Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2016; 8(3); 125-129

ISSN: 0975-5160

Research Article

EGCG: The Shield Against Genotoxicity Caused by Cisplatin

Choudhary A[1], Kaur B[1], SinghV S[3], Shah K[3], Kalsi V[2], Suttee A[2*]

[1]*Department of Pharmacology, School of Pharmaceutical Sciences, Lovely Professional University, Punjab, India*
[2]*Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Sciences, Lovely Professional University, Punjab, India.*
[3]*Department of Toxicology, Nicholas Piramal Pvt Ltd, Mumbai, Maharashtra, India*

Available Online:10th June, 2016

ABSTRACT
Genotoxicity studies are of great interest because of the wide spread and often chronic use of modern medicinal products, food products as well as environmental chemicals. Cisplatin is platinum based potent chemotherapeutic agent used to treat a variety of solid tumor, however, it also known to interact with specific biological molecules and produce several side effects such as genotoxicity, nephrotoxicity and ototoxicity. Cisplatin produces genotoxicity by generating oxygen/nitrogen free radicals during chemotherapy and causes DNA damage. Hence, to overcome such side effects antioxidants are employed. Epigallocatechin gallate (EGCG), a polyphenolic compound is a potent antioxidant obtained from green tea. In the present experiment protective effect of EGCG against cisplatin induced genotoxicity was evaluated by the bacterial reverse mutation assay (Ames test) and bone marrow micronucleus assay. It has been observed that EGCG significantly reduces the number of revertant colonies induced by cisplatin in bacterial reverse mutation assay. Whereas pretreatment with EGCG significantly reduces the number of micronucleated polychromatic erythrocytes in micronucleus test. The present result suggests that EGCG provides significant protection against cisplatin induced genotoxicity.

Keywords: Genotoxicity, Cisplatin, EGCG, Ames test, Micronucleus test

INTRODUCTION

Many anticancer drugs have been shown to be mutagenic, teratogenic and carcinogenic in experimental system and secondary malignancies are also shown to be associated with several specific chemotherapeutic treatments. Cisplatin is an effective anticancer agent with a wide spectrum activity against various solid tumours such as testicular, ovarian, bladder, cervical, lung, esophageal, head and neck tumour[2,4,6,19]. In spite of these therapeutic applications, the usage is limited due to the side effects such as genotoxicity, neurotoxicity, ototoxicity, nephrotoxcicity[19]. The genotoxic potential of cisplatin was proved in various animal species by means of chromosomal damage as well as micronucleus formation[4,10]. Cisplatin increases the oxidative/ nitrosative stress by generating free reactive oxygen as well as nitrogen species and reducing endogenous antioxidant enzyme level that ultimately increases the risk of development of secondary tumours in animals and patients[2,4,16,17,18,20]. Some reports show that cisplatin-induced ROS formation is also responsible for the severe side effects such as nephrotoxicity and hepatotoxicity[5]. Therefore, it is of clinical importance to find a protective therapeutic approach to prevent genotoxicity and provide safe treatment to patients. In recent years, antioxidants have gained a lot of importance because of their potential to provide prophylactic and therapeutic effects in diseases related to oxidative stress. Epigallocatechin gallate (EGCG) is a polyphenolic compound found in green tea leaves, having potent free radical scavenging activity. It is presumed that it prevent genotoxicity by combating oxidative/ nitrosative stress induced by cisplatin. In the present manuscript, protective effect of different concentration of EGCG against cisplatin induced genotoxicity is evaluated *in vitro* and *in vivo* by means of bacterial reverse mutation test and micronuclei test respectively.

MATERIALS AND METHOD

Cisplatin and EGCG were purchased from Sigma Aldrich, Banglore, India.

In vitro analysis

Ames test: The antimutagenic potency of EGCG was determined by Ames test. The Ames test was performed by using *Salmonella typhimurium* TA98 and TA100 and *E. coli* strains[1]. The mutagenic substance cisplatin was used at 1µg and 2µg concentration per plate for each TA98, TA100 and *E. coli* strain. The antimutagenic effect of EGCG was determined at three different concentrations (3µg, 30µg, 300µg) for strains TA98, TA100 and *E. coli*. For determination of protective effect of EGCG, its concentrations (3µg, 30µg, 300µg) were mixed with individual concentration of mutagen. For the metabolic activation, S9 liver homogenate was used[1]. Mutagenic activity was determined in presence (+S9) and absence (-S9) of metabolic activation system. The mutagen, antimutagen and combination of both these agents were

**Author for Correspondence*

Table 1: Animal treatment for MN test

Group	Treatment	Day 1	Day 2	Day 3
Group 1	Control	Saline	Saline	Sacrifice
Group 2	CDDP	5 mg/kg	5 mg/kg	Sacrifice
Group 3	EGCG	50 mg/kg	50 mg/kg	Sacrifice
Group 4	EGCG	100 mg/kg	100 mg/kg	Sacrifice
Group 5	EGCG + CDDP	50 + 5 mg/kg	50 + 5 mg/kg	Sacrifice
Group 6	EGCG + CDDP	100 +5 mg/kg	100 +5 mg/kg	Sacrifice

Table 2: Antimutagenic effect of EGCG on *Salmonella typhimurium* TA98 strain

Cisplatin + EGCG (µg/plate)	*S. typhimurium* TA98 (-S9)			*S. typhimurium* TA98 (+S9)		
	No. of revertants	SD	% inhibition	No. of revertants	SD	% inhibition
Control	24	2	-	30	2	-
1 + 0	38*	3	-	60*	6	-
2 + 0	74*	2	-	74*	7	-
0 + 3	19	3	-	32	3	-
0 + 30	27	3	-	26	2	-
0 + 300	22	4	-	22 *	1	-
1 + 3	34	3	9	53	6	12
1 + 30	31	2	18	49	6	19
1 + 300	22^{a}	2	42	41^{a}	4	32
2 + 3	69	2	7	71	7	4
2 + 30	61^{b}	3	18	66	5	11
2 + 300	52^{b}	2	30	58	4	22

SD- Standard deviation, Superscript describes statistically significant difference at $p \leq 0.05$. (*) when compared with control, (^{a}) when compared with cisplatin 1µg, (^{b}) when compared with cisplatin 2µg.

Table 3: Antimutagenic effect of EGCG on *Salmonella typhimurium* TA100 strain

Cisplatin + EGCG (µg/plate)	*S. typhimurium* TA100 (-S9)			*S. typhimurium* TA100 (+S9)		
	No. of revertants	SD	% inhibition	No. of revertants	SD	% inhibition
Control	103	13	-	100	6	-
1 + 0	302*	5	-	317*	19	-
2 + 0	433*	17	-	464*	11	-
0 + 3	86	4	-	101	3	-
0 + 30	86	7	-	94	6	-
0 + 300	80	4	-	95	4	-
1 + 3	292	6	3	310	22	2
1 + 30	199^{a}	5	34	243	29	23
1 + 300	116^{a}	6	45	150^{a}	18	53
2 + 3	420	18	3	444	17	5
2 + 30	338^{b}	6	22	344^{b}	17	26
2 + 300	194^{b}	8	55	261^{b}	13	44

SD- Standard deviation, Superscript describes statistically significant difference at $p \leq 0.05$. (*) when compared with control, (^{a}) when compared with cisplatin 1µg, (^{b}) when compared with cisplatin 2µg.

tested in three separate strains with three plates in each test. Colony counting was done by Synoptics colony counter. Data was analysed by unpaired student t-test.

In vivo analysis

Animal treatment: Experiment was performed with healthy Swiss albino mice of 6-7 weeks, they were kept in standard environmental condition (Temperature: 22 ± 3°C, Relative humidity: 50 ± 20%) under 12 hours light/dark cycle. For the time-course mice were divided into six groups consisting of 5 animals in each group. Their dosing schedule is given below.

MN test: The rodent bone marrow erythrocyte micronucleus test provides an *in vivo* method for detection of clastogenicity or genotoxicity of any chemical. Immediately after animal sacrifice, bone marrow from femur and tibia was flushed out in foetal bovine serum with the help of syringe. The bone marrow cell suspension was centrifuged at 1000 rpm for 10 min. The supernatant was discarded and a small drop of the viscous suspension was smeared and dried then the smear was fixed in absolute methanol for 5-10 min and air dried. After, it was stained with 10% Giemsa for 15 min. The slides were analysed for normochromatic erythrocyte, polychromatic erythrocyte, micronuclei polychromatic erythrocyte. Data was analysed by one way ANOVA followed by bonferroni test.

Table 4: Antimutagenic effect of EGCG on *E.coli* strain

Cisplatin + EGCG (µg/plate)	*E. coli* (-S9)			*E. coli* (+S9)		
	No. of revertants	SD	% inhibition	No of revertants	SD	% inhibition
Control	26	1	-	39	1	-
1 + 0	42*	1	-	57*	3	-
2 + 0	71*	2	-	84*	2	-
0 + 3	27	1	-	41	5	-
0 + 30	29	1	-	41	6	-
0 + 300	28	1	-	39	5	-
1 + 3	41	1	2	55	3	3
1 + 30	40	1	5	54	3	5
1 + 300	39	0	7	53	2	7
2 + 3	69	2	3	80	3	5
2 + 30	67	2	6	80	2	5
2 + 300	66	2	7	77	3	8

SD- Standard deviation, Superscript describes statistically significant difference at $p \leq 0.05$. (*) when compared with control.

RESULTS

Ames test

The result of Ames test is shown in tables 2-4 as a number of revertant colonies and percentage inhibition by the EGCG at three different concentration in comparison with mutagen, cisplatin alone in presence (+S9) and absence (-S9) of metabolic activation. The result is expressed as mean ± S.D. It has been observed that Cisplatin (1µg and 2µg) shows a significant ($p \leq 0.05$) increase in number of revertant colonies in all three strains, whereas the antimutagenic potential of EGCG was observed significantly ($p \leq 0.05$) in TA100 strain at 30µg and 300µg concentrations. The effect was dose dependant. Morover, EGCG (3µg) shows antimutagenic effect with cisplatin (1µg and 2µg), the effect was not significant. In case of *Salmonella typhimurium* TA98 strain the data was significant in some cases whereas in case of *E. coli* strain EGCG shows antimutagenic effect but the effect was not significant.

Micronucleus test

Genotoxic potential of any chemical entity is determined by calculating number of micronuclei polychromatic erythrocytes formed among 2000 polychromatic erythrocytes in the bone marrow cells collected from animal. In the present experiment when mice were treated with cisplatin 5mg/kg showed significant ($p \leq 0.001$) increase in MNPCE from 68 ± 4 in the control group to 475 ± 9 which is shown in fig. 1. When mice exposed to EGCG at 50mg/kg and 100mg/kg alone did not show any increase in number of MNPCE compared with the control group. EGCG was given as pre-treatment at 50mg/kg and 100mg/kg followed by cisplatin 5mg/kg after one hour showed significant ($p \leq 0.001$) reduction in MNPCE from 475 ± 9 to 448 ± 7 and 354 ± 12 respectively. Our data shows that EGCG pre-treatment at 100mg/kg dose shows significant ($p \leq 0.001$) reduction in MNPCE when compared with cisplatin and EGCG 50mg/kg pre-treatment group. (Fig. 1). The cytotoxic potential of any chemical entity is determined by calculating number of polychromatic erythrocytes in 200 polychromatic erythrocyte and normochromatic erythrocytes in bone marrow cells collected from animal. The control group showed 109±3 PCE in 200 calculated cells which reduced significantly ($p \leq 0.001$) on treatment with cisplatin 5mg/kg to 39±2. The groups treated with EGCG at 50mg/kg and 100mg/kg showed 92±5, 101±4 PCE in calculated cells. Pre-treatment with EGCG at 50mg/kg and 100mg/kg groups showed significant ($p \leq 0.001$) prevention in reduction in PCE from 39±2 to 69±4 and 81±3 respectively.

DISCUSSION AND CONCLUSION

Cisplatin is a potential anticancer agent used in various solid tumours. However, it is also reported to produce severe side effects like secondary tumour formation, genotoxicity, nephrotoxicity and hepatotoxicity by inducing oxidative/ nitrosative stress in body and leads to ROS generation. The formation of ROS such as superoxide anion, hydroxyl radical and peroxynitrite depends on the concentration of cisplatin administered[4, 12]. On transportation of cisplatin across the cell membrane, the attached chlorine atoms are displaced by water and forms reactive complex which further interact with DNA molecules. They form intra- and inter-strand cross linking between N^7 and O^6 of the adjacent guanine molecules and leads to local denaturation of the DNA chain[9]. Based on the earlier findings, the genotoxicity and related side effects caused by free radical generation can be blocked by using potent antioxidant. Epigallocatechin gallate was reported to have antioxidant activity[3]. EGCG exhibited significant dose-dependent antimutagenic activity against N-methyl-N-nitro-N-nitrosoguanidine (MNNG), N-nitroso-N-methylurea (MNU), 9-aminoacridine (9-AA), 4-nitroquinoline-N-oxide (4-NQNO) as assessed by Ames test. EGCG also suppressed aflatoxin B1-induced chromosomal aberrations in rat bone marrow cells *in vivo* when administered 24 hr prior to the carcinogen injection[8]. Moreover, it has been reported to have anti hepatotoxic activity[7]. Therefore, taking these findings into consideration, the protective effect of EGCG against cisplatin induced genotoxicity was evaluated by *in vitro* and *in vivo* methods in the present study. The *in vitro* test

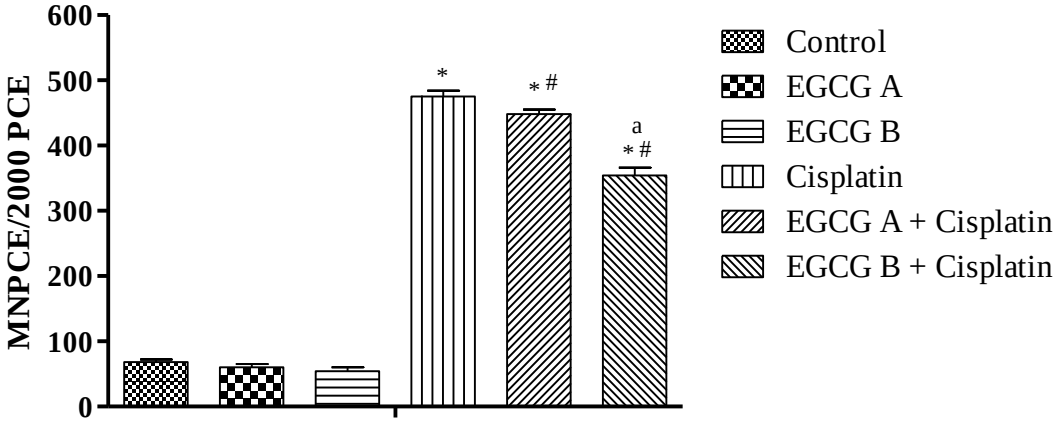

Figure 1: Number of multinucleated polychromatic erythrocytes (MNPCE) calculated among 2000 polychromatic erythrocytes (PCE) after different treatments with EGCG A (50mg/kg), EGCG B (100mg/kg), Cisplatin (5mg/kg), EGCG A + Cisplatin and EGCG B + Cisplatin. Values are expressed as mean ± SD (n=5). Superscript character indicate significant difference at p≤0.001. (*) indicate significantly different from control group, (#) indicate significantly different from cisplatin group, (a) indicate significant difference from EGCG A + Cisplatin group.

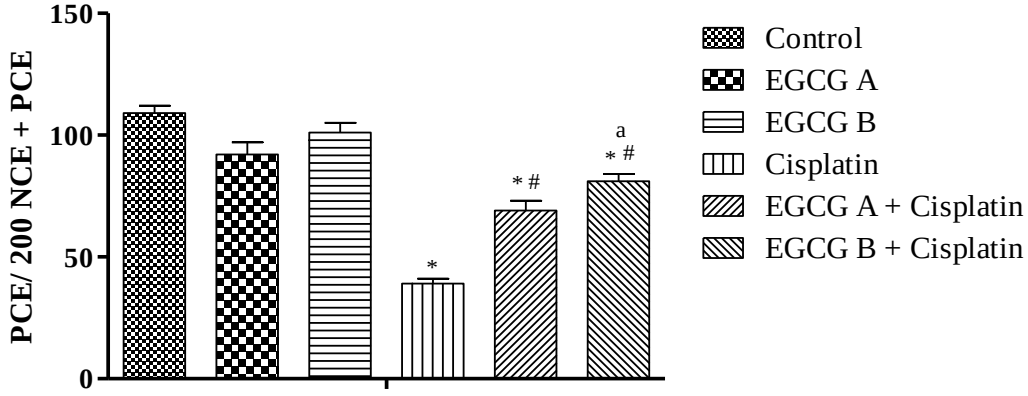

Figure 2: Number of polychromatic erythrocyte (PCE) per 200 normochromatic erythrocyte (NCE) and polychromatic erythrocyte (PCE) calculated after different treatments with EGCG A (50mg/kg), EGCG B (100mg/kg), Cisplatin (5mg/kg), EGCG A + Cisplatin and EGCG B + Cisplatin. Values are expressed as mean ± SD (n=5). Superscript character indicate significant difference at p≤0.001. (*) indicate significantly different from control group, (#) indicate significantly different from cisplatin group, (a) indicate significant difference from EGCG A + Cisplatin group.

including bacterial reverse mutation test (Ames test) uses amino-acid (histidine) requiring strains of *Salmonella typhimurium* (TA98 and TA100) and *Escherichia coli* to detect point mutations. The objective of this test is to evaluate the antimutagenic potential of EGCG on different strains of *Salmonella typhimurium*. This study involves the mixture of S9 as metabolic activation system because *Salmonella typhymurium* is only a prokaryote (basic cell living structure) and cannot represent the human being as a perfect model; therefore the addition of the exogenous liver enzyme could enhance the quality of the result. A positive response such as an increase in number of revertant colonies in any single bacterial strain either with (+S9) or without metabolic activation (-S9) is sufficient to designate a substance as genotoxic[13]. The top agar is the most critical medium components in Ames test because it contains the trace amount of histidine (0.05 mM) for limited growth of *Salmonella* strains. It also contains biotin at a concentration of 0.05 mM which is in excess of what is needed for the growth of *Salmonella* strains. After 48 hours incubation, the revertants colonies of *Salmonella typhymurium* were counted by using Computerized Colony Analyzer (ProtoCol.). The specificity and sensitivity was fixed to 96% with 8 cm diameter of countered area. This measurement was done homogenously to all plates. In the present experiment it has been observed that Cisplatin is potentially genotoxic *in vitro* at higher doses in presence and absence of metabolic activation. Furthermore, EGCG treatment significantly (p≤0.05) reduces the number of revertant colonies in *Salmonella typhimurium* TA100 strain at 30µg and 300µg doses in presence and absence of metabolic activation. The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the chemical agent to the chromosomes. The purpose of the micronucleus test is to identify substances that can cause cytogenetic damage and leads to the formation of micronuclei. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome lagging at anaphase or from acentric chromosomal fragments. They provide a quantifiable measure of recent DNA injury that result from when acentric fragments or whole chromosomes are left behind the main nucleusat telophase. An increase in the

prevalence of MN in a population of cells indicates that chromosome damage has occurred as a result of an exposure that caused either clastogenic or an aneuploidogenic effect[21]. A total of 2000 polychromatic erythrocytes were scored per animal for MNPCE and 200 erythrocytes were counted for the PCE:NCE ratio according to the OECD guideline for testing of chemicals. An increase in MNPCE and decrease in the PCE:NCE ratio has shows genotoxic effect of chemicals. In the present test, it has been observed that cisplatin treatment showed a significant ($p \leq 0.001$) increase in MNPCE per 2000 PCEs formation, however, EGCG pre-treatment at 100 mg/kg showed a significant ($p \leq 0.001$) reduction in increased MNPCE among 2000 PCE from mice bone marrow. Based on the obtained results EGCG pre-treatment provided the best protection against cisplatin-induced micronuclei in mice bone marrow cells and all free radical generation related side effects.

ACKNOWLEDGEMENT

The authors are thankful to Piramal for allowing us to work in their research centre.

REFERENCES

1. Ames BN, Mc Cann HO, Yamasaki E (1975). Methods for detecting carcinogens and mutagens with *Salmonella*/mammalian microsome mutagenicity test. Mutation research 31: 347-364.
2. Chirino YI, Chaverri JP (2009). Role of oxidative and nitrosative stress in cisplatin-induced nephrotoxicity. Experimental and Toxicologic Pathology 61: 223–242.
3. Dona M, Dell'Aica I, Calabrese F, Benelli R, Morini M, Albini A, Garbisa S (2003). Neutrophil restrainst by green tea: inhibition of inflammation associated angiogenesis and pulmonary fibrosis. Journal of Immunology 170: 4335-4341.
4. Evangelista CMW, Antunes LMG, Francescato HDC, Bianchi MLP (2004). Effects of the olive, extra virgin olive and canola oils on cisplatin-induced clastogenesis in Wistar rats. Food and Chemical Toxicology 42: 1291–1297.
5. Florea AN, Busselberg D (2011). Cisplatin as an anti-tumor drug: Cellular mechanisms of activity, drug resistance and induced side effects. Cancers 3: 1351-1371.
6. Hannan MA, Al-dakan AA, Hussain SS, Amer MH (1989). Mutagenicity of cisplatin and carboplatin used alone and in combination with four other anticancer drugs. Toxicol 55: 183-191.
7. Hasegawa R, Takekida K, Sai K, Umemura T, Tanimura A, Inoue T, et al. (1998). Inhibitory effect of green tea infusion of hepatotoxicit y. Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku 116: 82-91.
8. Hour TC, Liang YC, Chu IS, Lin JK (1999). Inhibition of eleven mutagens by various tea extracts, (-)-epigallocatechin-3-gallate, gallic acid and caffeine. Food Chem Toxicol 37(6): 569-579.
9. Jamieson ER, Lippard SJ (1999). Structure, recognition, and processing of cisplatin-DNA Adducts. Chem Rev 99: 2567-2498.
10. Kirkland D, Reeve L, Gatehouse D, Vanparys P (2011). A core *in vitro* genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. Mutat Res 721: 27-73.
11. Klaunig JE, Xu Y, lsenberg JS, Bachowski S, Kolaja KL, Jiang J (1998). The Role of Oxidative Stress in Chemical Carcinogenesis. Env Health perspectives 106: 289-285.
12. Masuda H, Tanaka T, Takahama U (1994). Cisplatin generates superoxide anions by intraction with DNA in cell-free system. Biochem Biophys Res Commun 203(2): 1175-1180.
13. OECD 471 (1997). Bacterial Reverse Mutation Test. 1-11.
14. OECD 474 (1997). Mammalian Erythrocyte Micronucleus Test. 1-10.
15. Preston RJ, Hoffmann GR (2008). Non-Organ Directed Toxicity: Genetoxic Toxicology. In: Klaassen CD (ed). Toxicology, The Basic Science of Poisons. The McGraw-Hill, pp 381-414.
16. Rjiba-Touati K, Ayed-Boussema I, Skhiri H, Belarbia A, Zellema D, Achour A, Bacha H (2012). Induction of DNA fragmentation, chromosome aberrations and micronuclei by cisplatin in rat bone-marrow cells: Protective effect of recombinant human erythropoietin. Mutat Res 747: 202-206.
17. Santos GCD, Mendonça LM, Antonucci GA, Santos ACD, Antunes LMG, Bianchi MLP (2012). Protective effect of bixin on cisplatin-induced genotoxicity in PC12 cells. Food Chem Toxicol 50: 335-340.
18. Tohamy AA, El-Ghor AA, El-Nahas SM, Noshy MM (2003). β-Glucan inhibits the genotoxicity of cyclophosphamide, adriamycin and cisplatin. Mutat Res 541: 45–53.
19. Vijayalaxmi KK, D'Souza MP (2004). Studies on the genotoxic effects of anticancer drug Carboplatin in *in vivo* mouse. Int J Hum Genet 4(4): 249-255.
20. Weijl NI, Cleton FJ, Osanto S (1997). Free radicals and antioxidants in chemotherapy induced toxicity. Cancer Tret Rev 23: 209-240.
21. Gonsebatt ME, Vega L, Salazar AM, Montero R, Guzman P, Blas J, et al (1997). Cytogenetic effects in human exposure to arsenic. Mutat Res.386(3):219–228