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MUTAGENIC EVALUATION OF PRANOPROFEN, A NEW ANTI-INFLAMMATORY AGENT.

Hwa Hwey Moon, Pu Young Kim, Tai Bo Yun, Dae Hyun Jo,
Han Soo Park, Ok Soon Heo, Soon Han Kim and Suk Hee Choi
Department of Safety Research, National Institute of Health, Seoul 122, Korea.

ABSTRACT: *The mutagenicity of pranoprofen, a new anti-inflammatory agent primarily used in Japan, was evaluated by employing several different methods such as the Ames test, micronucleus test, and the sister chromatid exchange test. For the Ames test, various doses of pranoprofen (5 and 1 mg, 100, 10, and 1 μg per plate) were applied, with or without the mammalian liver S-9 fraction, to the S. typhimurium LT2. For the micronucleus test, 24 hours after administering the various doses of pranoprofen (200, 100, and 50 mg/kg) to male mice by oral intubation, the femura of each group were isolated and the bone marrow samples were prepared. The micronucleated red cells and the ratio of the polychromatic versus the normochroomatic cells were counted. For the sister chromatid exchange test, the maximal non-cytotoxic concentrations (10 to 0.1 mM pranoprofen) were applied to the culture media of the Chinese Hamster Ovary (CHO) cells for 24 hrs. The numbers of revertant colonies did not increase with the increasing doses of pranoprofen when teseted with various strains of S. typhimurium. In the micronucleus test employing mice, the pranoprofen was identified to be a non-clastogen and a non-spindle poison. In the sister chromatid exchange test employing the cultured CHO cells, the pranoprofen did not increase the incidences of chromosomal abnormality. Based on these results, pranoprofen was found to have no mutagenic activity.*

INTRODUCTION

There is a growing awareness about the presence of genetically active agents in the human environment. These concerns have been borne out from the laboratory results obtained by using small animals and single-cell systems. Thus, considerable amounts of work have been devoted to establishing the mutagenicity of environmental chemicals. As a continuing effort to evaluate the safety of drugs used in Korea, our laboratory has conducted a battery of mutagenicity tests on pranoprofen.

Pranoprofen is a slightly bitter tasting crytalline powder and is a newly developed non-steroidal anti-inflammatory drug. It has a tricyclic base and is a 2-(5H-(1) benzopyrano (2,3-b) pyridin-7-yl) propionic acid. Pranoprofen blocks the biosynthesis of prostaglandin by inhibiting the prostaglandin synthetase. This action is similar to other anti-inflammatory agents like indomethacin (Piper and Vane, 1969; Vane, 1971). Thus, pranoprofen reduces the levels of

prostaglandins in peripheral tissues.

Every one of the established methods for testing the mutagenicity of environmental chemicals has both merits as well as demerits. Therefore, the selection of a particular testing method from the available menu depends primarily upon the choice of the involved investigator. However, it is often difficult to find the one perfect and ideal mutagenicity testing system suitable for all occasions. Thus, in the present study, we have chosen to use a battery of available test systems in evaluating the mutagenicity of pranoprofen. These tests include the Ames test, micronucleus test, and the sister chromatid exchange test.

Conventionally, the Ames salmonella/microsome plate assay is the most widely used in vitro mutagenicity testing system (Ames *et al.*, 1975 a,b). However, the accuracy of this test system for predicting the genetic toxicity was found to vary between 50 to 90% (Andrews *et al.*, 1978 a,b and Coombs *et al.*, 1976). Furthermore, as this bacterial test system cannot, in principle, detect the altered chromosomal events which occur in the mammalian tissues as carcinogenesis and as germ cell line mutations. These particular draw-backs can be overcome by conducting the in vivo mutagenicity tests. However, while the in vivo mutagenicity tests are more physiological, they appear to be relatively insensitive due to the inability to achieve the sufficiently high plasma levels without causing acute direct toxicity. In any case, we have included the micronucleus test to detect the clastogenic activity of pranoprofen. In an effort to overcome the problem of insensitivity of the in vivo testing system and as an adjunct to the physiological micronucleus test, we have also conducted the in vitro mammalian cell mutagenicity test, namely the sister chromatid exchange test. This in vitro assay detects the chromosomal abberations rather sensitively, but ignores the aspect of the protective mechanisms present in the physiological whole animal test system.

Thus, we have tested the mutagenicity of pranoprofen by employing all three test systems which complement each other. This is done to provide a more precise evaluation on the safety of pranoprofen.

MATERIALS AND METHODS

Ames Salmonella / Microsome Plate Assay

The Salmonella bacterial tester strains used in this study were obtained from Dr. Ames of the University of California. The test substances employed as positive mutagens were the 4-nitroquinone-N-oxide (Aldrich Chem. Co.), sodium azide (Wako Pure Chem. Co.), 9-aminoacridine (Aldrich Chem. Co.), and 2-aminoanthracene (Wako Pure Chem. Co.). Pranoprofen (Yoshitomi Co.) was dissolved in dimethyl sulfoxide (DMSO) before applying to the petri dishes.

For the preparation of the S-9 fractions required in the activated testing system, male Sprague-Dawley rats weighing about 200 g were used. These rats were provided by the Breeding Center of the Korean NIH. For the induction of liver microsomal enzymes, we have used phenobarbital and β-naphthoflavone as described by Matsushima et al (1976) and the detailed methods are shown in Fig. 1. The liver S-9 homogenates and the complete S-9 mixture were made up according to the method published by Ames *et al.* (1975b).

After testing for the bacteriocidal effects, the standard testing doses of pranoprofen (5 and 1 mg, 100, 10, and 1 μg per plate) were selected. These were dissolved in DMSO before adding to petri dishes. All of the experiments were conducted by the pre-incubation method as published by Ames *et al.* (1982). Complete test for each testing dose consisted of a non-activated (without the S-9 mix) and an activated (with the S-9 mix), each with appropriate

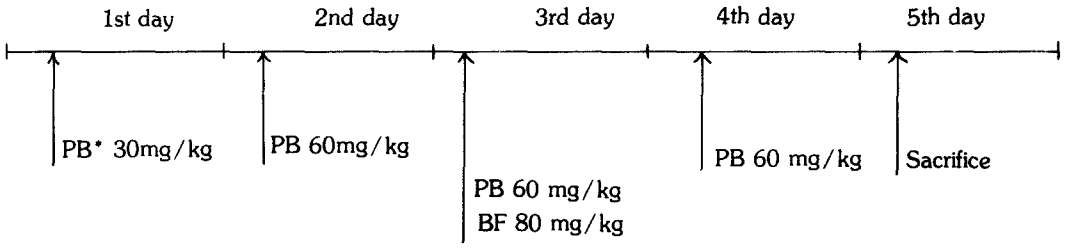


Fig. 1. The procedure of microsomal enzyme induction with S.D. male rats.
*PB: phenobarbital BF: β-naphthoflavone

Table 1. The experimental conditions and used positive controls in Ames test

Test strain	Positive control		Treatment
	−S-9	+S-9	
TA 98	4-NQNO*(0.2μg)	2-AA(10μg)	1, 10, 100, 1000,
TA 100	4-NQNO(0.025μg)	2-AA(10μg)	5000μg pranoprofen/plate
TA 1535	sod. azide(0.5μg)	2-AA(10μg)	
TA 1537	9-AA (80μg)	2-AA(10μg)	
TA 1538	2-NF (50μg)	2-AA(10μg)	

* 4-NQNO; 4-Nitroquinoline-N-oxide
9-AA ; 9-Aminoacridine
2-NF ; 2-Nitroflourene
2-AA ; 2-aminoanthracene

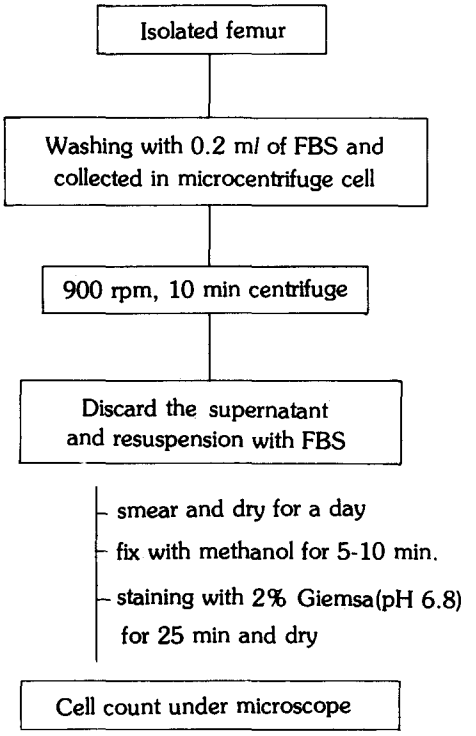
negative and positive controls as shown in Table 1.

Micronucleus Assay using Mouse

The male ICR mice (6 week old) were group-housed (10 mice per cage) and were fed commercial diet and tap water ad libitum. Varying doses (200, 100 and 50 mg/kg body weight) of pranoprofen dissolved in 0.5% CMC were administered as a single treatment by oral intubations. The highest dose used was the half of known oral LD_{50} value. Animals were sacrificed by cervical dislocation 24 hr after the treatment and femura were isolated. Bone marrow cells obtained from the femura were suspended in fetal bovine serum (CSL) and were stained by Giemsa (Gurr. PH 6.8) stain. These were then examined under a microscope. The detailed entire procedure is shown in Scheme 1. The negative control samples were obtained from the solvent treated mice and the positive samples from mice given 0.2 mg/kg mitomycin C (Sigma Chemical Co.) via a single intraperitoneal injection.

Sister Chromatid Exchange using Cultured Chinese Hamster Ovary Cells

An established CHO-K cells which have been derived as a proline-requiring subclone of CHO cells were kindly supplied by the Department of Virology of Korean NIH. The monolayer cultures of the cell line were grown at 37°C in a humidified CO_2 incubator on disposable tissue culture flasks (Nunclon Co.). The medium used in this study was the Eagle's Minimum Essential Medium (Sigma Chem. Co.) supplemented with 10% fetal bovine serum (CSL) containing 100 I.U./ml penicillin G and 100 μg/ml streptomycin (both from Sigma). The cultured cells



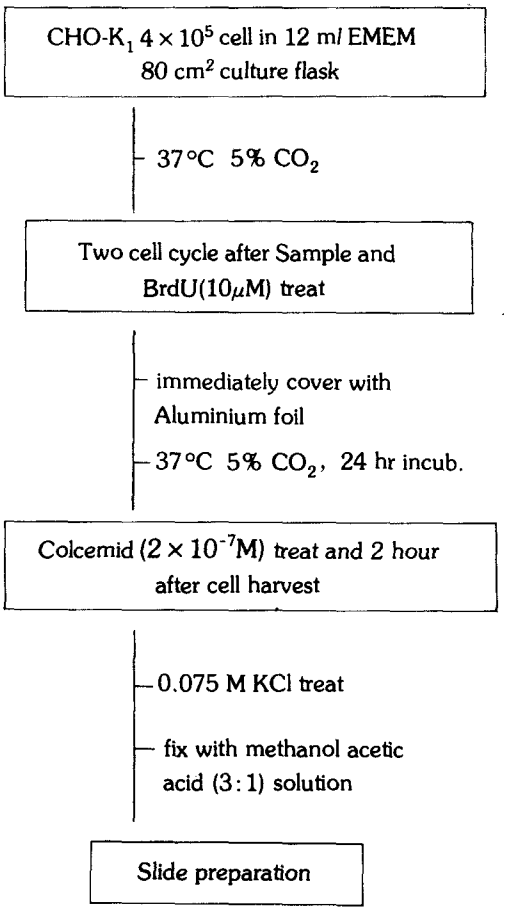
Scheme 1. The experimental procedure of micronucleus test.

were maintained in exponential growth by subculture for every two to three days using 0.25% trypsin-EDTA (Gibco) and phosphate buffered saline. The cultures were treated with varying doses of pranoprofen (10 to 0.1 mM) which have been dissolved in 10% DMSO. Cells were then stained by the method described by Goto *et al.* (1975). Control groups consisted of the negative control (solvent) and the positive control (7.5 x 10-8 M mitomycin C). The detailed entire procedure is as shown in Scheme 2.

RESULTS

Ames Test

In the pre-test with every tester bacterial strains, pranoprofen was found to have no antibiotic activity even with 500 μg per plate. Results shown in Table 2 demonstrate that, when tested without the S-9 activation system, the pranoprofen did not increase the numbers of revertant colonies with most of the tested strains. However, with the TA 98 strain treated with 100 μg of pranoprofen per plate, there were significant increases in the number of revertant colonies when compared with those of the control group. Results shown in Table 3 were obtained from the activated system. The TA 98 strain treated with 10 μg of pranoprofen and the TA 1538 treated with 1 mg pranoprofen showed significant increases in the number of revertant colonies. However, as shown by the results in Figures 2 and 3, these revertant colonies which have been induced by pranoprofen failed to demonstrate any dose-dependency.



Scheme 2. The experimental procedure of SCE test.

Table 2. The experimental results Ames test without S-9 mix.

Group	Treatment	Revertant/plate				
		TA 98	TA 100	TA 1535	TA 1537	TA 1538
Control	DMSO	42.8± 4.3	177.8± 3.5	18.3±3.5	8.0± 1.8	29.0±4.2
1μg		44.0± 5.1	180.3±10.9	20.8±1.1	5.3± 1.6	23.3±3.5
10μg		55.0± 6.9	187.8±10.6	17.8±1.1	9.0± 0.9	29.3±3.3
100μg	pranoprofen	56.6± 9.9	184.8±10.6	16.0±0.9	2.5± 0.3	29.8±2.5
1000μg	in DMSO	52.8± 3.8	174.0± 2.3	14.5±2.6	6.8± 0.9	25.5±2.5
5000μg		58.6±19.8	162.8± 6.1	15.8±1.7	8.8± 1.4	28.3±1.5
positive control	DMSO or water	108.5±8.4[a]	213.8± 5.0[b]	181.8±8.5[c]	64.0±10.3[d]	1000[e]

N=4, * vs control; $p<0.1$
a; 4-NQNO(0.2μg) b; 4-NQNO(0.025μg) c; sod. azide(0.5μg) d; 9-AA(80μg) e; 2-NF(50μg)
M±S.E.

Table 3. The experimental results of Ames test with S-9 mix.

Group	Treatment	Revertant/plate				
		TA 98	TA 100	TA 1535	TA 1537	TA 1538
Control	DMSO	23.0±4.7	163.3± 9.7	21.5± 0.9	105.5± 0.1	12.3±1.2
1μg		20.5±5.1	146.3±10.2	18.3± 2.6	9.5± 0.2	11.8±0.2
10μg		27.8±2.3[a]	148.0± 5.3	22.0± 1.5	9.3± 0.5	13.3±0.7
100μg	pranoprofen	23.3±5.7	152.0±11.5	20.3± 0.7	13.8± 1.0	10.0±0.2
1000μg	in DMSO	24.5±3.6	148.8± 6.7	22.3± 0.1	14.3± 1.5	15.3±1.6[b]
5000μg		21.8±2.2	141.8± 4.3	22.8± 1.1	13.0± 1.7	12.8±0.8
positive* control	DMSO	>1000	>1000	289.1±20.9	302.7±17.2	>1000

M±S.E.
N=4, * 2-amino-anthracene 10μg/plate
a vs control, b vs control; $p<0.1$

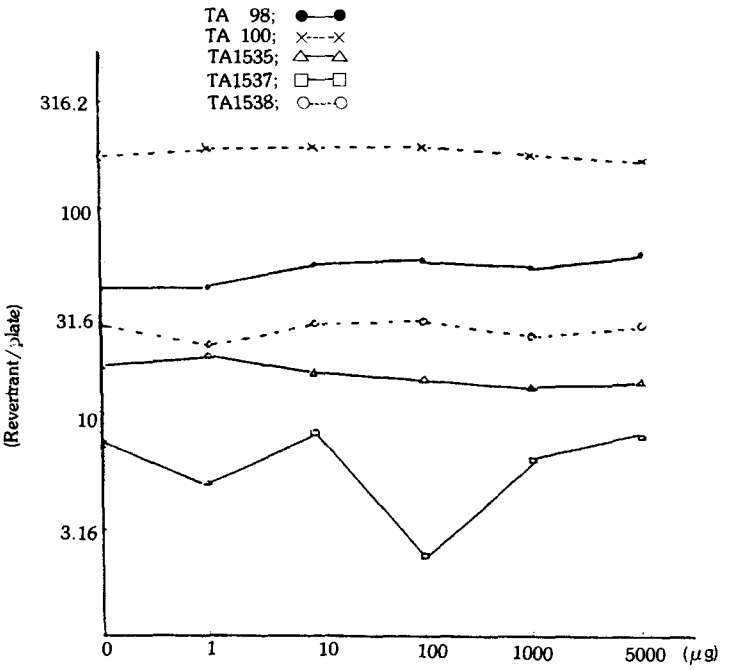


Fig. 2. The number of revertant colonies by treatment of pranoprofen without S-9 mix.

Micronucleus Test

Table 4 shows the incidences of micronucleated polychromatic erythrocytes. While the incidences were significantly increased in the mitomycin C treated group, there were no increases in the pranoprofen treated groups. The ratios between the normo-chromatic vs. polychromatic erythrocytes were not changed by the pranoprofen treatment. Although the result showed that the group treated with 100 mg/kg had a slightly increased incidences of micronucleated polychromatic erythrocytes, the increase was not statistically significant.

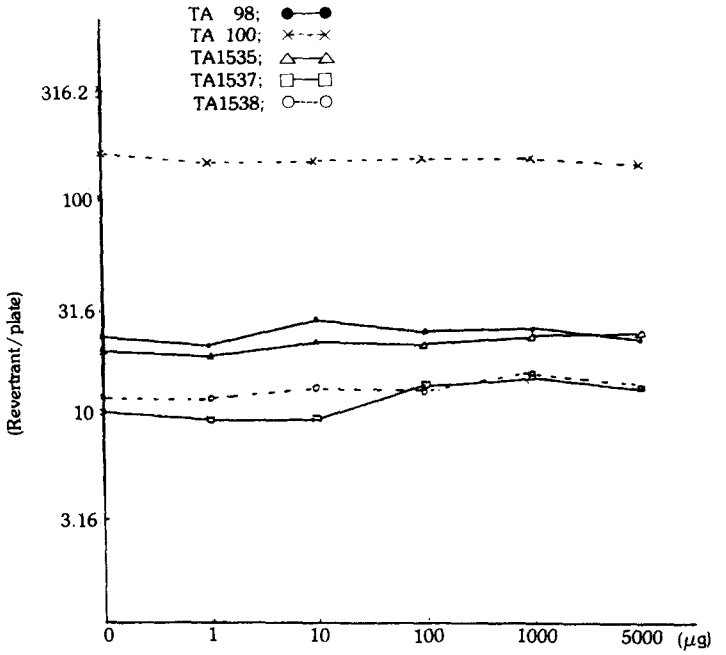


Fig. 3. The number of revertant colonies by treatment of pranoprofen with S-9 mix.

Table 4. The incidence of MNPCE after treatment of pranoprofen p.o.

Group	No. of mice	No. of PCE scored	MNPEC (%)
Non-treat	10	1000	0.21±0.14
Solvent-treat	10	1000	0.20±0.19
200 mg	10	1000	0.21±0.08
100 mg	10	1000	0.23±0.18
50 mg	10	1000	0.20±0.15
MMC 2.0 mg[a]	10	1000	6.72±1.21

M±S.E.
a; positive control

Table 5. The incidence of SCE/cell after treatment of pranoprofen.

Cell	Group	Treatment	No. of cell scored	No. of SCE
CHO-K_1	Control	0.5 ml of	20	6.2±1.4
	0.1 mM	10% DMSO	20	6.9±2.5
	0.5 mM	Pranoprofen	20	4.9±2.9
	1.0 mM	in 10% DMSO	20	6.1±0.2
	10 mM	7.5×10^{-8} M of	20	6.7±0.3
	MMC[a]	MMC in water	20	51.7±8.6

Mean±S.E.
a; Positive control

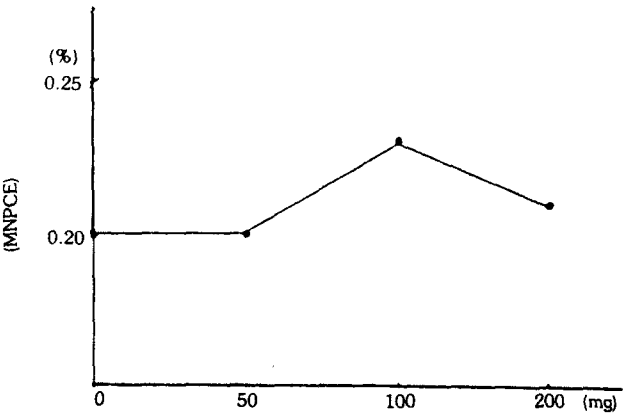


Fig. 4. The percentage of polychromatic erythrocyte vs Micronucleated polychromatic erythrocyte after treatment of pranoprofen in male ICR mice.

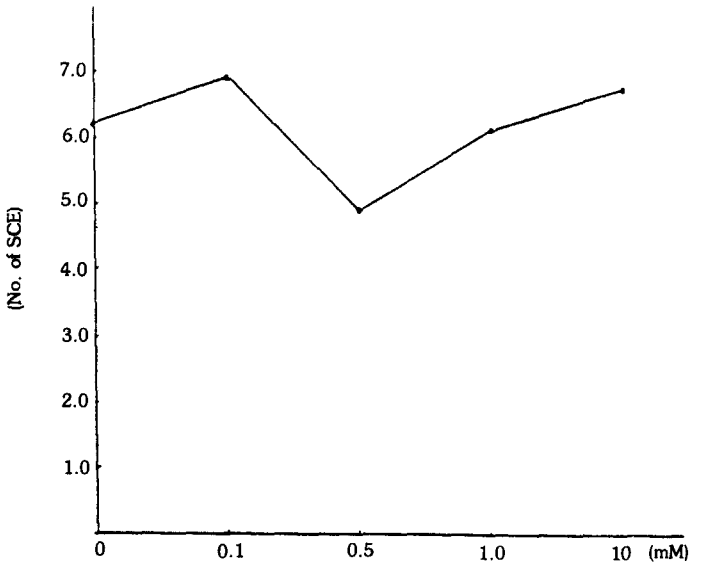


Fig. 5. The number of SEC/cell in CHO-K, cell after treatment of pranoprofen.

Sister Chromatid Exchange Test

Table 5 shows the results of SCE test with CHO cell, in pranoprofen treatment groups no significant changes were found compare with control group, but mitomycin C treatment group shows significant increased incidence of SCE. Dose-dependency was not found in SCE test on pranoprofen (Fig.5)

DISCUSSION

This study was conducted for establishing mutagenicity test system and evaluation of

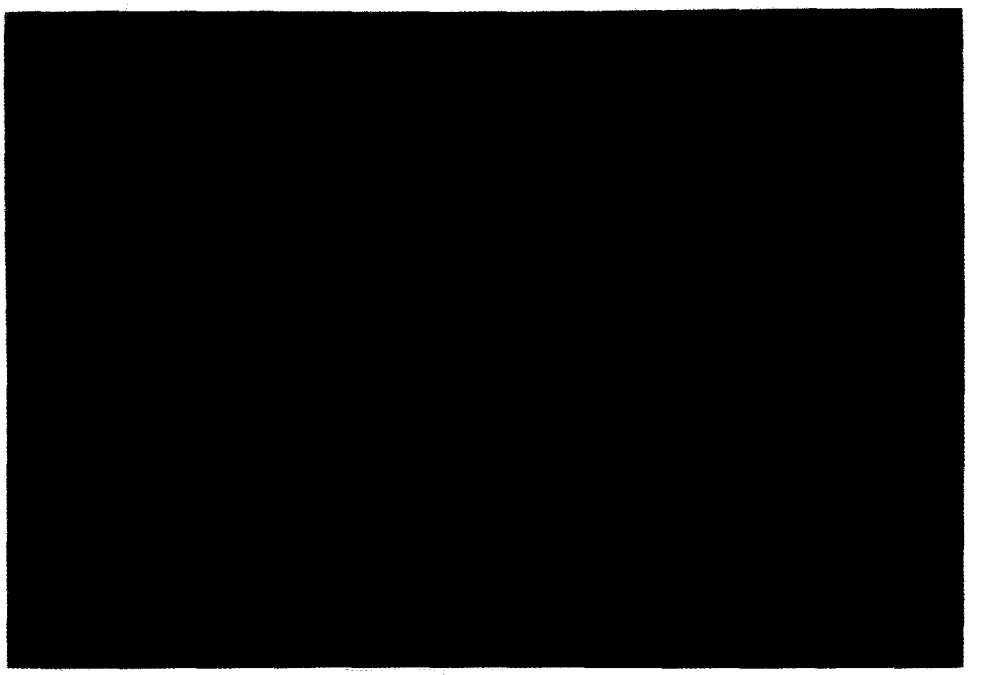


Photo. 1. The MNPCE of mitocycinc 2mg/kg treated group (Center), the reft cell is a NCE

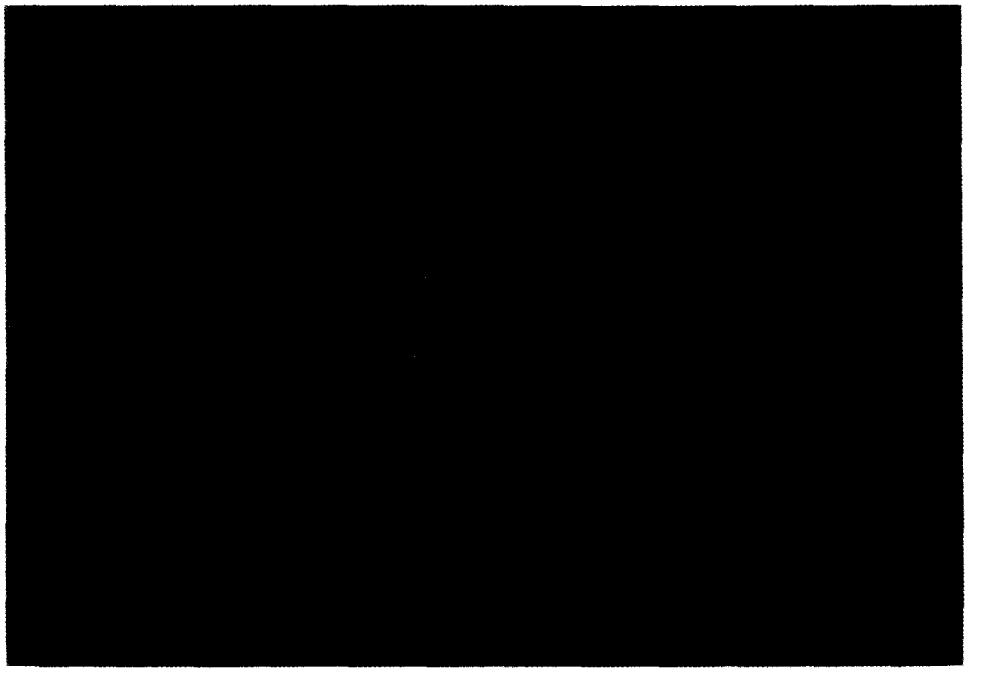


Photo. 2. Several micronuclei are present in single PCE in mitomycin c group.

Photo. 3. The SCE occured in control group.

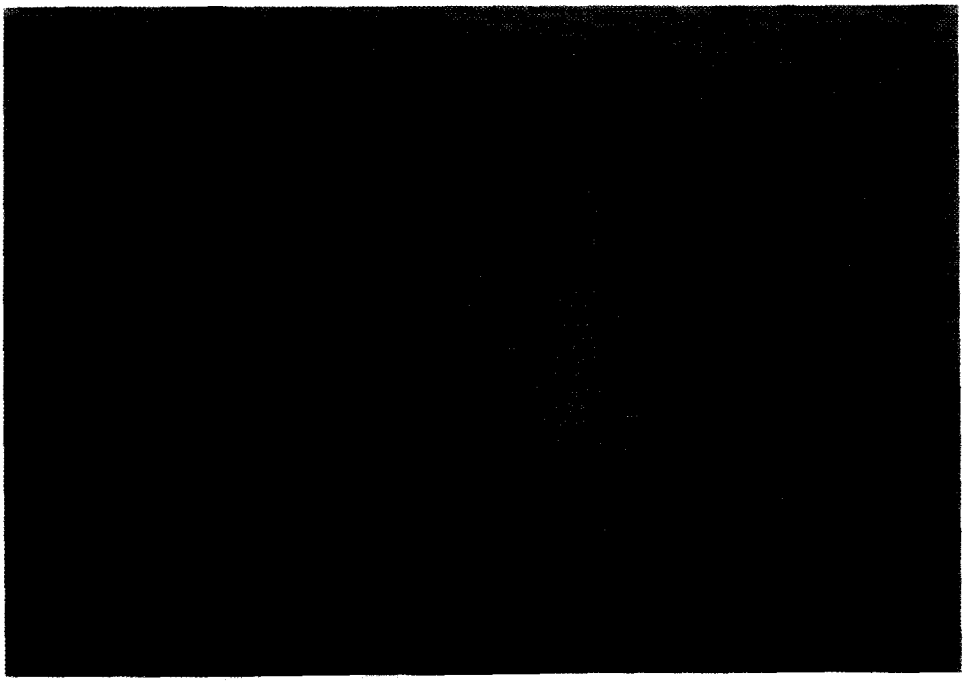


Photo. 4. The massive SCE occured in mitomycin c treated group.

mutagenicity on pranoprofen.

There are various mutagenicity assay methods to evaluation mutagenic activity and combination of these test item is inevitable because the results of an assay method are clearly 'positive' may show 'negative' in another methods.

Ames test was developed by Dr. Ames in 1972.

This test is highly efficient in detecting mutagen in paints of economy and time consuming but bacterial physiology is not similar to human.

Micronucleus test was developed by Heddle and schmid independently. (Heddle *et al.*, 1973; Schmid *et al.*, 1973.)

Micronuclei originate from chromatin which for different reasons has been lagging in anaphase. Lagging has two main cause chromosome breakage and malfunction of the spindle apparatus. In former case micronuclei in erythrocyte are smaller than latter. In our study, micronuclei in mitomycin C group are smaller than quater of erythrocyte diameter and than it was revealed as a 'clastogen' (Yam amoto et. al., 1981).

Micronucleus test has many advantageus in vivo system, but its limits are exist, mutagen which do not reach the target cell in bone marrow and specific point mutagens are not detected. SCE is a highly suitable assay system for the screening of mutagens and carcinogens because of its high sensitivity, but its conditions are far from physiological condition of human.

In this study the results of Ames test were 'negative' in all test strains. It was identified pranoprofen was 'non-clastogen' or 'non-spindle-poison' in micronucleus test and was 'negative' in SCE test. All above results represent that pranoprofen has no mutagenic effect.

REFERENCES

Ames, B.N., Kammen, H.O. and Yamasaki, E. (1975a); Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients. Proc. Nat. Acad. Sci. 72: 2423-2427.

Ames, B.N., Mccann, J. and Yamasaki, E. (1975b); Methods for detecting carcinogens and mutagens with the salmonella mammalian microsome mutagenicity test. Mutation Res. 31: 347-364.

Andrews, A.W., Thibault, L.H. and Lijinsky, W. (1978a); The relationship between carcinogenicity and mutagenicity of some polynuclear hydrocarbons. Mutation Res. 51: 311-318.

Andrews, A.W., Thibault, L.H. and Lijinsky, W. (1978b); The relationship between mutagenicity and carcinogenicity of some nitrosamines. Mutation Res. 51: 319-326.

Coombs, M.M., Dixon, C. and Kissonerghis, A.M. (1976); Evaluation of the mutagenicity of compounds of known carcinogenicity, belonging to the benz(α)anthracene, chrysene and cyclopenta (α) phenanthrene series, using Ames test. Cancer Res. 36: 4525-4529.

Dorothy, M. Maron and Ames, B.N. (1983); Revised methods for the Samonella mutavenicity test. Mutation Res. 113: 173-215.

Goto, K., Akematsu, T., Shimazu, H. and Sugiyama, T. (1975); Simple differential Giemas staining of sister chromatid after treatment with photosensitive dyes and exposure to light and the mechanism of staining. Chromosoma 53: 223-30.

Heddle, J.A. (1973); A rapid in vivo test for chromosomal damage. Mutation Res. 8: 187-190.

Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T. (1976); A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system, In Vitro metabolic Activation in Mutagenesis Testing de Serres, F.J., Fouts, J.R. Bend, J.R., Philpot, R.M. (Eds.) Elsevier/North-Holland Biomedical Press, Amsterdam; 85-88.

Piper, P.J. and Vane, J.R. (1969); Release of additional factors in anaphylaxis and its antagonism by anti-infiammatory drugs. Nature 223: 29-35.

Vane, J.R. (1971); Inhibition of prostaglandin synthesis as a mechanion of action of aspirin like drugs. Nature New. Biol. 231: 232-235.

Yamamoto, K.I. and Y. Kikuchi (1981); Studies on micronuclei time response and on the effects of multiple treatment of mutagens on induction of micronuclei. Mutation Res. 90 163-173.