

DNA DAMAGE ANALYSIS IN MAMMALIAN CELLS BY SMA-DMSO COMPLEX USING COMET ASSAY

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ABSTRACT: SMA-DMSO complex (RISUG) is a potent anti fertility compound developed by Prof.S.K Guha at IIT-Kharagpur. It has been fabricated as a male contraceptive which works by lowering the pH of vas deference in males and hence damaging the sperms. Cytotoxicity of SMA-DMSO was assessed using COMET assay whereby concentration of SMA-DMSO ranging from .001 μM to 1 μM was tested for inducing any significant DNA damage in Mammalian (Chinese Hamster Ovary) cell line. 50 cells were scored for each concentration, with 25 cells scored from each of two replicate blind coded slides. Statistical significance of the results was assessed by means of one-tailed Student's t-test. Analysis of tail length and % DNA amount after treating the CHO cells with varying concentration of SMA-DMSO, ranging from 0.001 μM to 1 μM has shown no significant DNA damage and cytotoxicity. Results have shown RISUG to be absolutely safe for further usage in Mammalian cells.

KEYWORDS: SMA-DMSO, COMET Assay, CHO cell line, Cytotoxicity, Olive tail

I. INTRODUCTION

Many techniques have been developed to assess DNA damage at genetic, structural and nuclear level which includes prominent tests like Bacterial reverse mutation assay for genotoxicity assessment, micronuclei assay, chromosomal aberration test, Unscheduled DNA synthesis test, Sister chromatid exchange assay etc. However to assess DNA damage at single cell level, Ostling & Johanson (1984) were the first to develop a microgel electrophoresis technique whereby, cells embedded in agarose were placed on a microscope slide, the cells were lysed by detergents and high salt, and the liberated DNA electrophoresed under neutral conditions. Cells with an increased frequency of DNA double-strand breaks displayed increased migration of DNA toward the anode. The migrating DNA was quantitated by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern using a microscope photometer. The neutral conditions used greatly limited the general utility of the assay. A modified version was published by Singh and colleagues in 1988, which used alkaline conditions. The idea was to combine DNA gel electrophoresis with fluorescence microscopy to visualize migration of DNA strands from individual agarose-embedded cells. If the negatively charged DNA contained breaks, DNA supercoils were relaxed and broken ends were able to migrate toward the anode during a brief electrophoresis. If the DNA was undamaged, the lack of free ends and large size of the fragments prevented migration. Determination of the relative amount of DNA that migrated provided a simple way to measure the number of DNA breaks in an individual cell.

In 1990, Olive et al proposed a further modification of this assay which used lysis of DNA under alkaline conditions and named it as the "comet assay" after the appearance of the DNA from an individual cell. The comet head containing the high-molecular-weight DNA and the comet tail containing the leading ends of migrating fragments were measured in real time from digitized images using software developed for this purpose. Tail moment, a measure of both amount of DNA in

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 1, January 2014

the tail and distribution of DNA in the tail, became a common descriptor along with tail length and percentage of DNA in the tail. Since the introduction of the alkaline (pH . 13) Comet assay, the breadth of applications and the number of investigators using this technique have increased almost exponentially. Compared with other genotoxicity assays, the advantages of the technique include: (1) its demonstrated sensitivity for detecting low levels of DNA damage; (2) the requirement for small numbers of cells per sample; (3) flexibility; (4) low costs; (5) ease of application; (6) the ability to conduct studies using relatively small amounts of a test substance; and (7) the relatively short time period (a few days) needed to complete an experiment. During the last decade, this assay has developed into a basic tool for use by investigators interested in research areas ranging from human and environmental biomonitoring to DNA repair processes to genetic toxicology (Tice 1995, Fairbairne et al 1995, Anderson et al 1994, 1998, Rojas et al 1999).

Cytotoxicity of SMA-DMSO was assessed using COMET assay whereby concentration of SMA-DMSO ranging from .001 μM to 1 μM was tested for inducing any significant DNA damage in Mammalian (Chinese Hamster Ovary) cell line.

II. RELATED WORK

Genotoxicity of SMA-DMSO complex has been tested using Bacterial Reverse mutation assay and Chromosomal Aberration Assay. Mutagenicity has been found to be negligible after assessing impact of SMA-DMSO in various strains of *Salmonella typhimurium* TA97a, TA 98, TA 100 and TA102. Observation of Ames test result have shown that SMA-DMSO is not mutagenic and it can be considered further for safety studies. Data obtained from chromosomal aberration test indicates that the concentrations of SMA-DMSO upto 0.1M have no genotoxic potential in CHO cells under experimental conditions, whereas, the above concentrations (1M and 10M) were cytotoxic. As per ICH guidelines for safety testing of pharmaceuticals, minimal 3 test battery must be conducted to assess genotoxicity and cytotoxicity of pharmaceuticals. Comet Assay was conducted in current work to assess cytotoxicity in mammalian cell line after confirming negligible genotoxic potential in bacteria and CHO cell line.

III. METHODOLOGY

a) CELL CULTURE AND TREATMENT

Chinese hamster ovary (CHO) cells were obtained from National Centre for Cell Sciences, Pune, India and maintained in Ham's F12 medium (with 300mg/L L-glutamine and 2g/L sodium bicarbonate) supplemented with 10% heat inactivated FBS and 10ml/litre antibiotic/antimycotic solution. The cells were grown in 25 cm² flasks at 37°C in a humidified atmosphere of 5% CO₂ in air. Exponentially growing cultures were used for the study.

CHO cells were disaggregated using 1.5 ml of 0.25% trypsin and when all the cells detached, trypsin was quenched with 2 ml complete medium. The cells were centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and pellet resuspended in complete medium to give 10⁶ cells / ml solution. Cells were then seeded in 96 well-microtitre plate (10,000 cells per well) for MTT assay (cell viability) and 24 well plate (20,000 cells /well) for Comet assay, and grown for 24 hours at 37°C and 5% CO₂ in air.

b) TREATMENT PREPARATION

7 mg of SMA-DMSO was taken in a 50 ml centrifuge tube and the volume was made upto 8 ml with Ham's F12 medium without serum (IF12) and sonicated at 14 MHz till the clump dissolved completely. The pH was adjusted to 7.5 and the final volume was made upto 10 ml with IF12 and filter sterilized through 0.22 μm filter. This was 1 mM concentration of SMA-DMSO. Different concentrations were made from this stock solution by adequate dilution.

c) COMET ASSAY

After the 24 hours, medium was discarded and the cells were washed with incomplete medium. The cells were treated for three hours and six hours with SMA-DMSO (concentration 0.001 μM , 0.01 μM , 0.1 μM and 1 μM) Incomplete medium served as negative control and 2mM Ethyl methanesulfonate as positive control. DMSO (0.05%) was used as vehicle control. After treatment, wells were washed with Phosphate buffered saline (PBS). Cells were disaggregated using cold 0.05% trypsin, and

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 1, January 2014

resuspended in complete medium. Cells were centrifuged at 6000 rpm for 5 minutes. Duplicate slides were prepared for each concentration. Each experiment was conducted in triplicate.

Briefly, 100 μ l cell suspension was mixed with 100 μ l of 1% LMA at 37°C, out of which 80 μ l each was loaded onto two microscope slides, pre-coated with 1% normal melting agarose. Cover slips were then placed gently on the slides to allow even spreading of gel. The slides were kept on ice for 5 minutes for the gel to solidify. Cover slips were removed and a third layer of 0.5% LMA was added onto the slide and kept over ice for 5 min. Finally the cover slips were removed and the slides immersed in cold lysing solution containing 2.5M NaCl, 100mM EDTA, 10mM Tris (pH 10) with 1% TritonX-100 being added just before use. These slides were kept overnight at 4°C.

After lysis, the slides were placed side by side into a horizontal electrophoresis tank containing freshly prepared chilled electrophoresis buffer (1mM EDTA sodium salt and 300mM NaOH), avoiding air spaces, with the agarose ends nearest to the anode. Slides were left for 20 minutes for DNA unwinding and electrophoresis was performed at 0.7 V/cm and 300mA at 4°C for 30 minutes. Slides were then removed from electrophoresis buffer and Tris buffer (pH 7.4) was added drop wise to neutralize excess alkali. The slides were allowed to sit for 5 minutes and the procedure repeated three times. Slides were then dipped in chilled distilled water and stained with 75 μ l ethidium bromide (20 μ g/ml) for 5 minutes. The slides were again dipped in distilled water to remove excess dye and coverslips were placed. Slides were stored in a humidified slide box until scoring.

d) SLIDES SCORING

Slides were scored using an image analysis system attached to a fluorescence microscope equipped with appropriate filters (N2.1). The microscope was connected to a computer through a charge coupled device (CCD) camera to transport images to a software (Komet 3.1) for analysis. The final magnification was X 400. The parameters taken for the cells were, tail DNA (%), tail length (migration of the DNA from the nucleus; μ m), image intensity and Olive tail moment (arbitrary units).

e) ANALYSIS

50 cells were scored for each concentration, with 25 cells scored from each of two replicate blind coded slides. Statistical significance of the results was assessed by means of one-tailed Student's t-test.

IV. RESULTS

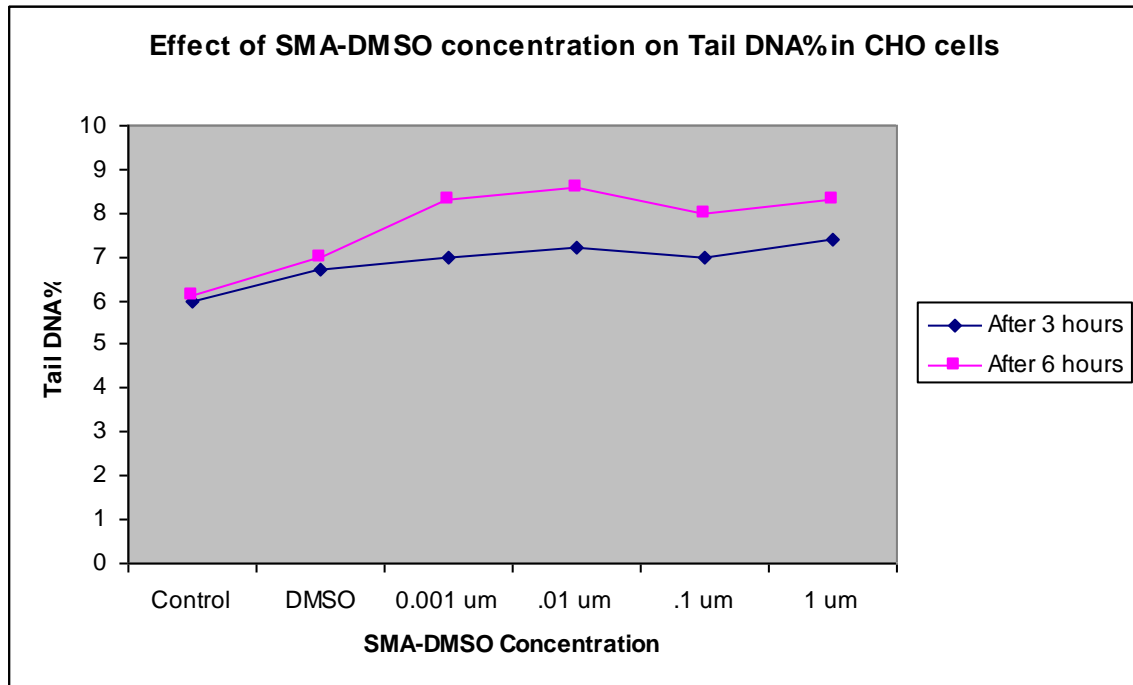


Figure 1

**International Journal of Innovative Research in Science,
Engineering and Technology**

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 1, January 2014

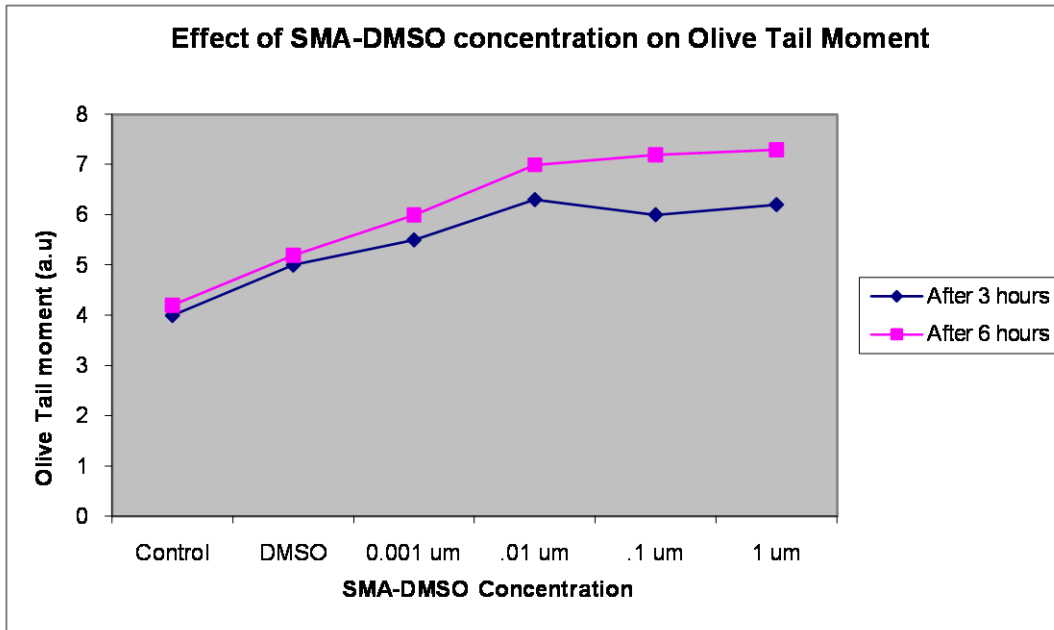


Figure 2

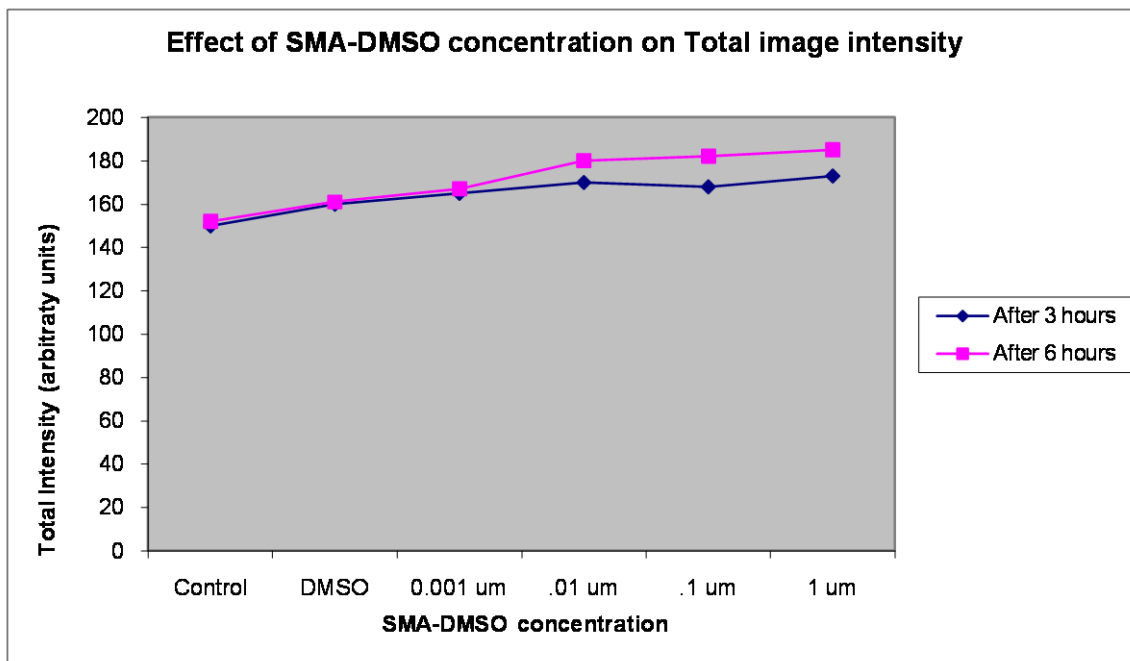


Figure 3

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 1, January 2014

In figures 1,2 and 3, Values are mean \pm SE of three experiments
*p<0.05 when compared to control

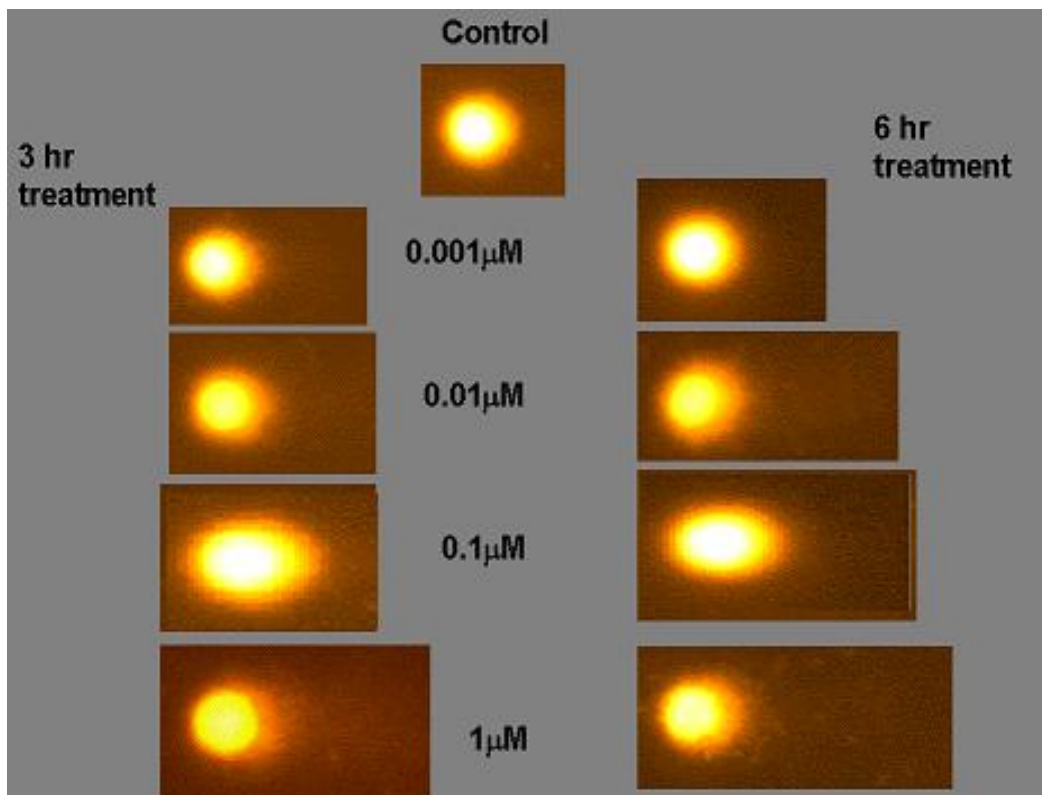


Figure 4: Images of CHO cells treated with different concentrations of SMA-DMSO showing DNA damage in the Comet assay

V. INFERENCE AND CONCLUSION

Analysis of tail length and % DNA amount after treating the CHO cells with varying concentration of SMA-DMSO ,ranging from 0.001 μ M to 1 μ M has shown no significant cytotoxicity as the intensity of image reflecting % DNA damage through tail protusion in comet assay is almost negligible. There is almost negligible DNA Damage by SMA-DMSO complex and it can be considered safe on human beings for futher usage. However further interrogative assays need to be carried to assess its genotoxic potential and allergenicity.

REFERENCES

1.Ostling, O. & Johanson, K.J.,Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.* 123, 291–298 ,1984

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 1, January 2014

2. Singh, N.P., McCoy, M.T., Tice, R.R. & Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175, 184–191, 1988
3. Olive, P.L. Cell proliferation as a requirement for development of the contact effect in Chinese hamster V79 spheroids. *Radiat. Res.* 117, 79–92, 1989
4. Olive, P.L., Ban ath, J.P. & Durand, R.E., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the “comet” assay. *Radiat. Res.* 122, 86–94, 1990.
5. Moller, P., Genotoxicity of environmental agents assessed by the alkaline comet assay. *Basic Clin. Pharmacol. Toxicol.* 96 (Suppl. 1), 1–42, 2005.
6. Tice RR. The single cell gel/comet assay: A microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In: Phillips DH, Venitt S, editors. *Environmental mutagenesis*. Oxford: Bios Scientific Publishers. p 315–339, 1995.
7. Fairbairn DW, Olive PL, O’Neill KL. The Comet assay: a comprehensive review. *Mutat Res* 339:37–59, 1995.
8. Anderson D, Yu T-W, Phillips BJ, Schmezer P. The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the Comet assay. *Mutat Res* 307:261–271, 1994.
9. Anderson D, Yu, T-W, McGregor DB. Comet assay responses as indicators of carcinogenic exposure. *Mutagen* 13:539–555, 1998.
10. Vasquez M, Tice RR. Detecting genotoxic activity against high molecular weight DNA using the alkaline single cell gel (SCG) assay. *Environ Mol Mutagen* 29(S28):53, 1997.
11. Rojas E, Lopez MC, Valverde M., Single cell gel electrophoresis assay: methodology and applications. *J Chromat B* 722:225–254, 1999.
12. Friauff, W., Hartmann, A. & Suter, W. Automatic analysis of slides processed in the Comet assay. *Mutagenesis* 16, 133–137, 2001.
13. Banath, J.P., Kim, A. & Olive, P.L., Overnight lysis improves the efficiency of detection of DNA damage in the alkaline comet assay. *Radiat. Res.* 155, 564–571, 2001.
14. Alapetite, C., Wachter, T., Sage, E. & Moustacchi, E., Use of the alkaline comet assay to detect DNA repair deficiencies in human fibroblasts exposed to UVC, UVB, UVA and γ rays. *Int. J. Radiat. Biol.* 69, 359–369, 1996.
15. Rydberg, B. Radiation-induced heat-labile sites that convert into DNA double-strand breaks. *Radiat. Res.* 153, 805–812, 2000.
16. Collins, A.R. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotechnol.* 26, 249–261, 2004.
17. Olive, P.L., Ban ath, J.P. & Fjell, C.D. DNA strand breakage and DNA structure influence staining with propidium iodide using the alkaline comet assay. *Cytometry* 16, 305–312, 1994.
18. Dorie, M.J. *et al.* DNA damage measured by the comet assay in head and neck cancer patients treated with tirapazamine. *Neoplasia*. 1, 461–467, 1999.
19. Speit, G. & Hartmann, A. (The comet assay: a sensitive genotoxicity test for the detection of DNA damage. *Methods Mol. Biol.* 291, 85–95, 2005.
20. Strauss, G.H., Peters, W.P. & Everson, R.B. Measuring DNA damage in individual cells of heterogeneous mixtures: a novel application of an immunological typing technique. *Mutat. Res.* 304, 211–216, 1994.
21. Santos, S.J., Singh, N.P. & Natarajan, A.T., Fluorescence *in situ* hybridization with comets. *Exp. Cell Res.* 232, 407–411, 1997.
22. Rapp, A., Hausmann, M. & Greulich, K.O., The comet-FISH technique: a tool for detection of specific DNA damage and repair. *Methods Mol. Biol.* 291, 107–119, 2005.
23. Hartmann, A. *et al.* Recommendations for conducting the *in vivo* alkaline Comet assay. 4th International Comet Assay Workshop. *Mutagenesis* 18, 45–51, 2003.
24. Albertini, R.J. *et al.*, IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. *Mutat. Res.* 463, 111–172, 2000.
25. Duez, P., Dehon, G., Kumps, A. & Dubois, J., Statistics of the Comet assay: a key to discriminate between genotoxic effects. *Mutagenesis* 18, 159–166, 2003.
26. Verde, P.E. *et al.*, Application of public-domain statistical analysis software for evaluation and comparison of comet assay data. *Mutat. Res.* 604, 71–82, 2006.
27. Sasaki YF, Tsuda S, Izumiyama F, Nishidate E., Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (Comet) assay. *Mutat Res* 388:33–44, 1997.
28. Sasaki YF, Sekihashi K, Itumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S., The comet assay with mouse multiple organs: comparison of comet assay results and carcinogenicity with 206 chemicals selected from the IARC monographs and U.S. NTP carcinogenicity database. *Crit Rev Toxicol*, submitted, 2000.
29. Sauvaigo S, Serres C, Signorini N, Emonet N, Richard MJ, Cadet J., Use of the single-cell gel electrophoresis assay for the immunofluorescent detection of specific DNA damage. *Analytical Biochemistry* 259:1–7, 1998.
30. Scott D, Galloway SM, Marshall RR, Ishidate M Jr, Brusick D, Ashby J, Myhr BC., Genotoxicity under extreme culture conditions: a report from ICPEMC Task Group 9. *Mutat Res* 257:147–204, 1991.