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Protective effect of caffeine against cyclophosphamide induced *in vivo* genotoxicity

Prakash G.

Mr. Prakash received his MSc., in Applied Zoology from the Kuvempu University, Shankaraghatta, Shimoga, Karnataka in 2004 and just submitted the thesis for award of PhD from the Kuvempu University, Shimoga. Currently, working as Associate Research Scientist at the Department of Mutagenicity and Teratology, Advinus Theraputics Ltd., Bangalore. Served as lecturer at the Department of Zoology, Sahyadri Science College, Shimoga for two years. He has been nominated by the CPCSEA as member of IAEC in few organizations.

Prakash G[1]*, Ingle A.D[2] and Hosetti B.B[1].

[1]Department of Applied Zoology, Kuvempu University, Shankaraghatta, 577 451, Karnataka, India.

[2]Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre (TMC), Kharghar, Navi Mumbai- 410 210, MS, India.

Corresponding author:
Prakash G, Associate Research Scientist, DSA, Toxicology, Advinus Therapeutics Ltd., Bangalore
Phone : 9620901066, email : prakash.geriyol@gmail.com

Abstract

Cyclophosphamide is a DNA non-intercalating agent with wide application against several human neoplasms. It is found that caffeine alters protection against cytogenotoxicity of different chemicals and physical mutagens including cyclophosphamide. Modulation of the clastogenic induced effect of cyclophosphamide by three different doses of caffeine (25, 50 and 100 mg/kg) was assessed through the chromosomal aberration and mitotic index studies in bone marrow cells. Micronucleus level were decreased significantly (28.8%) in 100 mg caffeine treated group at 30 h post treatment. Results revealed that caffeine decreased the cyclophosphamide induced chromosomal aberration and mitotic index in bone marrow cells. In the present study, the percentage of dividing cells were reduced more in the mice pre-treated with CAF at the dose rate of 100 mg/kg than with lower doses of CAF in all the three anticancer drugs tested. Thus, CAF modulation is expected to play an important role in the development of cancer chemotherapy. The protective effect of caffeine was found to be dose dependent. However, further study on the mechanism of quenching of free radicals and induction of apoptosis by caffeine are required to substantiate the results.

Key words: caffeine, chromosomal aberration, cyclophosphamide, cytogenotoxicity, mitotic index.

Introduction

Cancer is a scourge affecting mankind from the time immemorial. Since the last century, treatment of cancer remains an enigma till today (Srivastava *et al.* 2002). Since ancient time, the role of natural products has been recognized as a source for remedies (Fransworth *et al*. 1985; Cragg *et al*. 1997). Despite major scientific and technological progress in combinatorial chemistry of drugs derived from natural product, it can still make an enormous contribution to drug discovery today. This is relatively new branch of medicine that has expanded rapidly over the last twenty years. Caffeine is well known for its neurostimulator activity. But caffeine is known to exert protective function against genotoxic/ carcinogenic effects of environmental chemicals *in vitro* and *in vivo* assay systems (Abraham, 1989 and 1991; Aeschacher and Jaccud, 1990; IARC, 1991; Nehlig and Debry, 1994; Stavric, 1992). These findings indicate that caffeine is a chemo- preventive drug against environmental mutagens and carcinogens. The present study was undertaken to evaluate the modulation or regulation of caffeine (*Coffeea arabica*) on cyclophosphamide induced cytogenotoxicity in Swiss mice. The results were evaluated by analyzing the end points of number of chromosomal aberrations and mitotic index (Choudhury *et al*. 1995).

Materials and Methods

Chemicals and Reagents

Cyclophosphamide sulphate injection was procured from Cipla India, Pune, whereas Caffeine (CAF) was purchased from Sigma Co., St. Louis, USA. Colchicine, sodium chloride, sodium citrate, methanol, glacial acetic acid, ethyl alcohol, Giemsa stain, Sorensen's buffer, sodium dihydrogen phosphate, disodium hydrogen phosphate were purchased from HiMedia Chemicals, Mumbai, India.

Animals

Animal experiments were carried out taking appropriate measures to minimize the pain and discomfort in accordance with the guidelines of CPCSEA. Experimental procedures were carried out after obtaining permission from the Institutional Animal Ethics Committee. Swiss mice (6 weeks old) were procured from the Animal Facility of SCS College of Pharmacy, HarapanHalli, Karnataka and were acclimatized to 25 ± 2°C and 12 h-light / dark cycle. The animals were fed with balanced diet (Sai Feeds, Bangalore, India) and water was provided *ad libitum*.

Experimental Protocol

Mice weighing 20- 22 g were selected and divided into five groups with 6 (3 males and 3 females) animals in each group. Among five groups, group I served as control and administered orally with 0.5 ml of 0.9% sterile saline; group II as positive control and administered cyclophosphamide (40 mg/kg body weight); group III, group IV and group V were treated with of caffeine at the dose rate of 25, 50 and 100 mg/kg body weight (bw) respectively. After one h of oral administration, the caffeinated animals were treated with 1.5 mg/kg bw of cyclophosphamide and maintained for a period of 24 h. Later, all the groups except control were administered intraperitoneally with 0.02% of colchicine at the rate of l0 mg/kg bw. One h after the treatment, 3 female and 3 male mice from all groups were sacrificed for the preparation of slides for chromosomal aberration (CA) and mitotic index (MI) study from bone marrow. Further, 3 female and 3 male animals from each group were sacrificed for micronucleus test (MNT) and polychromatic erythrocytes (PCEs) after 30 h.

Mitotic metaphase chromosome aberration study

For mitotic metaphase chromosomal aberration study, slides were prepared from bone marrow cells as per the method described by Choudhury *et al*. (1995). Briefly, at 24 h post-treatment, six mice (3 females and 3 males) from each of the 5 groups were injected intraperitoneally with 0.02% colchicine at the dose rate of 1 ml/100 g bw. After one h, the mice were sacrificed by cervical dislocation, the femur and humerus bones were dissected and the adhered muscles were cleaned. Bone marrow cells from each of the femur and humerus were extracted separately for each of the animal in 0.9% sodium citrate pre-incubated at 37°C, aspirated thoroughly by Pasteur pipette to prepare a free cell suspension and incubated for 20 min at 37°C. The cell suspension was again centrifuged at 2000 rpm for 5 min, supernatant was discarded, freshly prepared fixative (methanol and glacial acetic acid 3:1 v/v) was added and aspirated thoroughly. The cell suspension was centrifuged at 2000 rpm for 5 min, the supernatant was discarded, fresh fixative added, and the cell suspension was aspirated and stored overnight in the refrigerator. Next day, the old fixative was replaced by a desired volume of fresh fixative following centrifugation at 2000 rpm for 5 min. The fixed cell suspension was dropped on clean, grease free pre-chilled slides in 50% ethyl alcohol and immediately warmed to complete the burning of alcohol. The slides were left overnight in dust free chambers for complete natural drying and then stained with 3% Giemsa stock diluted in Sorensen's buffer (pH 6.8) for 1 h and the stained slides were washed thoroughly under running tap water, left for natural drying before observing under binocular microscope. At least 150 well-spread metaphases from each animal were scanned and normal and aberrant metaphases with different types of chromosomal aberrations like chromatid, chromosome gaps, breaks, fragments, minutes, exchanges, pulverization etc. were recorded. The percentage of aberrant metaphases (including metaphases with only chromatid gaps and/ or chromosome gaps) and the aberrations (excluding gaps) per hundred metaphases were calculated and tabulated. The representatives from normal metaphases and aberrant metaphases with various types of chromosomal aberrations were photomicrographed.

Mitotic index study

Mitotic index study was undertaken from the same slides prepared for chromosomal aberration study. At least 1000 cells from each animal were taken into consideration for the calculation of the percentage of dividing cells.

Micronucleus test

Six mice (3 females and 3 males) from each group were utilized for MNT from bone marrow. A simple technique of Choudhury *et al.* (2000) which is based on the basic principles of Schmid (1982) was used for this test. Briefly, at 30 h of post-treatment, the mice were sacrificed by cervical dislocation and the bone marrow from femur and humerus was extracted in 1% sodium citrate at 5- 7°C and aspirated thoroughly by Pasteur pipette for the preparation of a cell suspension. The cell suspension was incubated for 5 min, at 5°C and then centrifuged for 5 min, at 1200 rpm. The supernatant was discarded and a desired volume of 1% sodium citrate at 5°C was added and aspirated gently by a Pasteur pipette to prepare a cell suspension. Small drops of the cell suspension was taken on clean, grease free, dry slides and smeared gently by a glass rod. Smeared slides were kept overnight in a humid chamber at 37°C, dipped in methanol for 5 min, and allowed for natural drying. The slides were then stained with 3% Giemsa stock diluted in Sorensen's buffer (pH 6.8) for 12 to 15 min, washed in tap water and left for natural drying before observation. At least 2000 tinge-blue colored polychromatic erythrocytes (PCEs) were scanned from each animal (the reddish brick coloured normochromatic erythrocytes (NCEs) were not taken into consideration). The

PCE with micro nucleus (MN) were recorded and the total MN per thousand PCE was calculated. Representative PCEs with or without MN were photomicrographed.

Coding and reading of slides

The slides prepared at different end points of various groups of mice were coded immediately with five digital random code numbers obtained from statistics books. After the completion of observation, slides were decoded and the data were recorded against the respective groups of mice. The data generated at different end points for different groups of mice were pooled group-wise and their averages with standard deviation were calculated.

Statistics

The differences between the groups were statistically evaluated and the significance of the differences at different levels were recorded following the tables of Kastenbaum and Bowman (1970). In any case, a difference was considered as statistically significant when $P \leq 0.05$ and $P \leq 0.01$.

Results

The possible protective effect, if any, of caffeine on cyclophosphamide induced cytogenotoxicity was investigated. It was found that the two higher doses of caffeine (50 mg and 100 mg/kg) significantly reduced the average percentage of aberrant metaphases ($P \leq 0.01$ and $P \leq 0.05$, respectively)

Table 1: Chromosomal aberrations in bone marrow cells of mice induced by caffeine and cyclophosphamide at 24 h post-treatment

Chemicals	Number of metaphases examined	Number of aberrant metaphases	Average percentage of aberrant metaphases ± SD	Types and number of chromosomal aberration					Total number of aberrations (excluding gaps)	Average percentage of aberrations (Excluding gaps) per 100 metaphases ± SD)
				Chromatic Type		Chromosome type		Frag-ment/ Minute		
				Gap	Break	Gap	Break			
Normal/Control	1061	30	2.79± 0.57	25	1	4/-	-	4	5	0.48±0.24
Cyclophosphamide/ Control	900	159	17.66± 2.21 ** [a]	111	75	-	-	21/9	114	12.60±1.16** [b]
Caffeine (25 mg) / Cyclophosphamide	900	137	15.21±2.12# [b]	95	62	2	4	26/11	118	13.10±2.51
Caffeine (50mg) / Cyclophosphamide	900	103	11.44±1.85** [b]	67	46	7	2	19/7	83	9.22±1.65** [b]
Caffeine (100mg) / Cyclophosphamide	900	124	13.77 ± 2.88 * [b]	62	42	3	5	19/15	101	11.22±1.76

Pooled data of 6 mice (3 females and 3 males), a Significant compared to vehicle control
SD = standard deviation b Significant compared to CP 40 mg/kg, * P <0 05 ** P ≤0.01 [two-tail t-test]

Table 2 : Protective effect of caffeine on mitotic index in bone marrow cells of mice

Chemical	Number of cells examined	Cells in dividing condition	% of dividing cells	Mean±SD
Normal/Control	7030	184	2.62	1171.7 ± 2.34
Cyclophosphamide/Control	6594	190	2.88	1099.0± 4.56
Cyclophosphamide/25 mg Caffeine	6237	203	3.25	1039.5± 8.94 *
Cyclophosphamide/50 mg Caffeine	6186	173	2.80	1031.0 ± 8.88**
Cyclophosphamide/100mg Caffeine	6323	167	2.64	1053.8 ± 14.18

Cyclophosphamide – 40 mg/kg, Significant compared to CP 40 mg/kg, * P ≤ 0.05, ** P≤ 0.01

Table 3. Induction of micronucleus in polychromatic erythrocytes of mice at 30 h post-treatment with caffeine and cyclophosphamide

Chemical	Total number of PCE examined	Number of PCE with MN	Total number of MN	Average MN per 1000 PCE ± S.D
Normal/Control	12593	34	35	2.82±1.16
Cyclophosphamide/Control	12150	418	458	37.64± 5.60 ** [a]
Caffeine (25 mg) /Cyclophosphamide	12480	429	446	35.72± 1.57
Caffeine (50 mg) /Cyclophosphamide	12368	387	414	33.44±2.68
Caffeine (100 mg) /Cyclophosphamide	12555	336	362	28.79 ± 2.57 ** [b]

Pooled data of 6 mice (3 females and 3 males) [a] Significant compared to vehicle control, Micronucleus = MN
SD = standard deviation [b] Significant compared to CP, ** P ≤ 0.01 Polychromatic Erythrocytes = PCE

induced by cyclophosphamide. The average CAs and excluding gaps induced by cyclophosphamide was also decreased in the groups of mice treated with caffeine at 50 and 100 mg/kg bw respectively and the assumed decrease was statistically significant ($P \leq 0.01$) in the mice pre-treated with caffeine 50 mg/kg bw alone. Markedly, the reduction in percentage of aberrant metaphases and CAs were more for the intermediate dose of caffeine **(**Table 1). In MI study, the average percentage of dividing cells induced by cyclophosphamide was decreased in mice treated with either caffeine 50 or 100 mg/kg bw. But the decrease in dividing cells was not statistically significant **(**Table 2**).** In MNT, only the highest dose of caffeine (100 mg/kg) significantly reduced the cyclophosphamide induced MN ($P \leq 0.01$), whereas the other two doses of caffeine (25 and 50 mg/kg) could only reduce the cyclophosphamide induced MN, but not as significant as the higher dose (Table 3).

Discussion

Irrespective of nature of the genotoxic agents and their mechanisms of action in the induction of the cytogenotoxic effects, caffeine is capable of providing protection to the cells from such effects, may be due to different genotoxic chemicals without interacting directly with the genotoxic agents. Therefore, it can be reasonably proposed that CAF does not give protection from the genotoxicants, rather it provides protection from their genotoxic effects, could be from the aftermath actions of the genotoxicants. Cyclophosphamide is a topoisomerase-II inhibitor and genotoxic to mouse *in vivo*. But the cytogenotoxic effects were decreased in caffeine pre-treated mice. Thus, CAF might not have interacted directly on drug, but it might have interacted upon a common material produced by the action, which was instrumental in causing cytogenotoxic effects. It is known that treatment with anticancer drugs like ethyl nitrosourea, methylnitrosourea, ethylmethanesulfonate and methylmethanesulfonate etc., creates a state of oxidative stress in the body (Noda and Wakasugi, 2001). A fall in the plasma antioxidants occurs following treatment with certain anticancer drugs (Weijl *et al*. 1998). The reactive oxygen species (ROS) generated by these drugs leads to lipid peroxidation and DNA lesions, which are implicated in the cytogenotoxic action of these drugs (Lown *et al*. 1982). Caffeine may also play an important role in protecting the cells against planar aromatic molecules such as intercalating agents (Traganos *et al.* 2008). This ROS triggers apoptosis via p53 and cytochrome release from mitochondria. The mechanism of action of most of the anticancer drugs involves active oxygen. Redox control is also involved in various tissues related to anticancer drug therapy (Noda and Wakasugi, 2001). Thus, oxidative stress is related to the treatment of most of the anticancer drugs, if not to all. On the other hand, CAF readily accepts electrons and acts as a free radical scavenger. It is a potent scavenger of hydroxyl radicals, which is comparable with that of other efficient hydroxyl radical scavengers (Shi *et al*. 1991; Devasagayam and Kesavan, 1996). It is a1so an effective inhibitor of lipid peroxidation (Lee, 2000). The possible mechanism involved in the antioxidant effects of CAF is the quenching of ROS (Devasagayam *et al*. 1996). Contrarily, CAF has a DNA-repair inhibiting effect and it enhances the cytocidal effects of anticancer drugs (Tsuchiya *et al*. 2000). It primarily inhibits replicative DNA synthesis, potentiates the cytotoxicity by intervening in DNA repair, overrides G2/M block by leaving less time for post-replication repair, or makes S-phase delay (Deplanque *et al*. 2000). Fernandez *et al*. (2003) also reported that the CAF is of critical importance because high doses of CAF induces apoptosis and low concentrations can act as antioxidants. There are reports supporting our findings that, post-treatment of different concentrations of caffeine significantly reduces the frequency of chromosomal aberrations induced by gamma-rays in mice (Farooqi and Kesavan, 1992). However, some investigators have reported that the caffeine alone induces a concentration and time-dependent inhibition of DNA synthesis and inhibit the entry of human fibroblasts into S-phase by 70–80% regardless of the presence or absence of wild type ATM or p53 as well as it also enhances the inhibition of cell proliferation induced by ultraviolet radiation in XP variant fibroblasts (Kaufmann *et al.* 2003).

The CAF was found weakly clastogenic to mouse bone marrow cells. Caffeine possibly may have played an important role in protecting the cyclophosphamide induced cytogenotoxicity in the somatic and male germ line cells of mice. Transmission of such effects in the male germ line was mostly significant for the highest dose of caffeine (100 mg/kg bw) except in the mitotic metaphase chromosomal aberration study, where caffeine 50 mg/kg bw reduced the toxicity of cyclophosphamide more efficiently. However, pre-treatment of each of the three doses of CAF has reduced the frequency of MTX 10 mg/kg-induced aberrant metaphases, CAs, MN and also the percentage of dividing cells, but significant reduction was only seen in the two higher doses of CAF. Thus, the higher doses of CAF were shown to protect mouse bone marrow cells from the cytogenotoxicity of MTX (Ramesh and Anil, 2004).

From the results of present study, it can be inferred that CAF met the challenges of the oxidative stress created following the treatment with the anti-neoplastic drugs in the cells of mice and thus, protected them to some extent from the cytogenotoxic effects caused through the generation of free radicals. As the higher doses of CAF induce apoptosis, thereby eliminating most of such affected cells and leaving less number of affected cells for observation. The higherdose of CAF tested here (50 and 100 mg/kg) was found to be more protective may be because of apoptosis induction and resulting in the incidence of less number of affected cells than that of the lower dose of CAF. The potentiation of genotoxicity by the higher dose of CAF might have also caused the grossly affected cells to undertake apoptotic pathway with the concomitant decrease in the effects observed from the surviving cells. Therefore, in the present MI study the percentage of dividing cells were reduced more in the mice pre-treated with CAF at the dose rate of 100 mg/kg than with lower doses of CAF. Thus, CAF modulation is expected to play an important role in the development of cancer chemotherapy. However, in-depth study is required to be undertaken to fully understand its potentials.

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