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Chitosan Oligosaccharide Inhibits $^{203}HgCl_2$-Induced Genotoxicity in Mice: Micronuclei Occurrence and Chromosomal Aberration

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The purpose of this study was to investigate the safety of chitosan oligosaccharide and the effects of chitosan oligosaccharide on mercury induced genotoxicity in mice using the micronuclei and chromosome aberration. The micronuclei test was performed by microscopic examination (×1,000, stained using a May-Grunwald solution) after administering 0.01, 0.1, and 1% (10 mg/mL) chitosan oligosaccharide for 7, 60, and 180 days *ad libitum* in mice. Total micronuclei of 1,000 polychromatic erythrocytes were recorded for each group. There was no difference between the untreated and experimental groups. The intake periods and concentrations of chitosan oligosaccharide did not affect the occurrence of micronuclei in bone marrow cells (P>0.05). The chromosomal aberration test was performed by microscopic examination (×1,000, stained using a 4% Giemsa solution) after administering the same concentration of chitosan oligosaccharide to mice, in F_1, F_2, F_3 generations and parents. The frequency of chromosomal aberrations was defined as [Ydr = (D+R)/total number of counted lymphocytes]. Similar to the micronuclei test, there was no difference between the untreated and treated groups. These results showed that the intake periods and concentrations of chitosan oligosaccharide did not affect chromosomal aberrations in bone marrow cells (P>0.05). To investigate the effect of chitosan oligosaccharide on mercury-induced chromosome aberration, mice in each condition were supplied with $^{203}HgCl_2$ and chitosan oligosaccharide *ad libitum*. Chitosan oligosaccharide significantly inhibited $^{203}HgCl_2$-induced chromosome aberration in mice. Based on the results of this study, it may be concluded that the chitosan oligosaccharide is a nontoxic material that could be used as a suppressor of heavy metal-induced genotoxicity.

Key words: Chitosan oligosaccharide, Mercury, Micronucleus, Chromosomal aberration

INTRODUCTION

Chitin is the second most abundant natural polymer, and can be found in various sources, especially in fungus, yeast, cuttle-fish, crabs, shrimp, animal-planktons and crustaceans. The structure is a linear β (1-4) homoglycan composed of 2-acetamido-2-deoxy-D-glucopyranosyl (GlcNAc) residue. However, there are restrictions on the commercial utilization of these materials. Chitosan is a deacetylated product of chitin with one of the polysaccharides having a free amino group at the C2 position of the glucose residue of cellulose (Kim *et al.*, 2001; Kim *et al.*, 1999; Kim *et al.*, 1997).

Chitin and chitosan have been called "the last biomass" and they have a number of applications. Chitosan has been used in various research fields including radioisotope chelator (Kim *et al.*, 1999; Kim *et al.*, 1997), artificial skin (Hirano *et al.*, 1991), anti-cancer (Okamoto *et al.*, 1995), wound healing (Tsurutani *et al.*, 1995), degradation of chitinase (Koga, 1993), cosmetics (Skjak *et al.*, 1998), drug delivery, cholesterol reducing agent (Tokura *et al.*, 1992), radioprotective effect (Nishimura *et al.*, 2003), and renal disease and regeneration (Yoon *et al.*, 2003; Yoon *et al.*, 2004).

Cytogenetic changes were induced by physical or chemical mutagens and carcinogens and could be investigated using cytogenetic tests including chromosome aberrations, micronuclei, sister chromatid exchange (SCE), or fluorescence *in situ* hybridization (FISH) (Heddle, 1973;

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Lawrence and Benford, 1991; Padovani *et al.*, 1997; Wolff, 1991).

Chromosomal aberrations are induced by a variety of mutagens, and were increased by misrepair or misreplication of lesions in the DNA (Natarajan and Obe, 1984).

Chromosome aberrations can be regarded as a very important and useful indicator of radiation or exposure to chemicals (Padovani *et al.*, 1997).

Light-microscopical analysis of structural chromosomal aberrations was developed 30 years ago and has become the standardized technique (Romm and Stwphan, 1990).

The micronucleus method applied to the lymphocyte culture system has the potential to also become a standard technique since measurement of the baseline number of micronuclei is simple and rapid, and can be used for population screening (Fenench and Morley, 1985).

The micronucleus test is based on the observation that mitotic cells have chromatid breaks or chromatid exchanges. Ed. Note: confirm wording. Micronuclei are found in a variety of different bone marrow cells of animals treated with chemical mutagens: myoloblasts, myelocytes, erythroblasts *etc.* The majority, however, are observed in polychromatic erythrocytes (Ledebur and Schmid, 1973). These fragments are always much smaller than the principal nucleus and are therefore called micronuclei. In addition, micronuclei typically found have a diameter of about 1/20 to 1/50 compared with erythrocytes (Schmid, 1975).

Micronuclei are counted separately in normochromatic and polychromatic erythrocytes. Polychromatic cells are advantageous for this test because the color of polychromatic cells is blueish instead of yellow (Ledebur and Schmid, 1973).

Micronuclei induction in polychromatic erythrocytes from treated mice is routinely used to screen chemicals for genotoxicity *in vivo* (Ramalho *et al.*, 1988).

The micronuclei test has been widely used as a measurement of chromosome aberration in mutagen-exposed cells. The micronucleus assay is a rapid, inexpensive and less laborious system for the evaluation of mutagenicity of environmental agents, and has been extended to use *in vitro* systems using cultured mammalian, including human, cells (Wakata and Sasaki, 1987).

During the past few years, many cases of mercury poisoning have been reported, including 25 infants from Minamata, a town in southern Japan, who were born with brain damage from 1953 to 1971. Neurologic symptoms of these infants included cerebral palsy, chorea, ataxia, tremors, seizures, and mental retardation. In addition, similar cases were reported in Sweden and New Mexico. Mercury is a chromosomal genotoxicant that elicits the formation of micronuclei in mammalian cells (Stoiber *et al.*, 2004). A previous report examined the genotoxic effects of mercury (II), and investigated interactions with the microtubule cytoskeleton (Thier *et al.*, 2003).

In this study, we investigated the safety of chitosan oligosaccharide and the effects of chitosan oligosaccharide on mercury induced genotoxicity in mice using the micronuclei and chromosome aberration tests.

MATERIALS AND METHODS

Animals

Male ICR mice (2535g, 810 weeks old) were purchased from the Samtaco Laboratory Animal Co. (Osan, Korea). Food and water were supplied *ad libitum*.

Chitosan oligosaccharide and $^{203}HgCl_2$

Chitosan oligosaccharide was purchased from Eco Bio Co. in Korea. The molecular weight was approximately below 10,000 with a 90% degree of deacetylation (DAC). Chitosan oligosaccharide was supplied *ad libitum* after being mixed with physical saline solution in the non-toxic concentrations. All mice were supplied with the same amount of drinking water everyday. When the concentration of chitosan oligosaccharide was 1%, the amount of chitosan oligosaccharide per day was 0.01 mg/day/g of BW. Radio mercury (specific activity: 1mCi, 37 MBq/mL, 1.0 mCi/mL, pH 1.4, 630 μgHg/mL, below $^{203}HgCl_2$) was purchased from Amersham Co. in England.

Experimental groups

The experimental group for the micronuclei test was divided into four subgroups with twenty mice in each subgroup. The control group was not treated with chitosan oligosaccharide. Subgroup 1 received 0.01% (10 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 7, 60 and 180 days. Subgroup 2 received 0.1% (1 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 7, 60 and 180 days. Subgroup 3 received 1% (0.1 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 7, 60 and 180 days.

The experimental group for the chromosome aberration test was divided into seven subgroups with twenty mice in each subgroup. The control group was not treated with chitosan oligosaccharide. Parent mice received 0.1% (1 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 30 days. F_1 generation mice were born to the parent mice. F_2 generation mice were born to the F_1 generation mice. F_3 generation mice were born to the F_2 generation mice. Subgroup 1 received 1% (10 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 7, 60 and 180 days. Subgroup 2 received 0.1% (1 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 7, 60 and 180 days. Subgroup 3 received 0.01% (0.1 mg/mL) chitosan oligo-

saccharide supplied *ad libitum* for 7, 60 and 180 days.

The $^{203}HgCl_2$ experimental group for the chromosome aberration test was divided into seven subgroups with twenty mice in each subgroup. Subgroup 1 was fed general food after being administered $^{203}HgCl_2$ orally. Subgroup 2 was administered $^{203}HgCl_2$ orally after the mice received 0.1% (1 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 30 days. Subgroup 3 was administered $^{203}HgCl_2$ orally after the mice received 0.5% (5 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 30 days. Subgroup 4 was administered $^{203}HgCl_2$ orally after the mice received 1% (10 mg/mL) chitosan oligosaccharide supplied *ad libitum* for 30 days. Subgroup 5 was injected with 0.1% chitosan oligosaccharide after the mice were administered $^{203}HgCl_2$ orally. Subgroup 6 was injected with 0.5% chitosan oligosaccharide after the mice were administered $^{203}HgCl_2$ orally. Subgroup 7 was injected with 1% chitosan oligosaccharide after the mice were administered $^{203}HgCl_2$ orally.

Micronuclei test

The bone marrow cells from the femur were placed in a centrifuge tube containing 2 mL of fetal bovine serum. The resulting cell suspension was centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in the remaining fluid and further dissociated by pipetting in and out of a Pasteur pipette. Slides were prepared from the cell button and stained using a May-Grunwald solution. A total of 1,000 polychromatic erythrocytes were scored for each group.

Chromosomal aberration test

The bone marrow cells from the femur were placed in a centrifuge tube containing 5 mL of RPMI 1640 medium, incubated for 2 h in a 5% CO_2 incubator, and then added to 0.2 mL colcemid (final concentration 0.1 ug/mL). Following the incubation, the cells were harvested, treated with an hypotonic solution containing 0.075 M KCl and fixed with a mixture of methanol: glacial acetic acid 3:1. Giemsa staining was applied to the slides on which metaphases were spread and finally counted for chromosomal aberrations at 100x using a standard light microscope. Number of ring-form (R) and dicentric (D) chromosomes was counted under the light microscope, and used to calculate the frequency of chromosomal aberrations, Ydr, which was defined as [Ydr = (D+R)/total number of counted lymphocytes]. A total of 500 chromosome cells were scored for each group.

Statistic analysis

Data was reported as mean ± S.D. of 20 mice per treatment subgroup. ANOVA was used to calculate *P*-values.

RESULTS

The effects of chitosan oligosaccharide on the frequency of Micronuclei occurrence

The effects of chitosan oligosaccharide on the frequency of micronuclei occurrence in bone marrow cells from mice are shown in Table I. The micronuclei test was performed by microscopic examination after administering 0.01, 0.1, and 1% (10 mg/mL) chitosan oligosaccharide for 7, 60, and 180 days *ad libitum*. There was no difference between the untreated and treated groups. The results showed that the intake periods and concentrations of chitosan oligosaccharide did not affect micronuclei occurrence in these experimental concentrations (P>0.05).

Chromosomal aberrations test by administration of chitosan oligosaccharide

To investigate whether the administration of chitosan oligosaccharide affects the frequency of chromosomal aberrations, mice were fed on chitosan oligosaccharide in the concentration dependent manner (0, 1, 0.1, and 0.01%), in the for various feeding periods of time (7, 60, and 180 days), and in each generation (Table II~III). There was no difference between the untreated and treated groups (P>0.05). These results were showed that the chromosomal aberrations frequency of the experimental groups did not differ as compared with the untreated group. To determine on the chromosomal aberrations of for each generation, mice were fed on 1% chitosan oligosaccharide for 30 days and were referred to as the 'Parent' mice. F_1 generation mice was were born to the parent mice, F_2 generation mice were born to the F_1 mice, and F_3 generation mice were born to the F_2 mice. The chromosomal aberrations frequency of each group was: Parent; 0.001 ± 0.000, F_1; 0.003 ± 0.001, F_2; 0.002 ± 0.000, F_3; 0.002 ± 0.000 (P>0.05). There was no difference between the untreated and treated groups. The results showed that the intake periods and concentrations of chitosan oligosaccharide, and each generation did not affect the chromosomal aberrations in these experimental concentrations (P>0.05).

Table I. Changes of micronuclei number after chitosan oligosaccharide treatment

Days Treatment	7[a]	60[b]	180[c]
Control	1.0±0.5	1.0±0.9	0.9±0.9
Group 1 (0.01%)	1.8±0.7	2.0±0.8	–
Group 1 (0.1%)	1.6±0.6	2.4±0.9	1.2±0.3
Group 1 (1%)	1.8±0.7	1.8±0.8	0.9±0.2

a: P>0.05, b: P>0.01, c: P>0.0 vs Control.

Table II. Changes of chromosomal aberration after chitosan oligosaccharide treatment

	Total cell number	Chromosomal aberration			Ydr
		D	R	A	
Control	1000	1	1	0	0.002±0.000
Group 1 (0.01%)					
7day[a]	1000	1	1	0	0.002±0.000
60 day[b]	1000	1	1	0	0.002±0.000
180 day[c]	1000	2	1	0	0.003±0.002
Group 2 (0.1%)					
7day[a]	1000	1	1	0	0.002±0.001
60 day[b]	1000	1	1	0	0.001±0.001
180 day[c]	1000	2	1	0	0.003±0.001
Group 3 (1%)					
7day[a]	1000	2	0	0	0.002±0.000
60 day[b]	1000	2	1	0	0.003±0.001
180 day[c]	1000	2	1	0	0.003±0.002

a: P>0.05, b: P>0.05, c: P>0.05. vs Control
D: dicentric chromosome, R: ring chromosome, A: acentric chromosome
Ydr = (D+R)/total number of counted lymphocytes

Table III. Chromosomal aberration after the treatment of chitosan oligosaccharide in each generation mice

	Total cell number	Chromosomal aberration			Ydr
		D	R	A	
Control	1000	1	1	0	0.002±0.000
Parent	1000	1	0	0	0.001±0.000
F_1	1000	2	1	0	0.003±0.001
F_2	1000	1	1	0	0.002±0.000
F_3	1000	1	1	0	0.002±0.000

P>0.05 as compared with control
D: dicentric chromosome, R: ring chromosome, A: acentric chromosome
Ydr = (D+R)/total number of counted lymphocytes

Blocking the chromosomal aberration by chitosan oligosaccharide after contaminated radiomercury ($^{203}HgCl_2$) in mice

We investigated whether chitosan oligosaccharid inhibited radio-mercury induced chromosomal aberrations in mouse bone marrow cells (Table IV). In three subgroups mice received chitosan oligosaccharide (0.1, 0.5, and 1%) supplied *ad libitum* for 30 days before oral administration of radio-mercury (subgroup 2, 3, and 4). In the other three subgroups mice were administered the radio-mercury orally, and then received the chitosan oligosaccharide (0.1, 0.5, and 1%) (subgroup 5, 6, and 7). After 6 h, Ydr value of subgroup 1 (only radio-mercury treated group) increased from 0.002 ± 0.000 to 0.106 ± 0.012 compared with untreated group. However, Ydr value of chitosan pre and post feeding groups decreased significantly compared with the control group (subgroup 2: 0.028 ± 0.004, subgroup 3: 0.044 ± 0.008, subgroup 4: 0.084 ± 0.012, subgroup 5: 0.050 ± 0.012, and subgroup 6: 0.034 ± 0.004). The results showed that chitosan oligosaccharide inhibited radio-mercury induced chromo-somal aberrations in mice (P<0.01).

Table IV. Blocking the chromosomal aberration by chitosan oligosaccharide after contaminate with radiomercury ($^{203}HgCl_2$) in mice at 6 h

	Total cell number	Chromosomal aberration			Ydr
		D	R	A	
Group 1	500	50	3	0	0.106±0.012
Chitosan prefeeding					
Group 2[+]	500	12	2	0	0.028±0.004
Group 3[+]	500	19	3	0	0.044±0.008
Group 4	500	39	3	0	0.084±0.012
Chitosan postfeeding					
Group 5[+]	500	23	2	0	0.050±0.012
Group 6[+]	500	12	5	0	0.034±0.004
Group 7	500	65	2	0	0.134±0.005

[+] P<0.01 as compared with control
Group 1. General food was fed after per oral contamination of radiomercury ($^{203}HgCl_2$).
Group 2. 0.1% chitosanoligosaccharide was supplied by feeding *ad libitum* for 30 days before per oral contamination of radiomercury ($^{203}HgCl_2$).
Group 3. 0.5% chitosanoligosaccharide was supplied by feeding *ad libitum* for 30 days before per oral contamination of radiomercury ($^{203}HgCl_2$).
Group 4. 1% chitosanoligosaccharide was supplied by feeding *ad libitum* for 30 days before per oral contamination of radiomercury ($^{203}HgCl_2$).
Group 5. 0.1% chitosanoligosaccharide was supplied by feeding after per oral contamination of radiomercury ($^{203}HgCl_2$).
Group 6. 0.5% chitosanoligosaccharide was supplied by feeding after per oral contamination of radiomercury ($^{203}HgCl_2$).
Group 7. 1% chitosanoligosaccharide was supplied by feeding after per oral contamination of radiomercury ($^{203}HgCl_2$).

DISCUSSION

Chitosan has been used in the various research fields. However, the safety of chitosan has not been researched in cytogenetic fields. In this study, we investigated the safety of chitosan oligosaccharide and the effects of chitosan oligosaccharide on mercury induced genotoxicity in mice using the micronuclei and chromosome aberration tests.

Schmid *et al.* (1975) reported that the frequency of

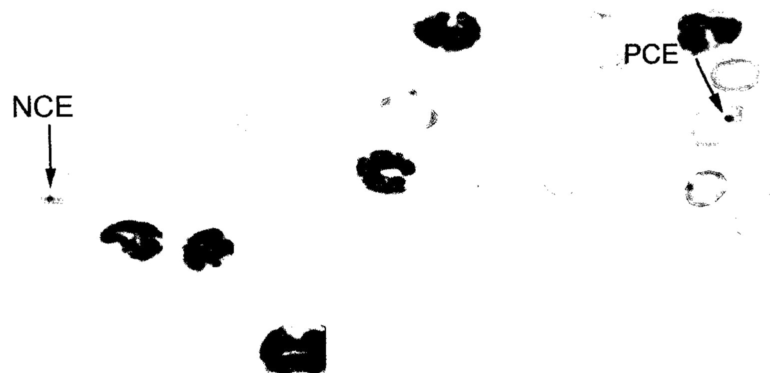

Fig. 1. A photomicrograph of mouse bone marrow cells stains with wright-giemsa method. Micronucleated polychromatic erythrocyte (PCE), and normochromatic erythrocyte (NCE) are indicated by arrows (×1,000).

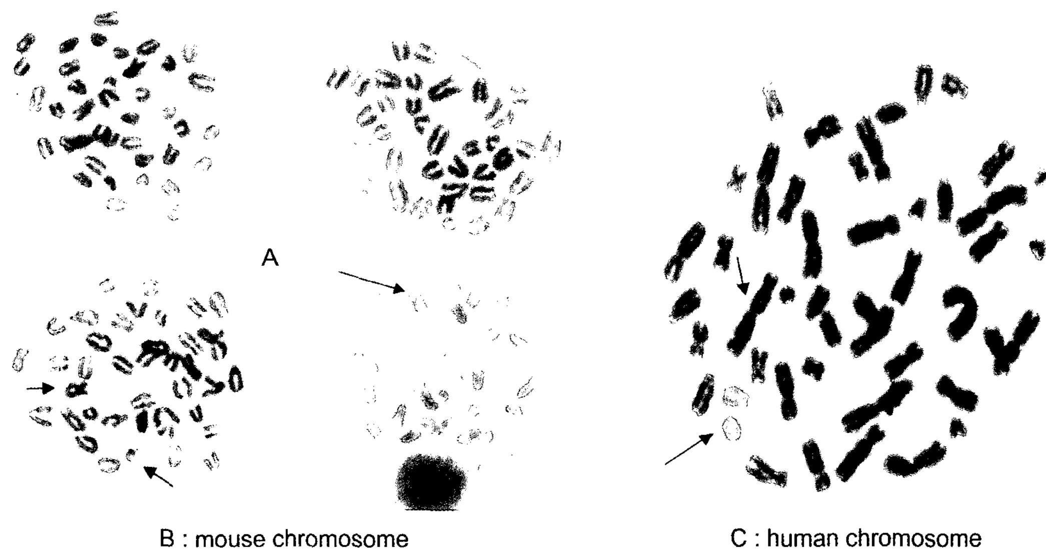

Fig. 2. Chromosomal aberration in metaphase (A: normal chromosomes, B~C: aberrant chromosomes)

micronuclei was 0-5/1,000 in normal mice. French *et al.* (1985) reported that the frequency of micronuclei was 4.4 ± 2.6 in normal human peripheral blood. Also, Ledebur *et al.* (1973) reported that trenimon (2,3,5-trisethyleneimino-benzoquinone-1,4) increased the frequencies of micronuclei at 24 h. Sudharsan and Heddle (1980) investigated the frequency of micronuclei in Chinese hamster ovary cells (CHO) after 8 different chemicals (acriflavin, acridine yellow, actinomycin D, 5-bromo-2-deoxy uridine, chloroquine, daunomycin, ethidium bromide and propidium iodide) were mixed to 5, 10, 50, and 100 μM in CHO. Except for chloroquine and daunomycin, all the other chemicals increased the frequency of micronuclei as the concentrations of the chemicals increased. Darina and Josef (1995) reported that carboxymethyl-chitin-glucan did not cause any cytogenetic damage to micronuclei of female ICR mice. In this study, chitosan oligosaccharide was supplied in a concentration dependent manner *ad libitum* in mice and we investigated the toxicity of chitosan oligosaccharide compared with each concentration and administration days. The chitosan subgroups were not significantly difference compared with the untreated control group (P>0.05). In addition, there was no difference in the results based on various concentrations of chitosan oligosaccharides.

Guadano *et al.* (1998) reported that rotenone, a type of pesticide, increased the frequency of micronuclei because of a delay in the cell cycle.

Rauscher *et al.* (1998) reported that it was extracts carotenoids lycopene, canthaxanthin, lutein, β-cryptoxanthin

from fresh fruits and vegetables. They observed that the frequency of micronuclei decreased about 50% after administering benzo [α] pyrene (BaP) and cyclophosphamide (CP) in mice bone marrow cells.

Jagetia and Aruna (1999) investigated the frequency of micronuclei after the acyclovir [9-(2-hydrox yethoxy methyl) guanine, ACV], which has possible anticancer compounds and very low toxicity, was mixed with the HeLa cell line. The reported that a concentration of 100 μM (0.55 mg/kg) increased the frequency of micronuclei higher than other concentrations. In addition, a concentration of 250 μg/mL increased chromosomal aberrations.

Lin *et al.* (1998) investigated the frequency of micronuclei based on increasing doses of sodium nitroprusside (SNP).

Goncharova *et al.* (2001) administered 1,4-dihydroisonicotinic acid (1,4-DHINA) by intraperitoneal injection to mice. The ethyl methane-sulfonate decreased the frequency of micronuclei in the mice.

In this study, we investigated the frequency of micronuclei based on administration dates and concentrations. However, there was no change in the frequency of micronuclei compared with control.

Robbiano *et al.* (1998) investigated the effects of halothane, enflurane, isoflurane, sevoflurane, chloroform and trichloro-ethylene, which are types of anesthesia, administered orally. Except for halothane and trichloroethylene, all the others increased the frequency of micronuclei.

Cytostatic agents, such as adriamycin and peptichemio, have been used as anti tumor therapecutic agents. Stacher *et al.* (1974) investigated chromosomal aberration after adriamycin and peptichemio were added to human peripheral blood. In the case of adriamycin, concentrations of 0.01 and 0.1 μg/mL resulted in rates of 17 and 93%, and in the case of peptichemio, concentrations of 0.5 and 5 μg/mL resulted in rates of 11 and 58%.

Ethylene oxide has been used in cosmetics, medicines and germicidal agents. Garcia *et al.* (2001) investigated genetic toxicity induced by ethylene oxide in rats after chronic inhalation for 4 weeks. Concentrations of from 50 to 200 ppm did not induce micronucleus and chromosome aberrations. On the other hand, in the case of acute toxicity by intraperitoneal injection, concentrations of from 50 to 100 ppm increased micronucleus and chromosome aberrations.

In the study, we confirmed that chitosan oligosaccharide did not affect the development frequency of micronuclei, chromosomal aberrations (P>0.05). However, in the 60 days feeding group, the development frequency of micronuclei increased somewhat, and in the 180 day feeding group, it recovered. Based on cytogenetic tests including micronuclei and chromosome aberration it may be concluded that chitosan oligosaccharide is a nontoxic material.

It was not report that chitosan oligosaccharide affected toxic material-induced chromosome aberration. In this experiment, chromosome aberration was induced by $^{203}HgCl_2$ in mice and mice were supplied with chitosan oligosaccharide *ad libitum* in each condition. Thier *et al.* (2003) examined the genotoxic effects of mercury (II), and investigated interactions with the microtubule cytoskeleton.

Bala and Rao (1993) reported that gamma-linoleic acid, an essential derived-fatty acid, significantly inhibited methyl mercury chloride (MMC)-induced chromosome aberration in human peripheral blood. In another study, it was reported that $^{203}HgCl_2$-chelated chitosan was excreted in the feces of mice (Yoon *et al.*, 2005). In this study, chitosan oligosaccharide significantly inhibited $^{203}HgCl_2$-induced chromosome aberration in mice. These data showed that chitosan oligosaccharide could be used to suppress heavy metal-induced genotoxicity because mercury, which was chelated with chitosan oligosaccharide, was excreted in the feces. The effects of 1% chitosan oligosaccharide on $^{203}HgCl_2$-induced chromosome aberration were not clearly understood. In order to understand this interaction more clearly more cytotoxic tests need to be performed.

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