Environmental Mutagens & Carcinogens 21-2 : 89-94 (2001)

Optimal Conditions of Single Cell Gel Electrophoresis (Comet) Assay to detect DNA single strand breaks in Mouse Lymphoma L5178Y cells

Jae-Chun Ryu*, Oh-Seung Kwon and Hyung-Tae Kim

Toxicology Laboratory, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, Korea

(Received October 23, 2001 / Accepted November 20, 2001)

ABSTRACT : **Recently, single cell gel electrophoresis, also known as comet assay, is widely used for the detection and measurement of DNA strand breaks *in vitro* and *in vivo* in many toxicological fields such as radiation exposure, human monitoring and toxicity evaluation. As well defined, comet assay is a sensitive, rapid and visual method for the detection of DNA strand breaks in individual cells. Briefly, a small number of damaged cells suspended in a thin agarose gel on a microscope slide were lysed, unwinded, electrophoresed, and stained with a fluorescent DNA binding dye. The electric current pulled the charged DNA from the nucleus such that relaxed and broken DNA fragments migrated further. The resulting images which were subsequently named for their appearance as comets, were measured to determine the extent of DNA damages. However, some variations could be occurred in procedures, laboratories's conditions and kind of cells used. Hence, to overcome and to harmonize these matters in comet assay, International Workshop on Genotoxicity Test Procedure (IWGTP) was held with several topics including comet assay at Washington D.C. on March, 1999. In spite of some consensus in procedures and conditions in IWGTP, there are some problems still remained to be solved. In this respect, we attempted to set the practical optimal conditions in the experimental procedures such as lysis, unwinding, electrophoresis and neutralization conditions and so on. First of all, we determined optimal lysis and unwinding time by using 150 μM methyl methanesulfonate (MMS) which is usually used concentration. And then, we determined optimal positive control concentrations of benzo(a)pyrene (BaP) and MMS in the presence and absence of S9 metabolic activation system, respectively.**

Keywords : Single Cell Gel Electrophoresis, Comet, Tail Moment, Mouse lymphoma L5178 cell, DNA single strand breakage, Methyl Methanesulfonate, Benzo(a)pyrene

Introduction

Since the tens of thousands of man-made chemicals that have been introduced into the environment in the last few decades must also be tested for their damaging effect on DNA, the agents that cause this damage must be identified. The establishment of toxicity that may pose a genetic hazard in our environment is subjects of great concern at present. Generally, the carcinogenicity including genotoxicity is one of the potential toxicity that may consider for the human health.

Several assay systems having rapidity and reliability have been introduced for this purpose, such as reversion test with bacterial gene mutation (Maron and Ames, 1983), chromosomal aberration assay with mammalian cells (Ishidate and Odashima, 1977) and micronucleus assay with rodents (Hayashi *et al.*, 1990; Schmid, 1975). Practically, several short term methods have been applied (Maron and Ames, 1983; Mersch-Sundermann *et al.*, 1991) for predicting the carcinogenicity of chemicals and also been introduced to the evaluation of genotoxicity (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979; Radman *et al.*, 1982; Hayashi *et al.*, 1990; Ryu *et al.*, 1993a, 1994, 1996a,b, 1998a,b, 2001b) and of antimutagenicity (Sato *et al.*, 1991; Ryu *et al.*, 1993b, 2001c). Cytogenetic studies on mammalian cells in vitro (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979) as well as *in vivo* (Schmid, 1975; Hayashi *et al.*, 1990, 1994; Heo *et al.*, 1997) have also been widely used as a screening method for DNA-attacking substances. These assay systems are frequently adopted as an index of genotoxicity methods in worldwide and as a screening probe for the detection of possible carcinogenic substances in our environment.

Moreover, many scientists attempt to develope more precise, convenient and sensitive techniques for the detection of DNA damages as an index of carcinogenicity. As one of the mechanisms of carcinogenicity, induction of DNA damage was ascertained by a comet assay, which is widely used for the detection and measurement of DNA strand

*To whom correspondence should be addressed

breaks. Since Ostling and Johanson (1984) introduced micro electrophoretic technique, Singh *et al.* (1988) have modified and improved the micro gel electrophoresis technique to evaluate DNA damage in single cells under alkaline conditions. The single cell gel electrophoresis (SCGE, comet, micro gel electrophoresis) assay is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells. It can be noticed that there are at least 3 major basic protocols: the first of Ostling and Johanson (1984), the second of Singh *et al.* (1988, 1991a,b, 1994, 1995a,b) and one of Olive *et al.* (1993, 1994). Each protocol follows the principle of embedding cells in agarose, lysing the cytoplasmic material and exposing the remaining cell nuclei to a weak electric field. Large differences of each version can be found in the physical and chemical conditions.

If the agent can cause the strand breakage, we can see the extent of tail from the head (nucleus) like comet with staining of fluorescent dyes such as ethidium bromide, acridine orange and propidium iodide and so on. However, there are some variations in procedure, conditions among laboratories and kind of cells used. So, to harmonize these variations, International Workshop on Genotoxicity Test Procedure (IWGTP) was held at Washington D.C. on March, 1999 by Environmental Mutagen Society supported with OECD. Our laboratory (Ryu *et al.*, 1997, 1999, 2001a) also involved in this procedure and published the preliminary form for OECD guideline with Tice *et al.* (2000). Furthermore, comet assay is very worthwhile to use in various fields such as radiation, human monitoring in work place and toxicity evaluation of some chemicals and so on. General reviews on this technique have been published by Tice *et al.* (1991), Fairbairn *et al.* (1995), Anderson *et al.* (1998) and Speit and Hartmann (1995, 1999).

The practical optimal condition in the experimental procedure was established by using L5178Y mouse lymphoma cells that suspended in a thin agarose gel on a microscope slide after chemical treatment and were lysed, unwinded, electrophoresed, and then stained with ethidium bromide. We analyzed the results by using the software (Komet 3.1) of image analyzer for this comet assay. We determined optimal treatment time of lysis and unwinding solution. And also, the positive responsible concentrations of benzo(a)pyrene (5-100 μM) in the presence of S-9 metabolic activation system and methyl methanesulfonate (10-300 μM) in the absence of that was determined.

Materials and Methods

Chemicals

Benzo(a)pyrene (BaP) and methyl methanesulfonate (MMS) were obtained from Sigma-Aldrich Co. (St. Louis, USA). MMS and BaP were dissolved and diluted in distilled water and in DMSO before use, respectively. The final concentration of DMSO in the incubation mixture was below 1%. The S9 mixture was prepared by adding 10 mM glucose-6-phosphate and 5 mM NADP to the S9 fraction (Maron and Ames, 1983). The final concentration of S9 mix in the incubation mixture was 10% (4 mg protein/ml). All doses were used in this experiment exceeded 90% cell viability.

Cell culture and viability test

The mouse lymphoma L5178Y (*tk*+/– 3.7.2c) cell line was subjected for this experiment. Cells were grown in 90% RPMI 1640 (Life Technologies, MD, USA) with 1 mM sodium pyruvate, 0.1% pluronic supplemented with 10% heat-inactivated horse serum and antibiotics in a humidified 5% CO_2 incubator at 37°C. For the determination of cell viability, about 10^6 cells were treated for 2 hr with test chemicals. After the staining of 0.4% trypan blue (Life Technologies, MD, U.S.A), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer. The average number of cells per section was calculated. Cell viability of treated cultures was related to controls that were treated with the solvent and S9 mixture. All experiments were repeated twice in an independent experiment.

Single cell gel electrophoresis (comet) assay

Preparation of agarose coated glass with L5178Y cells: For the comet assay, 8×10^5 cells were seeded into 12 wells plate (Falcon 3043) and then treated with test compounds. In the experiments with metabolic activation, parallel cultures were performed in the absence or presence of S9 mixture. After 2 hr chemical treatment, cells were centrifuged for 5 min at 100×g (about 1,200 rpm), and gently resuspended with PBS and 100 μl of the cell suspension was immediately used for the test. Cells were mixed with 0.1 ml of 1% low melting point agarose (LMPA, Gibco BRL, Life Technologies, Inc., MD, USA) and added to fully frosted slide (Cat. No., 12-544-5, Fisher Scientific, PA, USA) which had been covered with a bottom layer of 100 μl of 1% normal melting agarose (Amresco, OH, USA). The cell suspension was immediately covered with cover glass and the slides were then kept at 4°C for 5 min to allow solidification. After removing the cover glass gently, the slides were covered with a third layer of 100 μl of 0.5% LMPA by using a cover glass and then the slide were kept again at 4°C for 5 min.

Alkaline unwinding and alkaline electrophoresis: The procedure follows the method described by Singh *et al.*, (1988, 1994) with minor modification. The cells embedded in the agarose on slides were lysed for 1.5 hr in reaction mixture of 2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris-HCl (pH 10), 1% Triton X-100 at 4°C. Slides were then placed in 0.3 M NaOH containing 1 mM Na_2-EDTA (approximately pH 13) for 20 min to unwind DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

Evaluation of DNA damage: After the electrophoresis, the slides were washed gently to remove alkali and detergents which would interfere with ethidium bromide staining, by placing vertically and flooding them three times slowly with 0.4 M Tris (pH 7.5) for 10 min. The slides were stained by 50 μl of ethidium bromide in distilled water solution on each slide, and then covering the slide with a cover glass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed from each sample. All experiments were repeated in an independent test. Measurement was made by image analysis with Komet 3.1 (Kinetic Imaging Limited, Liverpool, UK) system, determining the mean of the 50 cells tail moment (percentage of DNA in the tail times tail length). Differences between the control and the other values were tested for significance by using one way of analysis of variance (ANOVA).

Results and Discussion

It is well known that carcinogenicity is the most serious effect of toxic chemicals in human health. As one of the mechanisms of carcinogenicity, induction of DNA damage play an important role in initiation of carcinogenicity. For the detection and measurement of DNA strand breaks in individual cells, comet assay is widely used in recent (Singh *et al.*, 1988, 1991a,b, 1994, 1995a,b). Since many laboratories adopted for various applications, there are several versions of comet assay in different laboratories.

Singh *et al.*, (1991a, b, 1995a, b) demonstrated the analysis of DNA damage in cells adhered to frosted slides. We attempted to make cells adhered to frosted slides but it was not successful. It gave us to concern that we need consider in developing a specialized technique to establish this method properly and it might be helpful for performing this comet assay more simply and promptly.

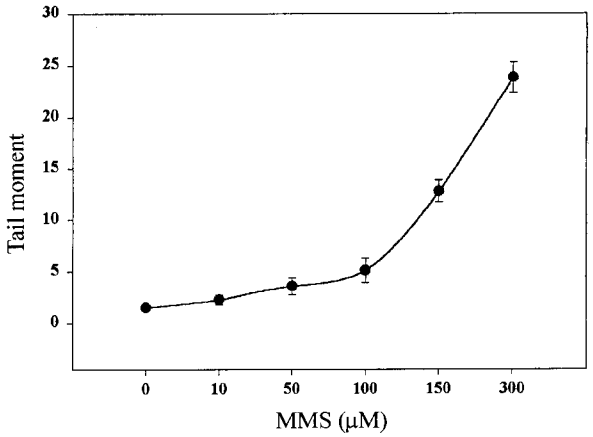

Fig. 1. Response of L5178Y cell to increasing concentration of methyl methanesulfonate (MMS) by comet assay. Mean tail moment indicates DNA damage of cell by MMS. Values are means±SE from three experiments. In each experiment the tail moment index had been assessed from 50 seperately calculated cells.

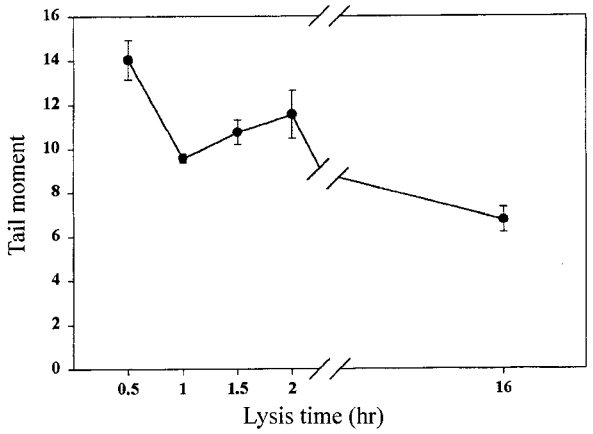

Fig. 2. Tail moment of cells exposed to 150 μM MMS in different lysis time. Values are means±SE from three experiments. In each experiment the tail moment index had been assessed from 50 separately calculated cells.

To determine the optimal conditions as recommended by IWGTP (Tice *et al.*, 2000), we used L5178Y mouse lymphoma cells in the comet assay. The dose-dependent typical DNA migration patterns was induced by MMS, which was well adopted as positive control in comet assay (Fig. 1). The optimal concentration of MMS (Fig. 1) in the absence of S-9 metabolic activation system was determined as 150 μM in this result.

No remarkable differences were observed in lysis time (Fig. 2) and unwinding time (Fig. 3) of the comet assay in mouse lymphoma cells. So, it led us to decide the optimal lysis time as 1.5 hr and unwinding time as 20 min in

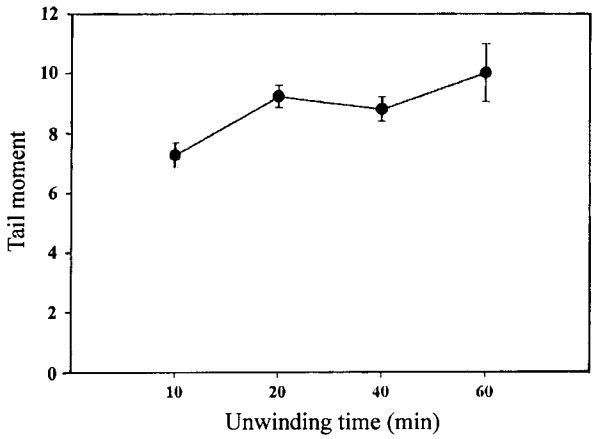

Fig. 3. Tail moment of cells exposed to 150 μM MMS in different unwinding time. Values are means±SE from three experiments. In each experiment the tail moment index had been assessed from 50 separately calculated cells.

mouse lymphoma cell as reported previously (Singh *et al.*, 1988, 1994; Tice *et al.*, 1991).

Using cell lines has some defects of lacking metabolic system, many chemicals need S9 mixture as a metabolizing enzyme system *in vitro*. In usual, S9 mixture must treat within 3-6 hrs because of its cytotoxicity. In order to determine whether S9 mixture makes DNA damage during this period, S9 mixture was treated at the final concentration 4% (20% v/v). We could find baseline tail moment value was slightly increased, but there was no significant difference (data not shown). Based on this data, we treated BaP, DNA bulky adduct to investigate the concentration of positive control in the presence of S9 mixture. The response was

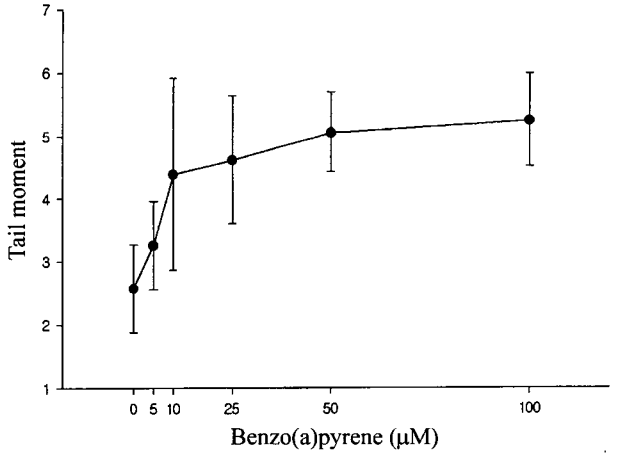

Fig. 4. Tail moment of cells exposed to benzo(a)pyrene in different concentrations. Values are means±SE from three experiments. In each experiment the tail moment index had been assessed from 50 separately calculated cells.

significantly different from control (Fig. 4) at a BaP concentration of 4×10^{-6} M. Similarly, Hartmann *et al.*, (1995) reported that significantly increase of tail length for BaP was at concentration of 5×10^{-5} M. We attributed the difference between laboratories to the parameter which we used, tail moment and protocol. It was reported that the tail length was proportionally less affected than other parameters (Hellman *et al.*, 1995), so tail length might not be fully reflected the effect of BaP.

From this results, the optimal conditions of lysis and unwinding time, and practical MMS and BaP concentrations using mouse lymphoma cell line in our laboratory were demonstrated and emphasized the utility, rapidity and applicability of comet assay for studying some chemicals to cause DNA damage.

In recent, comet assay is commonly used in the case of mutation research, radiation biology (Singh *et al.*, 1988, 1991a,b, 1994, 1995a; Muller *et al.*, 1994; Olive *et al.*, 1993), DNA damage (Green *et al.*, 1992, Gedik *et al.*, 1992), DNA cross linkages (Fuscoe *et al.*, 1996, Pfuhler *et al.*, 1996), oxidative damage (Collins *et al.*, 1995) and to examine the genotoxicity of unknown agent. (McKelvey-Martin *et al.*, 1993). The lymphocytes of human population was also subjected to identify the effect of smoking, age and sex (Betti *et al.*, 1994, 1995; Singh *et al.*, 1991a) because it need only 5-10 μl blood sample. Therefore, comet assay is very easy and convenient for human monitoring in the point of view of occupational and environmental toxicology.

References

Anderson, D. and Plewa, M.J. (1998): The international comet assay workshop. *Mutagenesis*, **13**, 67-73.

Betti, C., Tania, D., Liliana, G., Nicola, L. and Roberto, B. (1994): Microgel electrophoresis assay (comet assay) and SCE analysis in human lymphocytes from 100 normal subjects. *Mutat. Res.*, **307**, 323-333

Betti, C., Tania, D., Liliana, G., Nicola, L. and Roberto, B. (1995): Comparative studies by comet assay and SCE analysis in human lymphocytes from 200 healthy subjects, *Mutat. Res.*, **343**, 201-207

Collins, A.R., Ma, A.-G. and Susan, J.D. (1995): The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. *Mutat. Res.*, **336**, 69-77

Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995): The comet assay: a comprehensive review, *Mutat. Res.*, **339**: 37-59

Fuscoe, J.C., Afshari, A.J., George, M.H., Deangelo, A.B., Tice, R. R., Salman, T. and Allen, J. W. (1996): In vivo genotoxicity of dichloroacetic acid : Evaluation with the mouse peripheral blood micronucleus assay and the single cell gel assay, *Environ. Mol. Mutagen*, **27**, 1-9

Gedik, C.M., Ewen, S.W.B. and Collins, A.R. (1992): Single-cell gel electrophoresis applied to the analysis of UV-C damage and it's repair in human cells, *Int. J. Radiat.*, **62**, 313-320

Green, M.H.L., Lowe, J.E., Harcourt, S.A., Akinluyi, P., Rowe, T., Cole, J. Austey, A.V. and Arlett, C.F. (1992): UV-C sensitivity of unstimulated and stimulated human lymphocytes from normal and xerodermal pigmentosum donors in the comet assay: A potential diagnostic technique. *Mutat. Res.*, **273**, 137-144.

Hartmann, A., Kathleen, H., Michael, G. and Speit, G. (1995): DNA-damaging effect of Cyclophosphamide on human blood cells in vivo and in vitro studies with the single cell gel test (comet assay), *Environ. Mol. Mutagen.*, **25**, 180-187.

Hayashi, M., T. Morita, Y. Kodama, T. Sofuni and M. Ishidate Jr. (1990) The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides, *Mutation Res.*, **245**, 245-249.

Hayashi, M., J. Maki-Paakkanen, H. Tanabe, M. Honma, T. Suzuki, A. Matsuoka, H. Mizusawa and T. Sofuni (1994) Isolation of micronuclei from mouse blood and fluorescence in situ hybridization with a mouse centromeric DNA probe, *Mutation Res.*, **307**, 245-251.

Hellman, B., Vaghef, H. and Bostrom, B. (1995): The concepts of tail moment and tail inertia in the single cell gel electrophoresis assay, *Mutat. Res.*, **336**, 123-131.

Heo M.Y., Kim J.-H. and Ryu J.-C. (1997): Anticlastogenicity of beta-carotene and galangin using in vivo supravital staining Micronucleus Test, *Environ. Mutagen & Carcinogen*, **17**(2), 92-96

Ishidate, M. and S. Odashima (1977): Chromosome test with 134 compounds on chinese hamster cells in vitro-A screening for chemical carcinogens, *Mutation Res.*, **48**, 337-354.

Maron, D.M. and Ames, B.N. (1983): Revised methods for the Salmonella mutagenicity test. *Mutat. Res.* **113**, 173-215.

Matsuoka, A., M. Hayashi and M. Ishidate (1979): Chromosomal aberration test on 29 chemicals combined with S-9 mix *in vitro*, *Mutation Res.*, **66**, 277-290.

Mckelvey-Martin, V.J., Green, M.H.L., Schemezer, P., Pool-Zobel, B.L., De Meo, M.P. and Collins, A. (1993): The single cell gel electrophoresis assay (Comet assay): A european review. *Mutat. Res.*, **288**, 47-63.

Mersch-Sundermann, V.S., S. Kerekordes and S. Mochayedi (1991): Sources of variability of the Escherichia coli PQ 37 genotoxicity assay (SOS chromotest), *Mutation Res.*, **252**, 51-60.

Muller, W.U., Bauch, T., Streffer, C., Niedereichholz, F. and Bocker, W. (1994): Comet assay studies of radiation-induced DNA damage and repair in various tumor cell lines. *Radiat. Res.*, **65**(3), 315-319.

Olive, P.L., Garnet, F. and Judit P.B., (1993): Radiation-induced apoptosis measured in tk6 Human B lymphocytes cells using the comet assay, *Radiat. Res.*, **136**, 130-136.

Olive, P.L., Banath, J.P. and Fjell, C.D. (1994): DNA strand breakage and DNA structure influence staining with propidium iodide using the alkaline comet assay, *Cytometry*, **16**, 305-312.

Ostling, O. and Johanson, K.J. (1984): Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells, *Biochem. Biophy. Res. Commun.*, **123**, 291-298.

Pfuhler S. and Wolf, H.U. (1996): Detection of DNA - crosslinking agents with the alkaline comet assay, *Environ. Mol. Mutagen.*, **27**, 196-201.

Radman, M., P. Jeggo and R. Wagner (1982): Chromosomal rearrangement and carcinogenesis, *Mutation Res.*, **98**, 249-264.

Ryu, J.-C., S. Lee, K.-R. Kim, M. Kim, I.-M. Chang and J. Park (1993a): A study on the clastogenicity of trichothecene mycotoxins in chinese hamster lung cells, *Korean J. Toxicol.*, **9**(1), 13-21.

Ryu, J.-C., S.-H. Kim, K.-R. Kim, S. Lee, M. Kim, H.-K. Jung and J. Park (1993b): A study on the antimutagenic effect of cinnamic acid derivatives in Escherichia coli PQ 37 (SOS Chromotest) (I), *Environ. Mutagens & Carcinogens*, **13**(1), 8-17.

Ryu, J.-C., S. Lee, K.-R. Kim and J. Park (1994): Evaluation of the genetic toxicity of synthetic chemicals (I). Chromosomal aberration test on chinese hamster lung cells *in vitro*, Environ. *Mutagens & Carcinogens*, **14**(2), 138-144.

Ryu, J.-C., K.-R. Kim, H.-J. Kim, E.-K. Ryu. S.-Y. Lee, S.-O. Jung, J.-Y. Youn, M.-H. Kim and O.-S. Kwon (1996a): Evaluation of the genetic toxicity of synthetic chemicals (II). a pyrethroid insecticide, fenpropathrin, *Arch. Pharm. Res.*, **19**(4), 251-257.

Ryu J.-C., K.-R. Kim, E.-K. Ryu, H.-J. Kim, O.-S. Kwon, C.-E. Song, W. Mar and I.-M. Chang (1996b): Chromosomal aberration assay of taxol and 10-deacetyl baccatin III in chinese hamster lung cells *in vitro*, *Environ. Mutagens & Carcinogens*, **16**(1), 6-12.

Ryu, J.-C., Kim, H.-J., Seo Y.-R. and Kim K.-R. (1997): Single Cell Gel Electrophoresis (Comet Assay) to detect DNA damage and Apoptosis in cell level, *Environ. Mutagen & Carcinogen*, **17**(2), 71-77

Ryu J.-C., Kim K.-R., Kim H.-J., Myung S.-W., Kim G.-H., Lee M.-J. and Chang I.-M. (1998a): Genotoxicity Study of Bojungchisup-tang, an oriental herbal decoction-in vitro chromosome aberration assay in chinese hamster lung cells and in vitro supravital-staining micronucleus assay with mouse peripheral reticulocytes, *Arch. Pharm. Res.*, **21**(4), 391-397

Ryu J.-C., Kim K.-R., Kim H.-J., Jung S.-O., Kim, M.-K., Park H.-S. and Kim Y.-H. (1998b): Acute and Genetic Toxicity Study of DK 1002, a Drug Candidate for Analgesics, *J. Toxicol. Pub. Health*, **14**(3), 427-433

Ryu J.-C., Choi Y.-J., Kim Y.-J., Kim H.-T. and Bang H.-A. (1999): Recent advanced toxicological methods for environmental hazardous chemicals, *Kor. J. Environ. Toxicol.*, **14**, 1-12

Ryu J.-C., Seo Y.-R., Smith M.A. and Han S.-S (2001a): The Effect of methyl methanesulfonate (MMS)-induced excision repair on p53-dependent apoptosis in human lymphoid cells, Research Communications in Molecular Pathology and Pharmacology, **109**(1,2), 35-51

Ryu J.-C., Kim K.-R., Lee S. and Park J. (2001b): Evaluation of the genetic toxicity of synthetic chemicals (III), Chromosomal aberration assay with 28 chemicals in chinese hamster lung

cells in vitro, *Environ. Mutagens & Carcinogens*, **21**(1), 14-22

Ryu J.-C. and Park K.-Y. (2001c): Anticlastogenic effect of Baechu (Chinese cabbage) Kimchi and Buchu(leek) Kimchi in supravital staining micronucleus assay using peripheral reticulocytes of mouse, *Environ. Mutagens & Carcinogens*, **21**(1), 51-56

Sato, T., K. Chikazawa, H. Yamamori, Y. Ose, H. Nagase and H. Kito (1991): Evaluation of the SOS chromotest for the detection of antimutagens, *Environ. Mol. Mutagenesis*, **17**, 258-263.

Schmid, W. (1975): The micronucleus test, *Mutation Res.*, **31**, 9-15.

Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. (1988): A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184-191.

Singh, N.P., Danner, D.B., Tice, R.R., Pearson, J.D., Brant, L. J., Morrell, C. H. and Schneider, E. L. (1991a): Basal DNA damge in indicdual human lymphocytes with age, *Mutat. Res.*, **256**, 1-6

Singh, P.N., Tice, R.R. and Stephens, R.E. (1991b): A microgel electrophoresis technique for the direct quantitation of DNA and repair in individual fibroblasts cultured on microscope slides, *Mutat. Res.*, **252**, 289-296.

Singh, N.P., Stephens, R.E. and Schneider, E.L. (1994): Modifications of alkaline microgel electrophoresis for sensitive detection of DNA damage, *Int. J. Radiat. Biol.*, **66**, 23-28.

Singh, N.P., Graham, M.M., Singh, V. and Khan, A. (1995a): Induction of DNA single-strand breaks in human lymphocytes by low doses of γ-rays, Int. J. Radiat. Biol., **68**, 563-569.

Singh, N.P., Lai, H. and Khan, A. (1995b): Ethanol-induced single-strand DNA breakage in rat brain cells, *Mutat. Res.*, **345**, 191-196

Speit, G. and Hartmann, A. (1995): The contribution of excision repair to the DNA effects seen in the alkaline single cell gel test (comet assay), *Mutagenesis*, **10**(6), 555-559

Speit, G. and Hartmann, A. (1999): The comet assay (single-cell gel test). A sensitive genotoxicity test for the detection of DNA damage and repair. *Methods Mol. Biol.* **113**, 203-212.

Tice, R.R., Andrews, P.W., Hirai, O. and Singh, N.P. (1991): The single cell gel (SCG) assay: an electrophoretic technique for the detection of DNA damage in individual cells. *Adv. Exp. Med. Biol.* **283**, 157-164.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., and Sasaki, Y.F. (2000): Single cell gel/comet assay: guidelines for *in vitro* and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **35**, 206-221.