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Antimutagenic Effects of Ginsenoside Rb_1, Rg_1 in the CHO-K1 Cells by Benzo[a]pyrene with Chromosomal Aberration Test and Comet Assay

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Abstract

The usage and types of chemicals are advancing, specializing, large-scaled increasing, and new chemical exposed workers are concerning to occupational disease. The generation of reactive oxygen in the body from carcinogen, mutation and DNA damage in cancer is protected by natural antioxidants (phytochemicals) with antimutagenic effect. There were many reports of ginsenoside Rb_1, Rg_1 grievances of the genetic mutation to suppress the effect confirm the genetic toxicity test with chromosomal aberration test and the Comet (SCGE) assay confirmed the suppression effect occurring chromosomal DNA damage. We had wanted to evaluate the compatibility and sensitivity between the chromosomal aberration (CA) test and the Comet assay. We used the CA test and Comet assay to evaluate the anti-genotoxicity of ginsenoside Rb_1 and Rg_1, in CHO-K1 (Chinese hamster ovary fibroblast) cell *in vitro*, composed negative control (solvent), positive control (benzo[a]pyrene), test group (carcinogen+variety concentration of ginsenoside) group. The positive control was benzo[a]pyrene (50 μM), well-known carcinogen, and the negative control was the 1% DMSO solvent. The test group was a variety concentration of ginsenoside Rb_1, Rg_1 with 10^{-8}%, 10^{-6}%, 10^{-4}%, 10^{-2}%, 1%, 10%. In chromosomal aberration test, we measured the number of cells with abnormally structured chromosome. In Comet assay, the Olive tail moment (OTM) and Tail length (TL) values were measured. The ratio of cell proliferation was increased 8.3% in 10^{-8}%, 10^{-6}%, 10^{-4}%, 10^{-2}%, 1%, 10% Rb_1 treated groups, and increased 10.4% in 10^{-10}%, 10^{-8}%, 10^{-6}%, 10^{-4}%, 10^{-2}%, 1% Rg_1 treated groups. In the CA test, the number of chromosomal aberration was decreased all the Rb_1 and Rg_1 treated groups. In the Comet assay, the OTM values were decreased in all the Rb_1 and Rg_1 treated groups. To evaluate the compatibility between CA and Comet assay, we compared the reducing ratio of chromosomal abnormalities with its OTM values, it was identified the antimutagenicity of ginsenoside, but it was more sensitive the CA test than the Comet assay. Ginsenoside Rb_1 and Rg_1 significantly decrease the number of cells with chromosomal aberration, and decrease the extent of DNA migration. Therefore, ginsenoside Rb_1, Rg_1 are thought as an antioxidant phytochemicals to protect mutagenicity. The *in vitro* Comet assay seems to be less sensitive than the *in vitro* chromosomal aberration test.

Keywords: Mutagenicity, Genotoxicity, Phytochemicals, Ginsenoside Rb_1, Rg_1, Benzo[a]pyrene, Chromosomal aberration (CA), Comet assay, Olive tail moment (OTM), Tail length (TL)

Recently, it has being much attractive the chemoprevention to strengthen the defense capabilities to cancer with safe chemicals including phytochemicals originated from food. The chemoprevention of cancer is recognized a quite effective way unlike the existing chemotherapy for the elimination of cancer cells to administer anticancer agents, but block off or delay the progress of normal to cancer[1]. In this viewpoint, the phytochemicals contained in plants function not only eliminate reactive oxygen species directly binding to them, but also fundamentally reinforce the capability to lead antioxidative gene expression *in vivo*.

We verified the inhibition effect on chromosomal aberration and DNA damages with ginsenoside Rb_1, Rg_1 among the phytochemicals reported their cancer prevention or antioxidative effect, and compared chromosomal aberration test with Comet assay to evaluate genotoxicity. We selected the natural phytochemicals,

ginsenoside Rb_1 and Rg_1 which was known that inhibit the catalytic activity of CYP2C9 and CYP3A4 related to the process of oxidizing xenobiotics and endogenous compounds in microsome of human liver cell[2], and easily hydrolysis in stomach from the pharmacodynamic studies of ginsenoside Rb_1, Rb_2 and Rg_1 with rat[3].

It was reported the ginsenoside Rb_1, Rg_1 are mainly absorbed by simple passive diffusion process[4], the Rb_1, Rb_2, Rb_3 and Re have antioxidative effect in cardiovascular cell, but Rd do not have these effects[5]. The Rb_1 participate to Th1 immune response with autoimmunity, Th2 immune response with IgE-mediated allergies[6], and the Rb_1 functions as estrogen to bind and activate the estrogen receptor in study with MCF-7 human breast cancer cells[7]. In was confirmed that the Re, Rg_1, Rg_2 and Rh prevent the Ca^{2+} channels, the Rf functions in brain[8]. The Rg_1 cures dementia and improve the learning and memory[9], the Rg_1 relaxes the blood vessel in lung and activates the secretion of NO, a neurotransmitter[10]. Park *et al.* studied the effect of ginsenosides decreasing the repair of DNA strand breakage treat with benzo[a]pyrene by using of S-15 fraction as a metabolic activator in CHO-K1 cell[11]. In 18th century, benzo[a]pyrene became clear as a coal tar component, the occupational carcinogen caused scrotum cancer of chimney cleaning workers in England. It was easily found at diesel exhaust, the smoke of cigarette, marihuana and wood, the food roasted with charcoal fire, burned bread, steak with well-done, chicken with skin, hamburger, etc. It was confirmed the CYP1A1 is a major factor to defense the benzo[a]pyrene by using the cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1) knock-out mice. It is assumed that much benzo[a]pyrene are accumulated in the case of CYP1A1 elimination, biochemically activated (enzymatically metabolized) to benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide as a pro-carcinogen. This chemical binds covalently with N2 position of nucleophilic guanine bases, twists and changes the DNA structure[12]. It inhibits the DNA replication, causes mutation and carcinogenesis in this ways. It is similar to the carcinogenesis mechanism of aflatoxin with binding to guanine N7 position[13]. The p53 gene is a transcription factor related to self-defense, regulates cell division and inhibits the cancer, but the benzo[a]pyrene diol epoxide activates the cancer mechanism of p53 with G (guanine) → T (thymidine) transversion.

In this study, we performed to confirm the inhibitory effects of natural phytochemicals, the ginsenoside Rb_1, Rg_1 as ginseng extracts, on the genetic mutagenicity with two tests used in genotoxicity evaluation, the chromosomal aberration test and FLARE/Comet assay with treatment of benzo[a]pyrene.

Table 1. The cyto-toxicity test treated with ginsenoside Rb_1, Rg_1 in CHO-K1 cell.

Treatment	Ginsenoside Rb_1	Ginsenoside Rg_1
Control	1.084±0.125	1.172±0.117
10^{-10}%	1.170±0.099	1.312±0.119
10^{-8}%	1.161±0.106	1.279±0.145
10^{-6}%	1.155±0.142	1.285±0.158
10^{-4}%	1.197±0.148	1.293±0.165
10^{-2}%	1.178±0.155	1.279±0.183
1%	1.175±0.117	1.317±0.176
10%	1.185±0.130	1.062±0.062

Unit: Absorbance (Optical Density; OD) at 450 nm
All values are expressed as mean±S.D.

Cyto-toxicity of Ginsenoside Rb_1, Rg_1

In the cyto-toxicity test of ginsenoside Rb_1, Rg_1 with cell counting kit-8, we found the ratio of cell proliferation was increased 8.3% in 10^{-8}%, 10^{-6}%, 10^{-4}%, 10^{-2}%, 1%, 10% Rb_1 treated groups, but did not have statistical significance, and 10.4% increased in 10^{-10}%, 10^{-8}%, 10^{-6}%, 10^{-4}%, 10^{-2}%, 1% Rg_1 treated groups. According to the results of cyto-toxic test shown in Table 1, the test group with a variety concentration (10^{-8}%, 10^{-6}%, 10^{-4}%, 10^{-2}%, 1%, 10%) of ginsenoside Rb_1, Rg_1.

Chromosomal Aberration Reducing Effect of Ginsenoside Rb_1, Rg_1

In the CA test, the ratio of chromosomal aberration was decreased in all the Rb_1 and Rg_1 treated groups, and the effects of ginsenoside Rb_1 were shown in Table 2.

The polyploidy was not observed in both direct and metabolism activated method. Below 5% chromosomal aberration was observed in positive control and all the Rb_1 treated groups with direct (−S9 mix) method, especially in the positive control was so low to detect. The positive results were that 11.5% (*−gap*), 14.0% (*+gap*) chromosomal aberration in positive control group with metabolism activation (+S9 mix), especially more in chromatid exchange (*cte*) type than *gap* type. The negative results were that 1.5% (*−gap*), 3.0% (*+gap*) chromosomal aberration in negative control group, both 1.5% *gap* and chromatid exchange (*cte*) were observed. The chromosomal aberration of all the ginsenoside Rb_1 treated groups were less than positive control, but it was appeared 11.0% (*−gap*), 12.5% (*+gap*), the positive result in the 10^{-8}% Rb_1 treated group. It was reverse dose-response relations that the higher concentration leads to less chromosomal aberrations in all groups except the 10^{-8}%. So it was remarkably low in 1.0%, 10% Rb_1 treated group that we were able to confirm the preventive effect of Rb_1 in CA. It was not shown the big difference of

Table 2. The chromosomal aberration test treated with ginsenoside Rb_1 in CHO-K1 cell.

Treat.	Conc.	No. and ratio of chromosomal structure abnormality							Decision
		gap	Chromatid type		Chromosome type		Total		
		g	ctb	cte	csb	cse	−g	+g	
Direct (−S9)	−Control	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	−
	10^{-8}%	4(2.0)	2(2.0)	0(0.0)	0(0.0)	0(0.0)	4(2.0)	8(4.0)	−
	10^{-6}%	4(2.0)	3(1.5)	0(0.0)	0(0.0)	0(0.0)	3(1.5)	7(3.5)	−
	10^{-4}%	2(1.0)	1(0.5)	1(0.5)	0(0.0)	0(0.0)	2(1.0)	4(2.0)	−
	10^{-2}%	2(1.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(1.0)	−
	1.0%	3(1.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	3(1.5)	−
	10.0%	2(1.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(1.0)	−
	+control	2(1.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(1.0)	−
Metabolism activated (+S9)	−Control	3(1.5)	0(0.0)	3(1.5)	0(0.0)	0(0.0)	3(1.5)	6(3.0)	−
	10^{-8}%	3(1.5)	0(0.0)	22(11.0)	0(0.0)	0(0.0)	22(11.0)	25(12.5)	+
	10^{-6}%	5(2.5)	0(0.0)	13(6.5)	0(0.0)	0(0.0)	13(6.5)	18(9.0)	±
	10^{-4}%	7(3.5)	2(1.0)	13(6.5)	1(0.5)	0(0.0)	16(8.0)	23(11.5)	±
	10^{-2}%	3(1.5)	0(0.0)	15(7.5)	0(0.0)	0(0.0)	15(7.5)	18(9.0)	±
	1.0%	5(2.5)	0(0.0)	11(5.5)	0(0.0)	0(0.0)	7(3.5)	16(8.0)	−
	10.0%	5(2.5)	0(0.0)	5(2.5)	0(0.0)	0(0.0)	5(2.5)	10(5.0)	−
	+Control	5(2.5)	0(0.0)	23(11.5)	0(0.0)	0(0.0)	23(11.5)	28(14.0)	+

g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; ( ), the average

Table 3. The chromosomal aberration test treated with ginsenoside Rg_1 in CHO-K1 cell.

Treat.	Conc.	No. and ratio of chromosomal structure abnormality							Decistion
		gap	Chromatid type		Chromosome type		Total		
		g	ctb	cte	csb	cse	−g	+g	
Direct method	−Control	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0))	−
	10^{-8}%	2(1.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(1.0)	−
	10^{-6}%	4(2.0)	3(1.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4(2.0)	−
	10^{-4}%	4(2.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4(2.0)	−
	10^{-2}%	3(1.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	3(1.5)	−
	1.0%	4(2.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4(2.0)	−
	10.0%	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	−
	+Control	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	−
Metabolism activated method	−Control	2(1.0)	0(0.0)	2(1.0)	0(0.0)	0(0.0)	2(1.0)	4(2.0)	−
	10^{-8}%	9(4.5)	0(0.0)	5(2.5)	0(0.0)	0(0.0)	5(2.5)	14(7.5)	−
	10^{-6}%	7(3.5)	1(0.5)	7(3.5)	0(0.0)	0(0.0)	8(4.0)	15(7.5)	−
	10^{-4}%	6(3.0)	0(0.0)	8(4.0)	0(0.0)	0(0.0)	8(4.0)	14(7.0)	−
	10^{-2}%	7(3.5)	0(0.0)	7(3.5)	0(0.0)	2(1.0)	9(4.5)	16(8.0)	−
	1.0%	5(2.5)	0(0.0)	4(2.0)	0(0.0)	2(1.0)	6(3.0)	11(5.5)	−
	10.0%	7(3.5)	4(2.0)	0(0.0)	0(0.0)	0(0.0)	4(2.0)	11(5.5)	−
	+Control	16(8.0)	4(2.0)	10(5.0)	0(0.0)	8(4.0)	22(11.0)	38(19.0)	+ diploid 1(0.5)

g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; ( ), the average

1.5-3.5% *gap* type chromosomal aberration, the chromatid exchange (*cte*) was decreased to 2.5% in 10% Rb_1 treated group than positive control (11.5%).

Table 3 was shown that the effect of ginsenoside Rg_1 to chromosomal damage from benzo[a]pyrene. It was not observed a polyploidy in both direct and metabolism activated method with Rb_1 treated group, but 1 cell was detected as a diploid in metabolic activated positive control. Below 2% chromosomal aberration was observed in positive control and all the Rg_1 treated groups by direct (−S9 mix) method, it was not observed the effect of Rg_1 on the DNA damage with benzo[a]pyrene. By metabolism activated (+S9) method, the positive result was shown in positive control (benzo[a]pyrene) as 11.0% (−*gap*), 19.0 (+*gap*), respectively. Also, all of the Rg_1 treated groups were low-

Table 4. The effect (%) of ginsenoside Rb_1, Rg_1 to the chromosomal aberration decreased.

Treatment	Ginsenoside Rb_1	Ginsenoside Rg_1
10^{-8}%	4.3	77.3
10^{-6}%	43.5	63.6
10^{-4}%	30.4	63.6
10^{-2}%	34.8	59.1
1%	69.6	72.7
10%	78.3	81.8

Unit: %

er than the positive control group, especially the 10.0% Rg_1 treated group was so low that could not detect its preventive affects of chromosomal aberration with benzo[a]pyrene. It was not observed the dose-response relation in all Rg_1 treated group, but it could observe in 10^{-2}%, 1%, 10% treated groups.

Comparing the decreasing effect of chromosomal aberration between ginsenoside Rb_1 and Rg_1, the Rg_1 more inhibits chromosomal aberration than Rb_1 (Table 4).

Antioxidative DNA Damage Reducing Effect of Ginsenoside Rb_1, Rg_1

In the Comet assay, the OTM values were decreased in all the Rb_1 and Rg_1 treated groups (Table 5, 6). The OTM value of positive control were 2.8 times, and the TL values were 1.9 times more than the negative control, it was shown that the DNA damages was occurred with benzo[a]pyrene. The Rb_1 treated groups except 1% were shown the dose-response relations, and the preventive effect of DNA damage in 10^{-4}%, 10% Rb_1 treated groups, have statistical significance by Dunn's Method ($P<0.05$).

TL values with ginsenoside Rb_1 were not shown the dose-response relations, but smaller than those of positive control. Table 6 was shown the OTM and TL values in Rg_1 treated CHO-K1 cells. The OTM values of positive control were 4.8 times, and the TL values were 1.9 times more than the negative control, it was also shown that the DNA damages with benzo[a]pyrene. The Rg_1 treated groups show the dose-response relations, and preventive effect of DNA damage in 10^{-2}%, 10^{-4}%, 10% Rg_1 treated groups, have statistical significance with Dunn's Method ($P<0.05$). TL values with Rg_1 were smaller than those of positive control, but not shown the dose-response relations.

At the results of comparing the OTM decreasing rate with positive control for the inhibition of DNA damages between the ginsenoside Rb_1 and Rg_1, it was shown that the inhibition rate of DNA damage in 10% treated group of ginsenoside Rb_1 and Rg_1 as 62.0-72.2 %, appeared the higher inhibition effect to DNA damage (Table 7). Also, the difference of OTM decreasing rate between these two chemicals was not observed.

Table 5. The effect of ginsenoside Rb_1 to the inhibition of DNA damage (OTM & TL).

Treatment	Olive tail moment (a.u.)	Tail length (μm)
Negative-Control	5.55±3.01	69.44±38.32
Positive-Control	15.43±6.90	133.53±83.39
10^{-8}%	12.97±5.49	103.97±39.73
10^{-6}%	11.42±3.92	109.12±48.32
10^{-4}%	10.79±6.07*	127.48±72.91
10^{-2}%	10.69±3.61	80.55±28.48*
1%	11.24±3.54	107.04±36.04
10%	5.87±1.80*	80.46±40.58*

Unit: a.u. (arbitrary unit)
All values are expressed as mean±S.D
positive-control: benzo[a]pyrene 50 μM
negative-control: 1% DMSO
treatment: benzo[a]pyrene 50 μM+DMSO+Rb_1
*: compare with positive control $P<0.05$

Table 6. The effect of ginsenoside Rg_1 to the inhibition of DNA damage (OTM & TL).

Treatment	Olive tail moment values (a.u.)	Tail length (μm)
Negative Control	1.93±1.94	25.59±12.67
Positive Control	9.29±5.16	57.44±19.42
10^{-8}%	7.14±4.45	40.18±18.47
10^{-6}%	7.09±4.51	38.99±19.98
10^{-4}%	6.94±4.62	46.73±19.49
10^{-2}%	5.33±4.14*	31.55±15.41
1%	4.98±3.66*	37.50±16.53
10%	2.58±2.68*	30.36±15.66

Unit: a.u. (arbitrary unit)
All values are expressed as mean±S.D
positive-control: benzo[a]pyrene 50 μM
negative-control: 1% DMSO
treatment: benzo[a]pyrene 50 μM+DMSO+Rg_1
*: compare with positive control $P<0.05$

Table 7. The effect (%) of ginsenoside Rb_1, Rg_1 to the Olive Tail Moment decreased.

Treatment	Ginsenoside Rb_1	Ginsenoside Rg_1
10^{-8}%	15.9	23.1
10^{-6}%	26.0	23.7
10^{-4}%	30.0	25.2
10^{-2}%	30.7	42.6
1%	27.1	46.4
10%	62.0	72.2

The Compatibility and Sensitivity between Chromosomal Aberration Test and Comet Assay

To evaluate the compatibility between CA and Co-

met assay, we compared the reducing ratio of chromosomal abnormalities and OTM values, it was identified the antimutagenicity of ginsenoside, but the CA test was more sensitive than the Comet assay. To study on antimutagenic effects of ginsenoside Rb_1, Rg_1, we investigated these effects in the CHO-K1 cells which were treated with 50 μM benzo[a]pyrene.

Ginsenoside Rb_1 and Rg_1 significantly decrease frequencies of cells with chromosomal aberrations, and decrease the extent of DNA migration. Therefore, ginsenoside Rb_1 and Rg_1 are thought as an antioxidant phytochemicals to protect mutagenicity. The Comet assay seems to be less sensitive than the *in vitro* chromosomal aberration test.

From the results of cyto-toxicity test, cell proliferation was increased 8.3% in Rb_1 treated groups and 10.4% increased in Rg_1 treated groups. It match with the reports that no cyto-toxicity with ginsenoside-Rb_1 treated human cancer cell (breast cancer MCF-7, skin melanoma SK-MEL-2, human ovarian carcinoma B16)[14], not much cyto-toxicity with Rb_1, Rg_1 treated human cancer cell line (*in vitro*)[15]. Specially, it was reported the Rb_1 does not reduce cyto-toxicity in HepG2 cell but compound K, the metabolite of Rb_1 reduce cyto-toxicity[16].

Below 2% chromosomal aberration was observed in positive control and all the Rg_1 treated groups with both direct (−S9 mix) and metabolism activated (+S9) method, it was the positive result in positive control (benzo[a]pyrene) as 11.0% (*−gap*), 19.0 (*+gap*), respectively. All of the Rg_1 treated groups were lower than the positive control; especially in the 10.0% Rg_1 treated group was so low to detect its preventive effects. It refers benzo[a]pyrene could induce the mutation and carcinogenesis only if it is activated with electrophilic metabolites. It was reported the occurrence of chromosomal aberration with benzo[a]pyrene is reduced with the metabolites of ginseng saponin formed by intestinal bacteria of human[17].

With comparing the decreasing effect of chromosomal aberration between ginsenoside Rb_1 and Rg_1, the Rg_1 more inhibits chromosomal aberration than Rb_1. Ginsenoside Rb_1, Rg_1 significantly decrease the cell frequencies with chromosomal aberrations, and decrease the extent of DNA migration. So the ginsenoside Rb_1, Rg_1 are thought as an antioxidant phytochemicals to prevent cell mutation.

At the results of comparing the OTM decreasing rate for the inhibition of DNA damages, it was shown dose-response relation with the Rb_1 and Rg_1, the DNA damage inhibition rate in 10% Rb_1 and Rg_1 treated group was 62.0-72.2%, appeared the higher DNA damage inhibition effect. Lee *et al.* reported the orally injected *Panax ginseng* extracts defense DNA damage from intraperitoneally injected 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) with Comet assay of peripheral lymphocytes[18]. To evaluate the compatibility between CA and Comet assay, the ability of ginsenoside Rb_1, Rg_1 to protect against mutagenicity in the CHO-K1 cells was evaluated. The Comet assay seems to be less sensitive than the *in vitro* chromosomal aberration test.

In related with cellular signal transduction mechanism, it was reported the effect of total saponins from Korean red ginseng on the biosynthesis of inositol phospholipids *in vivo*, and also effects on the metabolic enzymes, such as phosphatidylinositol-specific phospholipase C (PI-PLC) and PI-kinases[19]. It was also reported the ginseng extract inhibits genetic mutation, chromosomal deletion, chromosomal recombination with MNNG (N-Methyl-N′-nitro-N-nitrosoguanidine) in *Drosophila melanogaster*[20].

From now on, the more researches will be necessary to find out the effects and their interaction among red pepper, garlic and cabbages for close examination of anticancer or antimutagenic mechanisms. Specially, the occupational cancer can occur from genetic mutation and disablement of its repair mechanisms with formation of reactive oxygen species in workers of the heavy chemical or chemical manufacturing industries. Taking the safe foods containing anticancer and antimutagenic ingredients like natural phytochemicals that it shall be raised the ability of anticancer with exposure to hazard chemicals among workers. And it could be verified the antimutagenic or preventive effects with so many natural phytochemicals by evaluation and comparing the toxicological data *in vitro* and verifying with the other *in vivo* tests.

Materials & Methods

Cells and Chemicals

The cell which used in test is the cultivated CHO-K1 (Chinese hamster ovary fibroblast) origins. The cell was obtained from the Korean Cell Line Bank (KCLB 10061). It was cultured in F-12 medium (GIBCO BRL, USA, Lot No. 1244774) with 5% CO_2 at 37°C, sub-cultured every 2-4 days. It was used 1% DMSO solvent as a negative control and the test group was with a variety concentration of ginsenoside Rb_1 and Rg_1 with 10^{-8}, 10^{-6}, 10^{-4}, 10^{-2}, 1 and 10%. For the metabolic activated system, the S9 (MOLTOX™, Annapolis, Maryland, USA, Lot No. 2151) was used within 6 months after manufactured.

Cyto-toxic Test and Determination of Concentration

It was used benzo[a]pyrene (Sigma-Aldrich, USA,

Lot No. 027K1425) as a positive control according to the reference[12]. The ginsenoside Rb_1 (Wako Pure Chemical Industries. Ltd., Osaka, Japan, Lot No. WKH7226) and ginsenoside Rg_1 (Wako Pure Chemical Industries. Ltd., Osaka Japan, Lot No. WKG7267) were used as antioxidative phytochemicals. It was performed the cyto-toxic test with cell counting kit-8 (CCK-8, DOJIDO LABORATORIES, Tokyo, Japan) according to the protocols. The cyto-toxicity was measured by absorbance with plate reader (DIALAB, DIALAB ELX 800 UV, Austria) at 450 nm.

in vitro Mammalian Chromosomal Aberration Test

This study was performed according to OECD guidelines for the testing of chemicals[21] (*In vitro* Mammalian Chromosomal Aberration Test. Ref. OECD TG473) and Ishidate's report[22]. For direct method (24 hour treatment), it was cultured for about 3 days from the aliquot with 2×10^4-4×10^4 cells in 60 diameter plate. For metabolic activated method (6 hour treatment), it was cultured with same as direct method. The slides of chromosomal sample were made from 5 media aliquot with 18 hour supplementary culture after removal of media and washing the cell layer with 5 fresh media. The main test was performed with dosages established by cell proliferation suppression/preliminary test. After 24 hour of benzo[a]pyrene (50 μM) treat to each plates, treat the 0.2/Colcemid® (GIBCO BRL, USA, Lot No. 1402494), separate the metaphase cell after 2 hours and take away with centrifuge at 1,000 rpm for 5 min. It was made the chromosome samples after 3 times fixing with the Carnoy's sol'n (acetic acid : ethanol=1 : 3) and counted the abnormalities after dyeing with 5% Giemsa (Merck, NJ, USA, Lot No. OB513429) sol'n for 5 min. Two samples were made in each plate.

It was observed 100 cell in metaphase per plate and classified as the 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 the others) and numerical abnormalities (*pol*). However the retest was performed in case that it was not confirmed the dosage dependency and the ratio of chromosomal aberration was unusually high in control solvent. It was not performed the statistical analyses of the results.

FLARE (Fragment Length Analysis with Repair Enzyme) Assay

FLARE analysis kit (Trevigen, MD, USA) with Fpg and Endo III enzymes was used in FLARE assay. FLARE assay was performed according to the method of Tice *et al.*[23]. Electrophoresis was performed at 25 V and 100 mA for 15 min in the dark. The ethidium bromide-stained electropherograms were examined with image analysis software Komet 5.0 (Kinetic Imaging, Ltd., Liverpool, UK). The Olive tail moment and tail length were used to quantitatively measure the extent of DNA damage. All results of FLARE assay are expressed as the average and standard deviation (mean ±S.D.). One way ANOVA test and Dunn's method (a=0.05) were used with SigmaStat 3.11.

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