RESEARCH COMMUNICATION

# Antimutagenic Effects of Piperine on Cyclophosphamide-induced Chromosome Aberrations in Rat Bone Marrow Cells

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## Abstract

**Piperine is a major pungent substance and active component of black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.). Both plants are used worldwide as household spices and condiments. They are also used as important ingredients in folklore medicine in many Asian countries. Therefore, it is of interest to study antimutagenic effects of piperine. In this study, its influence on chromosomes was investigated in rat bone marrow cells. Male Wistar rats were orally administered piperine at the doses of 100, 400 and 800 mg/kg body weight for 24 hours then challenged with cyclophosphamide at a dose of 50 mg/kg body weight by intraperitoneal injection. Twenty-four hours thereafter , all animals were sacrificed and bone marrow samples were collected for chromosomal analysis. The results demonstrated that piperine at a dose of 100 mg/kg body weight gave a statistically significant reduction in cyclophosphamide-induced chromosomal aberrations. In conclusion, piperine may have antimutagenic potential. The underlying molecular mechanisms now require attention.**

**Key Words:** Piperine - Cyclophosphamide - antimutagenic effects - chromosome aberrations

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## Introduction

The use of spices as additives to enhance the taste and flavour of food has been practiced widely since ancient times (Platel and Srinivasan, 2004). People in many parts of the world frequently and heavily consume several kinds of spices such as chili pepper, black pepper, garlic and caraway seed, onion and others in their foods. Pepper is the one of the oldest and the most important member of spices (Govindarajan, 1977), especially in Southeast Asian and Latin-American countries. Among the species, black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.), which belong to the plant family Piperaceae, are commonly used (Chopra and Chopra, 1955). Piperine (the trans-trans stereoisomer of 1-piperoyl piperidine) is the major pungent ingredient, active principle and also the principal alkaloid in these plants (Lewis, 1977).

Although, peppers have been used for a long time, toxicity reports or published data on their toxicity and anti-toxicity have been limited (Ames, 1983). Recently, attention has been focused on whether naturally occurring compounds can modify the effects of various mutagens and carcinogens. Here, we focused on effects on chromosomal aberrations induced by cyclophosphamide, an indirect-acting mutagen, using a cytogenetic method.

## Materials and Methods

*Animals*

Male Wistar rats, 5–7 weeks old and weighting 140-160 g were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed individually in stainless steel cages with wire mesh cover s(temperature 22-25˚C, relative humidity 65 ± 5% and 12 h light/dark cycle). They were fed with rat chow diets (C.P. Food, Bangkok, Thailand) and water *ad libitum*. Prior to the start each experiment, they were acclimatized to the animal room conditions for 1 week.

*Chemicals and selection of dose*

Based on the studies reported by Dalvi & Dalvi (1991) and Piyachaturawat et al (1983), the concentrations of 100, 400 and 800 mg piperine /kg body weight were chosen for the present study. These concentrations did not affect either the food intake or the body weight gain of the animals during treatment. Piperine (Aldrich, USA) at these doses were freshly prepared in the form of suspension in corn oil and mixed well before oral administration for 24 h (Preston et al., 1987).

Cyclophosphamide (CP; ASTA Medica AG, Frankfurt, Germany) was freshly prepared by dissolving 12 mg CP in 1 ml of sterile distilled water at a dose of 50 mg/kg

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body weight which used as standard mutagen. CP was injected intraperitoneally as a single agent to piperine-treated rats 24 h after the last piperine administration. The animals were sacrificed under ether anesthesia 24 h after mutagen administration.

*Bone marrow cell preparation*

Ninety minutes before sacrifice, the animals were injected intraperitoneally with colchicine (Sigma, St. Louis, MO), a metaphase arresting agent, at the dose of 3.5 mg/kg body weight. Their femurs were removed and the femoral marrow cells were flushed out with 3 ml of HBSS medium for chromosome analysis (World Health Organization, 1985; Preston et al., 1987). Bone marrow cells preparations were made by hypotonic solution (0.075 M KCl) and fixative (glacial acetic acid : methanol, 1 : 3). All slides were coded and stained with 10% Giemsa (Adler, 1984; Preston et al., 1987).

*Scoring criteria*

In this study, metaphase cells with one or more chromosome aberrations were scored from 50 well-spread metaphases per animal (250 metaphases per treatment group) at random. The types of aberration (gap, break, exchange and multiple aberrations) were scored and recorded strictly in accordance with the method of Ito and Ito (2001). The severity of chromosome aberrations included the aberrations with break, exchange and multiple aberrations, but not aberrations with gap (Ito and Ito, 2001).

*Statistical analysis*

The statistical software package, SPSS for windows version 12.0 was used for all statistical analysis. The study of chromosomal aberrations was analyzed and expressed as mean and the standard error of mean (X ± SEM). Each treatment group was compared with the control group. The one-way Analysis of Variance (ANOVA) was done to observe the significant differences between the individual groups and multiple comparison (LSD) was carried to compare the data of individual treated groups with the control group. The level of significance was established at $p < 0.05$ or $p < 0.01$.

## Results

The results of chromosomal analysis in male Wistar rat bone marrow cells at metaphase stage when oral administration only piperine at various doses (100, 400 and 800 mg/kg body weight) and piperine combined with CP 50 mg/kg body weight by intraperitoneally injection are summarized in Table 1. The mitotic index (M.I.) of piperine-treated groups was decreased in dose-related manner and showed statistically significant differences from the negative control group except at the lowest dose of piperine. In the combination groups, the M.I. was slightly decreased in a dose-related manner but did not show any statistically significant difference from the CP-treated group except with the lowest dose of piperine (Figure 1).

The percentage of aberrant cells with gap is shown in Figure 2. Only the lowest dose of piperine plus CP showed higher significant difference when compared with piperine-treated groups at equivalent dose or CP-treated group. In summary of this study, the M.I. of piperine-treated groups was decreased in the dose-related manner and showed significant decrease from corn oil-treated group except a dose of 100 mg/kg BW. This means that piperine at the doses of 400 and 800 mg/kg BW might have some cytotoxic effect on bone marrow cells. The M.I. of the combination groups was decreased in the dose-related manner but did not show significant increase from

**Table 1. Chromosome Aberration Rates in Male Wistar Rat Bone Marrow Cells Receiving Piperine and Piperine+CP at Various Doses**

| Group | Treatment (mg/kg BW) | M.I. ± SEM (%) | Types of Chromosome Aberration (%) | | | | Total cells with ab (%) | | Chromosome Damage per Cell |
|---|---|---|---|---|---|---|---|---|---|
| | | | Gap | Break | Exchange | Multiple ab | With Gap | Without Gap | |
| 1 | Corn oil (50mg/kgBW) | 7.54± 0.44[ehjlnp] (0%)* | 1.36± 0.27 | 0 | 0 | 0 | 1.36± 0.27 | 0 | 0 |
| 2 | Piperine100 | 6.74± 0.31[gjl] (10.6%) | 0.64± 0.16 | 0 | 0 | 0 | 0.64± 0.16 | 0 | 0 |
| 3 | Piperine400 | 5.94± 0.31[jn] (21.2%) | 0.64± 0.20 | 0.56± 0.24 | 0 | 0 | 1.20± 0.28 | 0.56± 0.24 | 0.0280± 0.012 |
| 4 | Piperine800 | 5.19± 0.26[jp] (31.2%) | 0.88± 0.39 | 0.16± 0.09 | 0 | 0 | 1.04± 0.39 | 0.16± 0.09 | 0.0080± 0.0049 |
| 5 | CP (50mg/kgBW) | 3.24± 0.21 (57.0%) | 0.56± 0.27 | 3.20± 0.87[bdfh] | 2.00± 0.73[aceg] | 5.92± 2.83[bdfhkmo] | 11.68± 1.77[bdfhl] | 11.12± 1.74[bdfhlmo] | 2.9250± 1.3180 |
| 6 | Piperine100 + CP | 4.20± 0.37[imo] (44.3%) | 1.76± 0.47 | 2.40± 0.22[ac] | 1.44± 0.24[ac] | 0.48± 0.23 | 6.08± 0.83[ad] | 4.32± 0.64[ac] | 0.7800± 0.1702 |
| 7 | Piperine400 + CP | 3.27± 0.23 (56.6%) | 1.20± 0.46 | 5.12± 0.77[bfil] | 1.84± [e] 0.56[a] | 1.60± 0.87 | 9.76± 0.95[bfk] | 8.56± 0.72[bfk] | 1.7000± 0.3958[ae] |
| 8 | Piperine800 + CP | 3.09± 0.14 (59.0%) | 1.44± 0.30 | 4.56± 0.37[bhik] | 2.48± 0.74[bh] | 1.60± 0.55 | 10.08± 1.50[bhk] | 8.64± 1.32[bhk] | 1.8800± 0.5472[ag] |

Data are Mean±SEM, n = 5, nalyzed statistically by one-way ANOVA.*Percentage Mitotic Index Reduction (MIR) Significant differences: [a]p<0.05, [b]p<0.01, from the negative control; [c]p<0.05, [d]p<0.01, from piperine 100 mg/kg; [e]p<0.05, [f] p<0.01, from piperine 400 mg/kg BW [g]p<0.05, [h]p<0.01, from piperine 800 mg/kg BW; [i] p<0.05, [j] p<0.01, from the CP-treated group;[k]p<0.05, [l]p<0.01, from piperine 100 mg/kg BW + CP; [m]p<0.05, [n]p<0.01, from piperine 400 mg/kg BW + CP; [o]p<0.05, [p]p<0.01, from piperine 800 mg/kg BW + CP

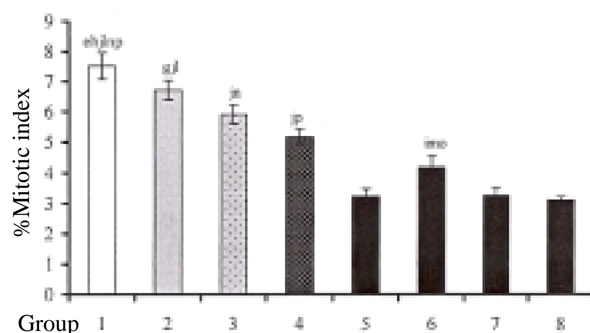


**Figure 1. Percentage Mitotic Indices of Male Wistar Rat Bone Marrow Cells.** Treatment was with piperine at doses of 100, 400 and 800 mg/kg BW, orally (Groups 2, 3 and 4, respectively) and piperine at the doses of 100, 400 and 800 mg/kgBW, orally combined with CP 50 mg/kgBW, i.p. (Groups 6, 7 and 8, respectively). The negative control (Group 1) received corn oil 10 ml/kgBW, orally and positive control (Group 5) received CP 50 mg/kgBW, i.p.Significant differences: [a]p<0.05, [b]p<0.01, from corn oil-treated group; [c]p<0.05, [d]p<0.01, from piperine 100 mg/kg; [e]p<0.05, [f]p<0.01, from piperine 400 mg/kg; [g]p<0.05, [h]p<0.01, from piperine 800 mg/kg; [i]p<0.05, [j]p<0.01,from CP-treated; [k]p<0.05, [l]p<0.01, from piperine 100 mg/kg + CP; [m]p<0.05, [n]p<0.01, from piperine 400 mg/kg + CP; [o]p<0.05, [p]p<0.01, from piperine 800 mg/kg + CP

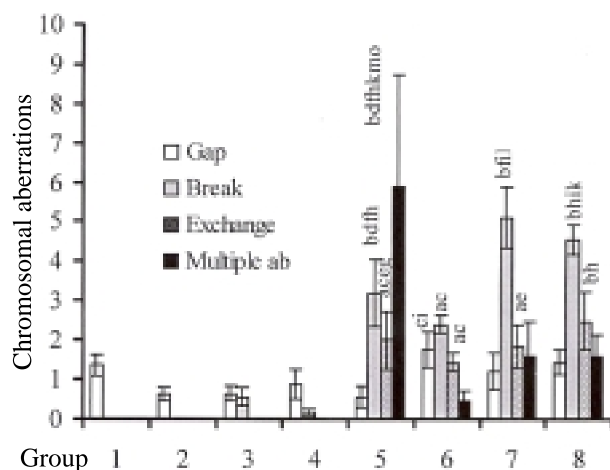


**Figure 2. Frequencies of Chromosome Aberrations.** Treated with piperine at the doses of 100, 400 and 800 mg/kgBW, orally (Group 2, 3 and 4, respectively) and piperine at the doses of 100, 400 and 800 mg/kgBW, orally combined with CP 50 mg/kgBW, i.p. (Group 6, 7 and 8, respectively). Negative control (Group 1) received corn oil 10 ml/kg BW, orally and positive control (Group 5) received CP 50 mg/kgBW i.p. Significant differences: [a]p<0.05, [b]p<0.01, from corn oil; [c]p<0.05, [d]p<0.01, from piperine 100 mg/kg; [e]p<0.05, [f]p<0.01, from piperine 400 mg/kg; [g]p<0.05, [h]p<0.01, from piperine 800 mg/kg; [i]p<0.05, [j]p<0.01, from CP; [k]p<0.05, [l]p<0.01, from piperine 100 mg/kg + CP; [m]p<0.05, [n] p<0.01, from piperine 400 mg/kg + CP; [o] p<0.05, [p]p<0.01, from piperine 800 mg/kg + CP

CP-treated group except a dose of 100 mg/kg BW plus CP. This means that piperine at a dose of 100 mg/kg BW might have partial anticytotoxic effect towards CP-induced damage. The major changes of chromosome that induced by piperine were gap and break which quite the same as in corn oil-treated group while the major changes of chromosome in CP-treated group and the combination groups were gap, break, exchange and multiple aberrations. There was no significant difference in chromosome damage per cell between piperine-treated groups and corn oil-treated group. This means that piperine at the given doses had no effect on chromosome damage of the rat bone marrow cells. However, the lowest dose of piperine plus CP (Group 6) showed significant decrease in chromosome damage per cell when compared with CP-treated alone. This means that piperine at a dose of 100 mg/kg BW might have partial inhibition of chromosomal damage induced by CP.

## Discussion

The relationship between food, nutrition and cancer, and the knowledge that cancer may be a preventable disease has resulted in an increased interest in studying the mutagenic or antimutagenic potential of some dietary constituents (Azevedo et al., 2003). Considerable emphasis has been laid down on the use of dietary constituents to prevent the mutagen induced mutation and/or chromosomal damage due to their relative non-toxic effects. CP (indirect-acting mutagen), a chemotherapeutic drug, damages chromosomes through generate of free-radicals and alkylating DNA thereby producing mutation (Povirk and Shuker, 1994). CP was often used as positive control in genotoxic test, both in laboratory animals or in cell cultures in the presence of liver S-9 fraction. The types of chromosomal aberrations induced by CP as a positive control were reported to be chromosome break, chromatid break, chromatid exchange, chromosomal exchange and ring chromosome (IARC, 1981). In the present study, the types of chromosomal aberrations (except ring chromosome) reports (Sharma, et al., 2001; Shukla and Taneja, 2002; Shukla et al., 2002).

CP generates alkylating metabolites following biological activation, resulting in formation of mutant cells (Vainio et al., 1992). The CYP450 system consists of over 50 related proteins that catalyze the oxidation of many structurally unrelated compounds of endogenous and exogenous origin. They play an important role in the activation and/or inactivation of many drugs. CYP450 activity displays high interindividual variability because of induction or inhibition by drugs and environmental, dietary factors, and genetic factors (Shimada et al., 1994). CP is activated and inactivated by different CYP450 enzymes. At least 6 different CYP450s play a role in CP metabolism. Among them, CYP2B6 is the major enzyme responsible for the bioactivation of CP (Lang et al., 2001).

Antigenotoxic agents especially those present in natural substances act through different cellular pathways involving endogenous sequestration of mutagens by various enzymes (Heddle, et al., 1999; Flora, 1998). Preventing the formation of carcinogens from precursors, blocking the metabolic activation of carcinogens by increasing the activation of detoxification enzymes might inhibit initiation of cancer (Dhuley, et al.,1993). The previous study demonstrated that alkaloids and flavonoids have chemopreventive effects against most of the carcinogens (Surh et al.,1998).

In the present study, the results showed that oral administration of piperine at a dose of 100 mg/kg body weight was found to reduce chromosomal aberrations induced by CP significantly. In the previous study, piperine has been reported as a potent unspecific inhibitor of drug metabolizing enzymes. It plays an important role in phase I as well as phase II drug metabolizing enzymes (Atal et

al., 1985; Singh et al., 1986). The enhancement of drug bioavailability by piperine might be a consequence of the observed reversible and non-competitive inhibition of NADPH-dependent CYP450 mediated monooxygenases (Atal et al., 1985). Piperine was reported as both inhibitory and stimulatory to the CYP450 mediated activities of the microsomal monooxygenases dependent on dose and route of administration (Dalvi and Dalvi, 1991). Earlier studies showed that piperine inhibits both constitutive and inducible CYP450-dependent drug metabolizing enzymes and inhibits CYP4502B1 mediated aflatoxin B1-induced cytotoxicity and genotoxicity in cell cultures (Reen et al., 1997; Koul et al., 2000). Piperine has been identified to play a major role in detoxification of lipid peroxidation in mice and can imply to its antioxidant activity (Selvendiran et al., 2003; Selvendiran et al., 2004; Singh, Sharad and Kapur, 2004). It also enhanced the GSH levels in rat intestinal model (Khajuria et al., 1998). GSH is the most important biomolecule against chemically induced toxicity and eliminate reactive intermediates by conjugation, hydroperoxide reduction or by direct quenching of free radicals (Kaplowitz, 1980; Burk, 1983). GSH is protective against drug cytotoxicity and reacts with toxic endogenous and exogenous substances, including free radicals and anticancer agents (Ali-Osman, 1989). It could modulate anticancer drug cytotoxicity by at least two different mechanisms. Firstly, the drug could be inactivated directly by binding to the sulfhydryl residuals of GSH, and secondly, GSH may reduce cytotoxicity by quenching DNA-drug monoadducts before they can rearrange to toxic bifunctional adducts (Sharma et al., 2001). Reactive mutagenic metabolites of CP have been suggested to undergo detoxification by GSH conjugation (Voskoboinik et al., 1997). Thus, increasing the level of GSH by piperine may increase the rate of CP detoxification.

Therefore, it is suggested that piperine at a dose of 100 mg/kg body weight reduces chromosomal aberrations by affecting to CYP450 mediated mutagenicity of CP. However, the effect of piperine on CP-induced chromosomal aberrations depends on dose of administration. The maximum inhibitory effect of piperine was found at a dose of 100 mg/kg body weight. Further increase in dose did not enhance this inhibitory effect (Table 1). The effect of piperine on mixed-function oxidases is depended on dose and route of administration (Dalvi and Dalvi, 1991). Piperine at the doses of 400 and 800 mg/kg body weight did not affect to CYP450 mediated mutagenicity of CP. Piperine may involve in scavenging potentially toxic mutagenic electrophiles and free radicals. Moreover, the modification of phase II enzymes and the enhancing of detoxification pathways can be involved (Selvendiran et al., 2003; Reen et al.,1996). It is proposed that piperine mediated preventing against the CP could be due to the induction of phase II enzymes involved in the detoxification pathways of CP and / or the inhibition of phase I enzymes responsible for activation of CP. In addition, our results correlated to the recent study by Selvendiran and his co-workers. They demonstrated that a signification suppression (26.7-72.5%) in the micronuclei formation induced by CP was reduced following oral administration of piperine at the doses of 25, 50 and 75 mg/kg body weight in mice (Selvendiran et al., 2005).

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