The Anti-mutagenic Activity of Piperine against Mitomycine C induced Sister Chromatid Exchanges and Chromosomal Aberrations in Mice

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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.). Regarding the variable biological activities of piperine, it is important to study its antimutagenic activity in somatic and germ cells *in vivo*. Swiss albino male mice were orally administered piperine at the doses of 5, 10 and 15mg/kg b. wt. for three consecutive days then treated with mitomycin C (MMC) interaperitonealy (i.p.) at a dose of 1mg/kg b. wt. Twenty-four hours thereafter, all animals were sacrificed and samples were collected from somatic and germ cells for chromosomal aberrations (CA) and sister chromatid exchanges (SCEs). Piperine inhibited the frequency of SCEs induced by MMC in bone marrow cells. This inhibition reached to 41.82% with piperine (15mg /kg b.wt.). The number of chromosomal aberrations induced by MMC in mouse splenocytes and spermatocytes decreased gradually with increasing the dose of piperine. The percentage of inhibition of chromosomal aberrations was 50% and 40.78% in splenocytes and spermatocytes respectively. In conclusion, the results of this *in vivo* study show that piperine has antimutagenic potential against carcinogens. Further investigations are required now to underlie the molecular mechanisms of piperine bioactivity. [Nature and Science 2009; 7(12): 72-78]. (ISSN: 1545-0740).

Keywords: Piperine, MMC, Chromosomal aberrations, SCEs, Spermatocytes, Mice.

1. Introduction

Piperine is an alkaloid found naturally in plants belonging to the Piperacese family, such as Piper nigrum Linn, known as black pepper, and Piper longum Linn, known as long pepper. It has been used extensively as a condiment and flavoring for all types of savory dishes (**Govindarajan, 1977**). Piper species have been used in folklore medicine for the treatment of various diseases, including seizure disorders (**Pei, 1983; Singh, 1992; D'Hooge et al., 1996**). Regarding its structure (Figure 1), piperine contains pentacyclic oxindole group which is effective for immunomodulation (**Reinhart and Uncaria 1999; Pathak and Khandelwal, 2008**).

Piperine is known to exhibit a variety of biological activities which include anti-pyretic (**Parmar et al., 1997**), anti-metastatic (**Pradeep and Kuttan 2002**), antithyroid (**Panda and Kar 2003**) and antidepressant (**Lee et al., 2005**). Piperine exhibits a toxic effect against hepatocytes (**Koul and Kapil, 1993**) and cultured hippocampal neurons (**Unchern et al., 1997**). Simultaneous supplementation with black pepper or piperine in rats fed high fat diet lowered thiobarbituric acid reactive substances (TBARS) and conjugated dienes levels and maintained superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione-S- transferase (GST) and glutathione (GSH) levels close to controls in rats (**Vijayakumar et al., 2004**). **Selvendiran et al. (2005a, b)** observed that supplementation of piperine caused inhibition of Phase I and II enzymes, elevation of glutathione metabolizing enzymes, reduction in DNA damage and DNA protein cross-links in benzo (a) pyrene induced lung carcinogenesis in mice.

The anti-apoptotic efficacy of piperine has also been demonstrated by **Choi et al. (2007)** against cisplatin induced apoptosis via heme oxygenase-1 induction in auditory cells. In another study, **Li et al. (2007)** showed that piperine could reverse the corticosterone induced reduction of brain derived neurotrophic factor (BDNF) mRNA expression in cultured hippocampal neurons. Also piperine has high immunomodulatory and antitumor activity (**Sunila and Kuttan, 2004**). This immunomodulation activity is due to its multi-faceted activities such as anti-oxidative (**Mittal and Gupta, 2000; Pathak and Khandelwal, 2008**), anti-apoptotic and restorative ability against cell proliferative mitogenic response; thymic and splenic cell population and cytokine release (**Pathak and Khandelwal, 2008**).
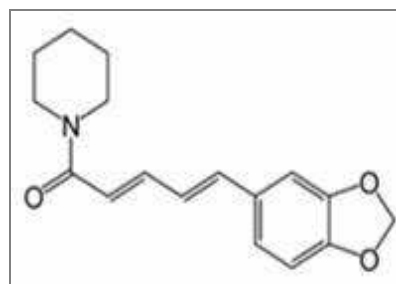

Figure (1): Piperine

Table 1: Frequency of sister chromatid exchanges (SCEs) in mouse bone-marrow cells treated with MMC and piperine with MMC.

Dose (mg/kg b. wt.)	No. of different types of SCEs/chromosome			Total No. of SCEs[a]	SCEs/Cell[b] Mean ±SE	Inhibitory Index (%)
	Single	Double	Triple			
I. Control	627	62	3	760	3.80±0.45	-
II. Piperine						
5mg	626	65	4	768	3.84±0.48	-
10mg	698	68	4	846	4.23±0.51	-
15mg	766	69	6	922	4.61±0.57	-
III. MMC						
1mg	4522	361	42	5370	26.85±0.53**	-
IV. piperine+MMC						
5mg +1mg	3784	352	38	4602	23.01±0.64•	16.65
10mg +1mg	3275	340	31	4048	20.24±0.58••	28.67
15mg +1mg	2854	333	28	3604	18.02±0.71••	38.30

a. The total number of chromosomes is 8000
b. The total number of scored cells is 200 (5 animals/ group)
** $p<0.01$: Significance compared to control; • $p<0.05$, •• $p<0.01$: Significance compared to treatment (t- test).

Mitomycine C (MMC) is one of the powerful mutagenic agents which have the ability to induce genotoxicity in a proliferating cell population such as mice bone marrow cells from the first division after treatment. Thus, it is widely used as positive control agent in genotoxic tests, both in laboratory animals or in cell cultures (**Russo *et al.,* 1992**). It damages chromosomes through generating of free radicals and alkaylating DNA thereby producing mutation (**Brookes, 1990**). Also, it has been demonstrated to inhibit mammalian DNA topo II (**Minford *et al.,* 1986**).

Considering all the above bioactivities of piperine, it was very important to assess the non toxic effect of piperine on somatic and germ cells *in vivo*. Also, this study was performed to determine the role of piperine in the inhibition of the mutagenic effects which could be induced by MMC in mice somatic and germ cells using two main cytogenetic parameters , i.e. chromosomal aberration and sister chromatid exchanges.

2. Materials and Methods
2.1. Animals:
Laboratory-bred strain Swiss albino male mice of 10-12 weeks old with an average weight of 27.5±2.5 g obtained from the National Research Center, Cairo, Egypt, were used. Animals were housed in groups (5 animals/ group) and maintained under standard conditions of temperature, humidity and light. The animals were given standard food and water *ad libitum*.

2.2. Chemicals:
Piperine and mitomycin C were purchased from Sigma, USA. All other chemicals used were of analytical grade.

2.3. Doses:
Animals were divided into 8 groups of 5 animals each. Group I were used as negative control. Group II as positive control, were treated with mitomycin C (MMC) at 1mg/kg b.wt. intraperitonealy (i.p.). Groups III, IV and V were treated orally with 5, 10 and 15mg piperine/ kg b.wt. for 3 consecutive days, respectively. Groups VI, VII and VIII were treated orally with 5, 10 and 15mg/kg b.wt. of piperine, respectively, for 3 consecutive days and with MMC 24h after the last dose of piperine for a single dose . Animals were sacrificed 24h after the last treatments.

2.4. Sister chromatid exchanges (SCEs):
The method described by **Allen (1982)**, for conducting *in vivo* SCE's induction analysis in mice was applied with some modifications. Approximately 55mg 5'-Bromodeoxyuridine (BrdU, Fluka AG, Buchs SG) tablets were inserted in mice subcutaneously (s.c.) 21-23h before sacrifice. Mice were injected intraperitonealy with colchicine at a final concentration of 3mg/kg body wt. 2hrs before sacrifice. Bone-marrow cells from both femurs were collected. The fluorescence-photolysis Giemsa technique was used (**Perry and Wolff, 1974**). 40 well spread metaphases were analyzed per mouse to determine the frequency of SCEs/cell .

Table 2: Number and mean percentage of chromosomal aberrations in mouse splenocytes after treatment with MMC and piperine with MMC.

Dose (mg/kg b. wt.)	No. of different types of chromosomal aberrations					Total aberrations (without gaps)		Inhibitory Index (%)
	Gaps	Fragment and/or Break	Deletion	Robertosonian translocation	Polyploidy	No.	(%)	
I. Control	**4**	**12**	**1**	**-**	**-**	**13**	**2.60**	**-**
II. Piperine								
5mg	**5**	**11**	**3**	**-**	**-**	**14**	**2.80**	**-**
10mg	**3**	**12**	**3**	**-**	**-**	**15**	**3.00**	**-**
15mg	**6**	**10**	**4**	**-**	**-**	**14**	**2.80**	**-**
III. MMC								
1mg	**21**	**67**	**25**	**8**	**3**	**103*****	**20.60**	**-**
IV. piperine+MMC								
5mg +1mg	**18**	**50**	**23**	**7**	**3**	**83 [n.s.]**	**16.60**	**22.22**
10mg +1mg	**17**	**44**	**23**	**4**	**2**	**73••**	**14.60**	**33.33**
15mg +1mg	**18**	**40**	**13**	**4**	**1**	**58•••**	**11.60**	**50.00**

The total number of scored cells is 500 (5 animals/ group)
*** $p < 0.001$: Significance compared to control
n.s.: not significant; •• $p < 0.01$, ••• $p < 0.001$: Significance compared to treatment (X^2- test).

2.5. Chromosome abnormalities in somatic and germ cells:

For somatic and germ cells preparations, animals from the different groups were injected i.p. with colchicines, 2-3h before sacrifice. Chromosome preparations from splenocytes (somatic cells) carried out according to the method of **Yosida and Amano (1965)**. 100 well spread metaphases were analyzed per mouse. Metaphases with gaps, chromosome or chromatid breakage, fragments, deletions, Robertsonian translocation as well as numerical aberrations (polyploidy) were recorded. Chromosome preparations from spermatocytes (germ cells) were made according to the technique of **Evans et al. (1964)**. 100 well spread diakinase-metaphase I cells were analyzed per animal for chromosomal aberrations. Metaphases with univalents and chromosome translocations were recorded.

Evaluation of the activity of piperine to reduce SCEs and chromosomal aberrations induced by MMC was carried out according to **Madrigal-Bujaidar et al. (1998)** formula as follows:

Inhibitory index (II) = [1- (piperine and MMC – control)/ (MMC- control)] X100

2.6. Statistical analysis:

The significance of the results from the negative control data and between piperine with MMC comparing to MMC alone was calculated using t-test for SCEs and Chi-square test (2X2 contingency table) for chromosomal aberrations.

3. Results
3.1. Sister chromatid exchanges:

Sister chromatid exchanges analysis is a rapid objective method of observing reciprocal exchanges between sister chromatids. In the present study, the frequencies of SCEs/cell induced with the different doses of piperine were not significant in comparing to control group. When mice treated with piperine three days prior to MMC, all piperine doses decreased the mean percentage of SCEs/cell induced by MMC alone. The mean percentage of SCEs/cell was reduced from 26.85 ± 0.53 with 1mg/kg b.wt. MMC to 23.01 ± 0.64, 20.24 ± 0.58 and 18.02 ± 0.71 after pretreatment with 5, 10 and 15mg piperine/kg b.wt. respectively. The percentage of inhibitory index increased from 16.65% with low dose to 38.30% with the high dose of piperine. The number and percentage of the different types of SCEs/ chromosome were recorded (Table 1 and Figure 2 a, b).

3.2. Chromosomal aberrations:
3.2.1. In somatic cells:

Table (2) and Figure (2c, d) show the number and percentage of the chromosomal aberrations induced in control and treated animals after three consecutive days of piperine. The percentages of aberrant cells in animals treated with piperine were statistically not significant in comparing to the control group. Piperine reduced the number of the chromosomal aberrations when administered before the positive control MMC in a dose dependent manner. This reduction of

Table 3: Number and mean percentage of diakinase metaphase I cells with chromosomal aberrations in mouse spermatocytes after treatment with MMC and piperine with MMC.

Dose (mg/kg b. wt.)	No. of different types of chromosomal aberrations					Total Aberrations		Inhibitory Index (%)
	XY univalent	Autosomal univalent	XY+ Autosomal univalent	Frag.	Chain (IV)	No.	(%)	
I. Control	8	7	-	-	-	15	3.00	-
II. Piperine								
5mg	10	7	-	-	-	17	3.40	-
10mg	13	6	-	-	-	19	3.80	-
15mg	12	8	-	-	-	20	4.00	-
III. MMC								
1mg	50	31	3	3	4	91***	18.20	-
IV. piperine+MMC								
5mg +1mg	41	30	1	2	2	76[n.s.]	15.20	19.73
10mg +1mg	44	25	-	1	-	70 [n.s.]	14.00	27.63
15mg +1mg	40	18	1	-	1	60••	12.00	40.78

The total number of scored cells is 500 (5 animals/ group); Frag. : Fragment
*** p<0.001: Significance compared to control
n.s.: not significant; •• p<0.01: Significance compared to treatment (X^2- test).

chromosomal abnormalities excluding gaps reached 50% which was highly significant (p<0.001) with 15mg piperine/kg b.wt. in comparing to MMC alone.

3.2.2. In germ cells:

There were no significant differences between the animals treated with piperine alone and the control group. The mean percentage of diakinesis metaphase I cells was 18.2% (p<0.001) with 1mg MMC/kg b.wt. compared to the control. This percentage was decreased gradually parallel to pretreatment with the different doses of piperine and decreased to 12.0% (p<0.01) with 15mg piperine/kg b.wt. (Table 3). Also, Table (3) illustrates the protective effect of piperine in reducing the different types of aberrations. The different types of chromosomal aberrations such as XY-univalents and /or autosomal univalents were recorded (Figure 2e, f).

4. Discussion

The relationships 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**). Also, 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 (**Wongpa *et al.,* 2007**). Depending on these ideas, it

was very important to study the cytogenetic effects induced by piperine in animal model and its bioactivity against MMC as a mutagen.

In the present study, the incidences of the cytogenetic parameters (SCEs and chromosomal aberrations in somatic and germ cells) were not significant when animals were treated with the different doses of piperine alone compared with the control group. This result is confirmation to the other studies which demonstrated that piperine appears to be a non-genotoxic chemical (**Singh *et al.,* 1994; Karekar *et al.,* 1996**). **Karekar *et al.* (1996)** studied the genotoxic potential of piperine using four different test systems, namely, Ames test using *Salmonella typhimurium*, micronucleus test, sperm shape abnormality test and dominant lethal test using Swiss albino mice. In the Ames test, six different doses of piperine, in the range of 0.005-10 mumol/plate, did not induce his+ revertants, with or without metabolic activation, indicating its nonmutagenic nature.

In the bone marrow micronucleus test using two doses in the range of therapeutic usage (10 and 20 mg/kg b. wt.), piperine itself was non-mutagenic. Like in somatic cells, piperine (10 and 50 mg/kg b. wt.) failed to induce mutations in male germ cells of mouse as assessed by using the sperm shape abnormality and dominant lethal tests.

The frequency of SCEs in bone marrow cells induced by MMC was reduced significantly when animals were pretreated with piperine. **Singh** *et al.* **(1994)** studied the effect of piperine on the cytotoxicity and genotoxicity of aflatoxin B1 (AFB1) in rat hepatoma cells H4IIEC3/G-(H4IIE) using cellular growth and formation of micronuclei as endpoints. They demonstrated that piperine reduced the AFB1-induced formation of micronuclei in a concentration-dependent manner. They also suggested that piperine is capable of counteracting AFB1 toxicity by suppressing cytochromes P-450 mediated bioactivation of the mycotoxin and markedly reduced the toxicity of the mycotoxin. **Reen** *et al.* **(1997)** investigated the potential of piperine for inhibiting the activity of cytochrome P4502B1 and protecting against aflatoxin B1 in V79MZr2B1 (r2B1) cells. They demonstrated that piperine was a potent inhibitor of rat CYP4502B1 activity, and piperine counteracted CYP4502B1 mediated toxicity of AFB1 in the cells offered a potent chemopreventive effect against procarcinogens activated by CYP4502B1.

Chromosomal aberrations assay was proved to be sensitive indicator for monitoring the genotoxicity of environmental chemicals (**Dulout** *et al.,* **1983; Tucker and Preston, 1996**).Thus using MMC as a genotoxic agent was important to detect the protective effect of piperine in reducing the number of chromosomal aberration induced by MMC in mouse splenocytes and spermatocytes *in vivo*. In the present study piperine reduced the percentage of chromosomal abnormalities induced by MMC in somatic and germ cells significantly ($p<0.01$) in a dose dependent manner.

Our results agreed with the recent study by **Selvendiran and his co-workers (2005c).** They demonstrated that a significant suppression (26.7-72.5%) and (33.9-66.5%) in the micronuclei formation induced by cyclophosphamide (CP) and benzo(a)pyrene respectively were reduced following oral administration of piperine at the doses of 25, 50 and 75 mg/kg b. wt. in mice. Also, **Wongpa** *et al.* **(2007)** observed that oral administration of piperine significantly reduced chromosomal aberrations induced by CP in rat bone marrow cells.

The mechanism of action of piperine may involve scavenging potentially toxic mutagenic electrophiles and free radicals. Moreover, the modification of phase II enzymes and the enhancing of detoxification pathways can be involved (**Reen** *et al*.**, 1996; Selvendiran** *et al*.**, 2003).**
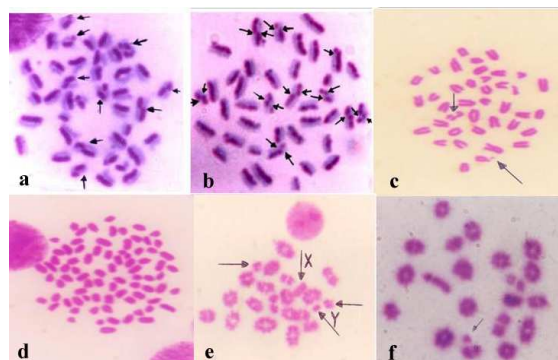

Figure (2): Metaphases from mice treated with piperine and mitomycin C showing (a, b) sister chromatid exchanges from mouse bone marrow cells; (c, d) metaphases from mouse splenocytes showing (c) break and fragment, (d) polyploidy; (e, f) metaphases from mouse spermatocyte cells showing (e) XY univalent and autosomal univalent , (f) fragment.

In conclusion, this study is a complementary survey to the biochemical and immunomodulatory studies and the few cytogenetic studies which were carried out upon piperine to investigate its cytogenetic activity directly on chromosomes by two main cytogenetic biomarkers chromosomal aberrations and sister chromatid exchanges. Using of MMC as a free radical generator and DNA alkylating agent makes severe mutations in chromosomes gives the ability to fairly judge on the cytogenetic activity of piperine in mice *in vivo*. This work explores the antigenotoxic activity of piperine, and its antimutagenic effects in reducing and preventing the DNA damages which can be induced by carcinogens in somatic and germ cells. The underlying molecular mechanisms now require attention.

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10/30/2009