduced NDMA-induced oxidative DNA damage and restored the cell growth inhibition, as well as decreased the proportion of G1 phase cells (Fig.8, Fig.9, Fig.10). These data suggest that NDMA-induced DNA damage and G1 arrest are caused by the production of ROS.

According to published report, the generation of ROS is known to play an essential role in cell cycle progression, signal

transduction cascades, protein ubiquitination, and degradation (Boonstra and Post, 2004; Verbon, Post et al., 2012). Several recent studies have shown that the p53 protein is known to play a key role in the arrest of cell growth and p53 over-expression has also been shown to cause the accumulation of ROS. The p53 protein induces $p21^{Waf1/Cip1}$, a necessary mediator of p53-induced cell cycle arrest, which inhibits cyclinD1/CDK4/6 complex that the first responding to mitogenic signals in G1 phase and eventually triggers G1 arrest (Boonstra and Post, 2004; Hseu, Lin et al., 2009; Kim, Oh et al., 2006; Liu, Wang et al., 2012). It can be assumed that NDMA-induced G1 arrest may also progress through the aforementioned process. Therefore, further research is required to be sure to evaluate the expression of G1 arrest-associated regulatory proteins in human lymphocytes exposed to NDMA.

It was reported that the maximum intake of NDMA in adult is approximately 5.15 ㎍/day/person in korea (Jung-Hye shin, 2002). It is much lower than concentrations in experiment and also less likely to evoke caner in health adult, but NDMA can be transported through perfused human placenta, and it is probable that the fetus is also exposed if the mother is exposed, with

potential fetal health effects. Recent epidemiological studies indicate that NDMA may induce brain tumors in children if the mother has consumed large quantities of cured meats during pregnancy. It is a real possibility that fetal exposure during pregnancy may evoke fetotoxicity including transplacental carcinogenesis (Annola, Heikkinen et al., 2009; Dietrich, Block et al., 2005). Thus our results indicate that NDMA-induced toxic effects in human lymphocytes can lead to an increased risk of cancer occurrence in vulnerable people including fetus.

In conclusion, NDMA induces DNA damage via ROS generation, which triggers cell-cycle arrest in human lymphocytes. Taken together, the present study manifests that NDMA causes cytotoxicity and genotoxicity in human lymphocytes. These findings can be provided significant data to toxicity assessment of NDMA restricted to hepatotoxicity.

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국문초록

사람 림프구에서 N-nitrosodimethylamine 의 세포 유전독성 평가

김 계 은

서울대학교 보건대학원

보건학과 분자역학 전공

지도교수 정 해 원

N-nitrosodimethylamine (NDMA) 은 가공된 고기류, 햄, 훈제 생선, 맥주 와 같은 식품에 포함되어 있으며, 강력한 간독성 물질로서 발암물질 과 돌연변이 유발물질로 알려져 있다. NDMA 는 식품 속에 포함된 아질산염 (NO_2) 과 secondary amine 인 demethylamine 이 반응하여 체내에서 발생할 수 있고, 연소가스를 이용한 식품의 건조나 가열하는 과정에서 발생할 수 있다.

NDMA 가 대사를 일으켜서 세포손상을 유발하는데 중요한 역할을

하는 Cytochrome P450 2E1(CYP450 2E1)은 여러 화학물질과 알코올의 독성을 유발하는데 큰 위치를 차지하며, 간세포에 많이 들어있는 것으로 알려져 있다. 최근에 이 효소는 간세포 이외에 사람 림프구 에서도 발현되는 것으로 보고되고 있으며, NDMA 가 사람 림프구에서 CYP2E1을 통해 혈액에서 대사되어 독성을 유발 할 가능성이 있다. 하지만 NDMA 에 의한 인체 독성 평가는 거의 간 독성에서 주로 이루어졌으며, 혈액독성에 의한 독성평가 및 기전에 관한 연구는 거의 이루어지지 않았다. 따라서, 본 연구는 사람 림프구에서 NDMA 에 의해 일어나는 세포 유전독성 정도를 소핵 분석, 단세포 전기 영동 법, 유세포 분석을 통한 세포 내 활성 산소 종의 측정, 세포 자사멸 및 세포주기 억류 측정 등 다양한 분자 독성 기법을 시옹하여 평가하고자 하였다.

사람 림프구에서 세포 생존률은 NDMA 의 농도가 증가함에 따라 감소하였고, 소핵분석에서 음성으로 나타났지만, 단세포 전기영동법 분석에서 DNA 손상이 양−반응적으로 증가함을 확인하였다. 또한 NDMA 에 의한 세포사멸은 나타나지 않았지만, G1 단계에서 세포주기 억류과정을 유발하는 것을 유세포 분석을 통해서 확인할 수 있었다. 또한, NDMA 를 처리한 세포에서 세포 내 활성산소종 수준이 현저히 증가하였고, 항산화제인 N-acetylcysteine (NAC)에 의하여 활성산소종의 발생이 감소함을 관찰 하였다. 그리고 NDMA 와 NAC 을 함께 처리한 세포에서 DNA 손상과 G1 단계의 세포주기 억류

정도가 현저히 줄어드는 것을 확인 함으로써, 사람 림프구에서 NDMA 에 의해 유발된 독성에 대한 NAC 의 보호효과를 확인할 수 있었다.

결론적으로 NDMA 는 사람 림프구에서 활성산소종을 발생시켜 DNA 손상을 증가 시킴으로써 세포주기 억류를 유발했다. 위와 같은 결과를 바탕으로 간세포에 한정되어 있는 NDMA 에 대한 독성평가에 중요한 자료로 제공될 수 있을 것으로 기대된다.

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주 요 어 : N-nitrosodimethylamine(NDMA); 사람 림프구;
DNA 손상; 활성산소종; 세포주기 억류

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