Objectives: In this study, the in vitro mammalian chromosomal aberration (CA) assay was conducted to gain additional information concerning the hazards associated with the use of cyclopentane and ammonium nitrate. While these two chemicals had already been tested by many methods, they had not been studied in the CA test.

Methods: The assay was performed using the ovarian infantile cell (CHO-K1 cell), by the direct method (-S9) and by the metabolic activated method (+S9 mix).

Results: Using the direct method, the 7 dosages in a 48 hour treatment group did not show that the frequency of CA is proportion to the dosage addition. The frequency of CA is not proportion to the dosage addition for a 6 hour treatment using the metabolic activated method.

Conclusion: From these findings, it was decided that the 2 chemicals do not induce chromosomal aberrations under the tested conditions.

Key Words: Cyclopentane, Ammonium nitrate, Chromosomal aberration, In vitro, Chinese Hamster Ovary

Introduction

The necessity for hazard assessment has increased, because the frequency of chemical exposure for workers is increasing as the chemical industries have been developed. Many chemicals that are used in industry represent current concerns since they may pose genetic hazards for humans. Since these substances are not limited to the original products, they have become widespread as environmental pollutants, thus leading to concerns about a variety of chemicals that possibly threaten the health of workers. In this respect, the regulation and evaluation of chemical hazards are important to human health and the working environment.

This study was conducted because insufficient information was available about the potential hazards of cyclopentane (CAS No. 287-92-3) and ammonium nitrate (CAS No. 6484-52-2), therefore an in vitro mammalian chromosomal aberration assay was performed to include in a hazard assessment. Moreover, toxicology information from this study can be used for a worker’s “right to know,” and to prepare or update the Materials Safety Data Sheet (MSDS) in many chemical industries.
Cyclopentane is a central nervous system depressant in humans. Because the industrial use of pure cyclopentane is limited and the commercial reagents and solvents that contain cyclopentane also contain other hydrocarbons, there are few toxicological data on the effects of exposure to the pure substance [1]. Exposure to cyclopentane can occur through inhalation, ingestion, and eye or skin contact. Cyclopentane is a central nervous system depressant in animals and application of cyclopentane to the skin of guinea pigs caused redness and dryness [2]. The minimal narcotic concentration of cyclopentane in mice is 38.3 ppm; this level of exposure may also cause loss of reflexes and death. Exposure to a cyclopentane concentration of 112 to 1,139 ppm for 6 hours/day for 3 weeks caused no effects in male and female rats; however, exposure to 8,110 ppm for 6 hours/day for 12 weeks caused a decrease in the rate of body weight gain in female rats. The threshold limit value for cyclopentane in the workplace (TWA) is 600 ppm [1]. Cyclopentane produced a slight erythema on the skin but no additional effects were reported in male and female rats.

Exposure of female rats to 8,110 ppm for 12 weeks (6 h/day) led to decreased body weight gain [2] and also caused a skin dry appearance, thickening of skin, and affected arginase activity in the epidermis. A slight increase in the excretion of β-microglobulin was observed, indicating minor tubulotoxicity [3]. It was also reported to be a weak sensitizer of the myocardium to the effects of adrenaline [4]. Symptoms of acute exposure to high concentrations of cyclopentane include excitement, loss of equilibrium, stupor, coma, and rarely, respiratory failure [5].

Cyclopentane has been detected in the blood of chemically-sensitive patients, as analyzed by chromatography-mass spectrometry (GC-MS) [6,7]. In a Danish study conducted to delineate risks due to exposure of humans to neurotoxic chemicals, cyclopentane has been assigned a risk index value of 2 on a 0 to 5 scale, i.e. “Chemicals with a small risk of damaging the nervous system during normal work with the substances” [8].

Occupational exposures to gasoline and petroleum products, including cyclopentane, were measured in numerous petroleum companies. Results of long-term samples (n = 79) were: median value of cyclopentane was 0.19 mg/m³, maximum 3.74 mg/m³, short-term samples (n = 105): median value of cyclopentane was 0.66 mg/m³, maximum 73.36 mg/m³ [9].

Ammonium nitrate (CAS No. 6484-52-2) uses include: a fertilizer in the agricultural industry, use in basic chemical synthesis, personal and domestic use, as an oxidizing agent in the chemical industry and in the production of pharmaceutical drugs, etc. Exposures can occur when handling and using chemicals on farms. Occupational exposure of workers, applicators, distributors, merchants and farm workers can occur during the manual handling of solid fertilizer products, or may occur by inhalation. The Good Laboratory Practice (GLP) reports the acute oral LD₅₀ values as 2,085 mg/kg in mice and 2,217 mg/kg in rats. Ellen et al. [10] administered single oral doses (0.15 g NH₄NO₃/kg) to 12 adult human volunteers with no measurable hematological effects, elevation of methaemoglobin or detection of circulating N-nitroso-compounds. One subject developed diarrhea after 7 hrs and one vomited after 12 minutes. Twelve other subjects, administered 9.5 g NaNO₃ in 750 mL water intravenously over a 1 hour period, showed no effects from the treatment.

Results from genotoxic evaluation, show that cyclopentane is negative for activity in the Ames assay with Salmonella [11] and ammonium nitrate is negative for activity in the micronucleus test [12].

The identification of toxicity that may pose a genetic hazard in our environment and workplace is currently of great importance [13], because there are many new chemicals used in chemical industry. Several assay systems demonstrating rapidity and reliability have been introduced for this purpose, such as the reversion test using bacterial gene mutation [14,15], chromosomal aberration assay using mammalian cells [16] and micronucleus assay using rodents [17,18].

It has been assumed that mutation represents at least one step in the mechanism of carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens [19], and for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency [20]. Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement [21] which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of cellular cancer genes [22].

In addition, -5,000 tons of cyclopentane are used every year by -2,700 workers, and -32,000 tons of ammonium nitrate are used by -34,000 workers in Korea. It is necessary to assess these chemicals’ hazards, because worker exposure frequency is increasing and there are great concerns about genetic hazards.

The chromosomal aberration assay, conducted using mammalian cells, is frequently used to evaluate the genotoxicity of chemicals and has been adopted as an index of genotoxicity worldwide. Furthermore, it is utilized as a screening probe for the detection of possible carcinogenic substances. Despite its increasing use, the available genotoxicity data for cyclopentane and ammonium nitrate are still controversial. Therefore, we evaluated their genotoxicity using the in vitro mammalian chromosomal aberration assay.
Several short term methods have been developed for predicting the carcinogenicity of chemicals and have also been introduced for the evaluation of genotoxicity [23,24] and antimutagenicity [25]. Cytogenetic studies on mammalian cells in vivo [26,27] and in vitro [23] have also been widely used as screening methods to identify DNA-attacking substances.

We used CHO cells in this experiment because it has been reported that there are no sensitivity differences between CHO and Chinese hamster lung (CHL) cells in the vitro chromosome aberration assay [28,29].

### Materials and Methods

#### Cells and chemicals

The cultivated CHO-K1 (Chinese hamster ovary fibroblast) cells used in this test were obtained from the Korean Cell Line Bank (KCLB 10061). Cells were cultured in F-12 medium (GIBCO BRL, NY, USA, Lot No. 507762) with 5% CO2 at 37 °C, and sub-cultured every 2-4 days.

Ethanol (Merck, NJ, USA, Lot No. K35091883538) was used as a negative control for cyclopentane (Sigma, MO, USA, 95%, Lot No. 10120721), and distilled water for ammonium nitrate (Sigma, MO, USA, > 99.5%, Lot No. 03416JH) and as a solvent according to results of a solubility test.

Mitomycin C (MMC) (Sigma, MO, USA, Lot No. 028K1815) and cyclophosphamide (CPA) (Sigma, MO, USA, Lot No. 076K1050) were used as positive controls in the study. For the metabolic activated system, the S9 (MOLTOX™, Annapolis, Maryland, USA, Lot No. 2374) was used within 6 months after manufacture.

#### In vitro mammalian chromosomal aberration test

This study was performed according to OECD guidelines for the testing of chemicals (OECD, 1997) (In vitro Mammalian Chromosomal Aberration Test.Ref. OECD TG473(632,584),(959,880)) and Ishi-date's report [30].

For the cell proliferation suppression test, 7 dosages of each chemical (0.003, 0.007, 0.01, 0.03, 0.06, 0.125 and 0.25 mM cyclopentane), and (0.156, 0.3125, 0.625, 1.25, 2.5, 5 and 10 mM ammonium nitrate) were used, respectively. For the metabolic activated method (6 hour treatment), the cells were cultured using conditions identical to the direct method. Slides for observation of chromosomal samples were made from 5 mL media aliquots, with 18 hour supplementary culture, after removal of media and washing of the cell layer with 5 mL fresh media.

The main test was performed using dosages established by the cell proliferation suppression / preliminary test. After 24 and 48 hours of exposure to test chemicals, plates were treated with 0.2 μg/mL Colcemid® (GIBCO BRL, NY, USA, Lot No. 453240) After 2 hours, the metaphase cells were separated and centrifuged at 1,000 rpm for 5 min.

The chromosome samples were produced by fixing (3×) with the Carnoy’s solution (acetic acid : ethanol = 1 : 3) and abnormalities were counted after 5 min of staining with 5% Giemsa solution (Merck, NJ, USA, Lot No. HX888942). Two samples were made from each plate.

Two hundred metaphase cells were observed per plate and classified according to structural abnormalities (gap of chromatid or chromosome;g, cutting of chromatid;ctb, exchange of chromatid;cte, cutting of chromosome;csb, exchange of chromosome;cse and others) and numerical abnormalities (pol). Statistical analysis of the results was not performed.

Results were evaluated as “positive” only when the percentage of chromosomal aberrations was > 10%.

#### Results

##### Test for suppression of cell proliferation

The ratios of cell proliferation for the dosages of cyclopentane were 86.31% and 87.29% at 0.003 mM and 0.06 mM respectively, for a 24 hour treatment using the direct method. Moreover cell proliferation ratios were 66.78%, 68.45%, 65.93%, 55.55% and 51.03% at 0.003 mM, 0.01 mM, 0.06 mM, 0.125 mM and 0.25 mM respectively, for a 48 hour treatment. For the direct method, it was confirmed to the guideline of GLP [31] that the maximum concentration is over 5 mg/ml in case of the cellular toxicity not being recognized.

Cell proliferation ratios were 89.90%, 64.74% at 0.007 mM and 0.25 mM for a 6 hour treatment using the metabolic activated method. However, ammonium nitrate showed no suppression of cell proliferation after 24 hours of treatment using the direct method (89.40-240.26% at each concentration), and it was also conformed to the guideline of GLP [31] to decide the maximum concentration.

##### Chromosomal aberration test

Duplicate samples of 100 cells per plate were observed in metaphase and classified for structural abnormalities (gap of chromatid or chromosome;g, cutting of chromatid;ctb, exchange of chromatid;cte, cutting of chromosome;csb, exchange of chromosome;cse, etc) and numerical abnormalities (pol). Results were evaluated as being positive only when the percentage of chromosomal aberrations was ≥ 10% (≥ 20 abnormalities in
200 cells observed). A statistical analysis of the results was not performed.

The ratios of chromosomal aberration using the direct method are shown in Tables 1-3 and 4. No diploid presence was observed at any concentration after 24 hour treatment. The structural chromosomal aberrations were < 5.0% in both without gap (-gap) and with gap (+gap) groups. No dependency between chromosomal aberrations and dosages was observed.
The ratios of chromosomal aberration using the metabolic activated method are shown in Tables 5 and 6. All results were the same as from the direct method. It was shown that the two chemicals do not induce any chromosomal aberrations, using either the direct method (24 hour and 48 hour treatment) or the metabolic activated method, (6 hour treatment) in CHO-K1 cells.

**Table 3. Chromosomal aberration test (direct method, 24 hr treatment) with ammonium nitrate**

| Treatment            | Time of treatment (hr) | Conc. of treatment (mM) | Obs. cell No. | No. of diploid | No. of chromosomal structure abnormality | No. and ratio of chromosomal structure abnormality |
|----------------------|------------------------|-------------------------|---------------|----------------|----------------------------------------|--------------------------------------------------|
| Control solvent (DW) | 24                     | 0                       | 200           | 0              | 0.5 0 0 0 0 0 0 0 0.5 0               | 0 0 0 0 0 0 0                                       |
| Test material        | 24                     | 0.156                   | 200           | 0              | 0 0 0 0 0 0 0 0 0 0                  | -                                                 |
|                      |                        | 0.313                   | 200           | 0              | 1 0 0 0 0 0 0 0 0 1                  | -                                                 |
|                      |                        | 0.625                   | 200           | 0              | 0 0 0 0 0 0 0 0 0 0                  | -                                                 |
|                      |                        | 1.25                    | 200           | 0              | 0 0 0 0 0 0.5 0 0 0.5 0              | -                                                 |
|                      |                        | 2.5                     | 200           | 0              | 0 0 0 0 0 1 0 0 1 1                  | -                                                 |
|                      |                        | 5                       | 200           | 0              | 2 0 0 0 0 0 0 0 0 2                  | -                                                 |
|                      |                        | 10                      | 200           | 0              | 0.5 0 0 0 0 0 0 0 0.5 0              | -                                                 |
| Positive control (MMC)| 24                     | 0.0004 (mg/mL)          | 200           | 0              | 18.5 14 32.5 1 4.5 0 5 70.5         | +                                                 |

Conc.: concentration, Obs.: observed, No.: number, g: gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, DW: distilled water, MMC: mitomycin C.
-: negative, +: positive, -g: without gap, +g: with gap.

**Table 4. Chromosomal aberration test (direct method, 48 hr treatment) with ammonium nitrate**

| Treatment            | Time of treatment (hr) | Conc. of treatment (mM) | Obs. cell No. | No. of diploid | No. of chromosomal structure abnormality | No. and ratio of chromosomal structure abnormality |
|----------------------|------------------------|-------------------------|---------------|----------------|----------------------------------------|--------------------------------------------------|
| Control solvent (DW) | 48                     | 0                       | 200           | 0              | 0 0 0 0 0 0 0 0 0 0                  | -                                                 |
| Test material        | 48                     | 0.156                   | 200           | 0              | 0 0 0 0 0 0 0 0 0 0                  | -                                                 |
|                      |                        | 0.3125                  | 200           | 0              | 0 0 0.5 0 0 0 0 0 0.5 0              | -                                                 |
|                      |                        | 0.625                   | 200           | 0              | 0 0 0 0 0 1 1.5 0 0 0 2.5 2.5         | -                                                 |
|                      |                        | 1.25                    | 200           | 0              | 0 0 0 0 0 0 0 0 0 0                  | -                                                 |
|                      |                        | 2.5                     | 200           | 0              | 0 0 0 0 0 0.5 0 0 0.5 0              | -                                                 |
|                      |                        | 5                       | 200           | 0              | 0.5 0.5 0.5 0 0 0 0 0 0.5 1          | -                                                 |
|                      |                        | 10                      | 200           | 0              | 0 0 0 0 0 0 0 0 0 0                  | -                                                 |
| Positive control (MMC)| 48                     | 0.0004 (mg/mL)          | 200           | 0              | 20.5 19 28.5 0 0 0 47.5 68          | +                                                 |

Conc.: concentration, Obs.: observed, No.: number, g: gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, DW: distilled water, MMC: mitomycin C.
-: negative, +: positive, -g: without gap, +g: with gap.
Chromosomal aberrations alter genomic sequence, and are mutagenic. More importantly, chromosomal aberrations may result in loss of tumor suppressor genes and/or activation of oncogenes. Chromosomal aberrations occur quantitatively at

**Table 5. Chromosomal aberration test (metabolic activated method, 6 hr treatment) with ammonium nitrate**

| Treatment                  | Time of treatment (ht) | Conc. of treatment (mM) | Obs. cell No. | No. of diploid | No. of chromosomal structure abnormality | Decision |
|----------------------------|------------------------|-------------------------|---------------|----------------|-----------------------------------------|----------|
|                            |                        |                        |               |                | Gap         | Chromatid | Chromosome | Etc. | Total      | -g | +g |
| Control solvent            | 24 (6 +18)             | 0                      | 200           | 0              | 0           | 0         | 0          | 0.5  | 0          | 0.5 | 0.5 | -  |
| Test material              | 24 (6 +18)             | 0.003                  | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.007                  | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.01                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.03                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.06                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.125                  | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.25                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
| Positive control           | 24 (6 +18)             | 0.01 (mg/mL)           | 200           | 0              | 9.5         | 11.5      | 78         | 0    | 0          | 50.5| 60 | +  |

Conc.: concentration, Obs.: observed, No.: number, g: gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, CPA: cyclophosphamide.

- negative, +: positive, -g: without gap, +g: with gap.

**Table 6. Chromosomal aberration test (metabolic activated method, 6 hr treatment) with cyclopentane**

| Treatment                  | Time of treatment (ht) | Conc. of treatment (mM) | Obs. cell No. | No. of diploid | No. of chromosomal structure abnormality | Decision |
|----------------------------|------------------------|-------------------------|---------------|----------------|-----------------------------------------|----------|
|                            |                        |                        |               |                | Gap         | Chromatid | Chromosome | Etc. | Total      | -g | +g |
| Control solvent            | 24 (6 +18)             | 0                      | 200           | 0              | 0           | 0         | 0          | 0.5  | 0          | 0.5 | 0.5 | -  |
| Test material              | 24 (6 +18)             | 0.003                  | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.007                  | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.01                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.03                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.06                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.125                  | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
|                            |                        | 0.25                   | 200           | 0              | 0           | 0         | 0          | 0    | 0          | 0   | 0   | -  |
| Positive control           | 24 (6 +18)             | 0.01 (mg/mL)           | 200           | 0              | 9.5         | 11.5      | 78         | 0    | 0          | 50.5| 60 | +  |

Conc.: concentration, Obs.: observed, No.: number, g: gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, DW: distilled water, CPA: cyclophosphamide.

- negative, +: positive, -g: without gap, +g: with gap.

**Discussion**

Chromosomal aberrations alter genomic sequence, and are mutagenic.
a much smaller scale compared to DNA sequence mutations, however, their biological consequences are generally more severe than most point mutations [32].

From this study, cyclopentane and ammonium nitrate did not induce chromosomal aberrations using either the direct method (24 hours and 48 hours treatment) or the metabolism activated method, (6 hour test treatment) with CHO-K1 cells.

The purpose of the in vitro chromosomal aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells [33]. Structural aberrations may be of two types, chromosome or chromatid. For the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosomal-type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events cause alterations in oncogenes and tumor suppressor genes of somatic cells which are involved in cancer induction in humans and experimental animals [33].

Tests conducted in vitro generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot entirely mimic the mammalian in vivo conditions. Care should be taken to avoid conditions that would lead to positive results, but do not reflect intrinsic mutagenicity, and may arise from changes in pH, osmolarity or high levels of cytotoxicity [34,35].

The frequency of chromosomal aberrations (CAs) in peripheral blood lymphocytes has been applied for decades as a biomarker for the early effects of genotoxic carcinogens in occupational and environmental settings (including biodosimetry of radiation) [36,37]. CAs in lymphocytes are thought to represent a surrogate endpoint for more specific chromosome alterations in target tissues of carcinogenesis. Assuming that the mechanisms of chromosome damage are similar in different tissues, the level of damage in lymphocytes can be expected to reflect the level of damage in cancer-prone tissues and to indicate cancer risk [38,39].

An association between high CA frequency and increased cancer incidence was originally detected by the Nordic Study Group on the Health Risk of Chromosome Damage in a collaborative project of 10 Nordic cytogenetic laboratories [40]. An independent study among 10 laboratories from Italy, based on cancer mortality data, also concluded that CA is predictive of cancer risk. The Nordic and Italian cohorts were subsequently combined by the European Study Group on Cytogenetic Biomarkers and Health for an updated analysis, which confirmed the cancer risk prediction of CA frequency, but suggested no such association for SCEs or MN [41-43]. Both chromatid-type and chromosome-type CAs were predictive of cancer risk. However, there is some evidence that chromosome-type CAs may have better predictive value than chromatid-type CAs. SCEs do not appear to be indicative of cancer risk [44-47].

Moreover, how these toxic chemicals behave inside the organism is one of the big issues that needs to be resolved. These toxic chemicals can trigger stress reactions that lead to inflammation and weaken the body’s defense against other pathogens. Some chemicals accumulate in organs; another concern is their potential interaction with biological pathways inside the body, which may affect the regulatory mechanisms of enzymes and other proteins. Many other chemicals have proved toxic to human tissue and cell cultures, by producing increased oxidative stress, inflammatory cytokine production and cell death. Studies have demonstrated the potential for chemicals to cause DNA mutation and resulting cell death. Internal phenomena such as metabolism, errors of DNA replication, inflammation, and oxidative stress may be of importance. Inflammatory diseases, oxidative stress, and radiation exposure have been associated with the generation of clastogenic factors which may be quite persistent and might play an important role in carcinogenesis and thereby, in the association between CAs and cancer. Furthermore, availability of micronutrients, such as folate, appears to be important for maintaining genome integrity [48].

The CA test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test, because they appear to act through mechanisms other than direct DNA damage.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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