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The impact of quercetin on cisplatin-induced clastogenesis and apoptosis in murine marrow cells

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The aim of the present investigation is to determine whether the quercetin in combination with cisplatin can ameliorate cisplatin-induced clastogenesis and apoptosis in the bone marrow cells of mice. The scoring of chromosomal aberrations, micronuclei and mitotic activity were undertaken in the current study as markers of clastogenicity. Apoptosis was analysed by the Annexin V–propidium iodide assay and the occurrence of a hypodiploid DNA peak. Oxidative stress markers such as bone marrow lipid peroxidation and reduced glutathione were assessed as a possible mechanism underlying this amelioration. Quercetin was neither clastogenic nor apoptogenic in mice at doses equivalent to 50 or 100 mg/kg for 2 days. Pre-treatment of mice with quercetin significantly reduced cisplatin-induced clastogenesis and apoptosis in the bone marrow cells and these effects were dose and time dependent. Prior administration of quercetin ahead of cisplatin challenge ameliorated oxidative stress markers. Overall, this study provides for the first time that quercetin has a protective role in the abatement of cisplatin-induced clastogenesis and apoptosis in the bone marrow cells of mice that resides, at least in part, in its antioxidant effects. Therefore, quercetin can be a good candidate to decrease the deleterious effects of cisplatin in the bone marrow cells of cancer patients treated with this drug.

Introduction

Cisplatin is currently used as a first-line chemotherapeutic agent for the treatment of testicular, ovarian, bladder and other carcinomas (1). The current accepted paradigm about cisplatin mechanism of action is that the drug induces its cytotoxic properties through reacting with nucleophilic bases in DNA and form intra- and interstrand cross-links (cisplatin–DNA adducts) and subsequent interference with normal transcription and/or DNA replication mechanisms (2). This has led to propose the involvement of multi-step and multi-level effects of cisplatin in the tumour cell/host during cisplatin-mediated cancer chemotherapy (3). However, full therapeutic efficacy of this drug is limited due to the development of acquired drug resistance by the cancer cells and various side effects in the host, including nephrotoxicity, damages the bone marrow cells and cumulative myelosuppression, the latter frequently presenting as severe anaemia. These adverse effects cause intolerable discomfort in cancer patients and worsen their quality of life, becoming the major difficulty in continuing cancer chemotherapy. The clastogenic potential of cisplatin has become of great interest because of its serious effects on the chromosomes of non-tumour cells. In patients treated with long-term cisplatin, genetic damage can be observed during chemotherapy or many years later (4–6). Oxidative stress is believed to be important mechanisms in the development of cisplatin toxicity (7).

Dietary supplements, mainly antioxidants, are broadly used not only by the population at large for health promotion but also by individuals diagnosed with cancer (8,9). They are capable of detoxifying free radicals or inactivating radicals' intermediates generated by anti-neoplastic drugs. Moreover, experimental observations have shown that some antioxidant agents, as for example certain flavonoids, are able to enhance the cytotoxic action of the chemotherapeutic drugs without damaging normal cells (10,11). The flavonoid quercetin that is in fruits and vegetables has been shown to induce cell death in human head and neck squamous cell carcinoma cells *in vitro* and synergistically potentiates the anti-proliferative effect of cisplatin in these cells (12). Importantly, the synergistic effects induced by quercetin appeared due to the augmentation of cisplatin-induced apoptosis in cancer cells (13). Quercetin has also been reported to have anti-tumour effects against variety of cancer cells *in vitro* (14–16). The anti-cancer effects of quercetin have been reported to induce cell growth inhibition and apoptosis in cancer cells (17). In mice bearing human tumour xenografts, a combination of quercetin and cisplatin led to a significantly reduced tumour growth compared to treatment with either drug alone (18). Quercetin has also been shown *in vitro* to protect normal renal tubular cells from cisplatin toxicity. Therefore, the combination of cisplatin with quercetin might be of therapeutic benefit.

Considering the widespread use of cisplatin in clinical oncology and the ability of quercetin to enhance the sensitivity of cancer cells to cisplatin prompts the investigation of whether non-toxic doses of quercetin has influence on the bone marrow toxicity induced by cisplatin in mice. In addition, the possible mechanism underlying this modulation was assessed. The bone marrow clastogenesis was assessed by the chromosome analysis and micronucleus assay. Apoptosis was analysed with the Annexin and propidium iodide (PI) methods using flow cytometry. In addition, the biochemical alterations characteristic of oxidative stress activity induced by cisplatin has been conducted by use of standard techniques.

Materials and methods

Animals

Adult male Swiss albino mice weighing 25–30 g (10–12 weeks old) were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were maintained under standard conditions of

humidity, temperature (25 ± 2°C) and light (12-h light/12-h dark). They were fed with a standard mice pellet diet and had free access to water. All animal experimentations described in the manuscript were conducted in accord with accepted standards of humane animal care in accordance with the National Institutes of Health guidelines and the legal requirements in Kingdom of Saudi Arabia.

Drugs

Cisplatin was supplied as vials (Platinol, Bristol Myers Squibb Co., Princeton, NJ, USA). The vial contents were thoroughly dissolved in saline and administered by intraperitoneal injection within 1 h following preparation at the dose levels of 4 and 8 mg/kg (19). Quercetin (Sigma Chemical Co, St. Louis, MO, USA) was administered by gavage in propylene glycol as a vehicle. Gavage administrations were made 24 h and 1 h prior to the cisplatin intraperitoneal injection. Control animals were given vehicles only. Quercetin was administered at the dose levels of 50 and 100 mg/kg. National dietary record-based cohort assessments of the intake of quercetin from the habitual diet indicated daily levels of quercetin as high as 200–500 mg may be attained by high-end consumers of fruits and vegetables, especially in cases where the individuals consume the peel portion of quercetin-rich fruits and vegetables, such as tomatoes, apples and onions (20). Upon conversion of animal dose to the equivalent human dose, a dose of 100 mg/kg quercetin in mice was found to be corresponding to 8.1 mg/kg in humans. Accordingly, for an average person weighing 60 kg, 486 mg quercetin would be needed. Based on the previously mentioned level range, 486 mg quercetin would be provided through a daily oral supplement of quercetin-rich diets. All other chemicals and reagents used were of analytical grade.

Experimental protocol

The mice were acclimatized for 2 days and divided according to the schedule presented in Table I into nine groups consisting of 10 mice each, set up as follows: Group 1: mice served as a control group and treated daily with the vehicle only for two consecutive days; Groups 2 and 3: mice were treated with quercetin in a dose of 50 or 100 mg/kg, respectively, once a day, for two consecutive days; Group 4: mice were injected with a single dose of 4 mg/kg cisplatin alone; Groups 5 and 6: mice were treated with quercetin at a dose of 50 or 100 mg/kg/day, respectively, once a day, for two consecutive days and 4 mg/kg of cisplatin was administrated on the Day 2, 1 hour after regular quercetin exposure; Group 7: mice were injected with a single dose of 8 mg/kg cisplatin alone and Groups 8 and 9: mice were treated with quercetin at a dose of 50 or 100 mg/kg/day, respectively, once a day, for two consecutive days and 8 mg/kg of cisplatin was administrated on the Day 2, 1 hour after regular quercetin exposure. Twenty-four or 48 h after cisplatin treatment, femurs bone marrow samples were collected in tubes containing foetal calf serum from the mice killed by cervical dislocation.

Chromosome analysis

At the specified time after cisplatin treatment, groups of five mice were intraperitoneally injected with colchicine at 4 mg/kg body weight, 90 min before sacrifice. The slides were prepared and stained as described by Adler (21). All slides were coded and scored under ×1000 magnification using a Nikon microscope. One hundred well-spread metaphase plates per mouse (500 metaphases for each group) were scored for both structural and numerical aberrations in bone marrow cells. Cells were classified, according to the damage severity, into seven categories: cells with chromatid gaps, chromatid breaks, chromosome gaps, chromosome breaks, acentric fragments, centric rings and polyploidy. Cells with gaps were not included in the percentage of total structural chromosomal aberrations due to their controversial genetic significance. From the same slides, 1000 cells from each animal were taken into consideration for the mitotic activity study. The mitotic index of bone marrow metaphase was evaluated by calculating the number of dividing cells in a population of 1000 cells.

Table I. Experimental groups and treatment protocol

Groups[a]	Pretreatment[b]	Treatment[c]
1	0.1 ml propylene glycol per 10 g mouse	0.1 ml saline per 10 g mouse
2	50 mg/kg quercetin	0.1 ml saline per 10 g mouse
3	100 mg/kg quercetin	0.1 ml saline per 10 g mouse
4	0.1 ml propylene glycol per 10 g mouse	4 mg/kg cisplatin
5	50 mg/kg quercetin	4 mg/kg cisplatin
6	100 mg/kg quercetin	4 mg/kg cisplatin
7	0.1 ml propylene glycol per 10 g mouse	8 mg/kg cisplatin
8	50 mg/kg quercetin	8 mg/kg cisplatin
9	100 mg/kg quercetin	8 mg/kg cisplatin

[a]Each group consists of 10 mice.
[b]Mice received quercetin or propylene glycol by gavage once a day, for two consecutive days.
[c]Mice intraperitoneally injected by a single dose of cisplatin or saline on day 2, 1 hour after regular quercetin or propylene glycol exposure.

Micronucleus test

Groups of five mice were sacrificed at 24 or 48 h after cisplatin treatment and both femurs were dissected out. The bone marrow cells were collected in tubes containing foetal calf serum, centrifuged at 1100 r.p.m. for 10 min, and the pellet was carefully resuspended in, as little supernatant as possible, before slide preparation. Two smears of bone marrow were prepared from each mouse. After air-drying, the smears were coded and stained by May-Gruenwald/Giemsa as described by Adler (21). From each animal, 1000 polychromatic erythrocytes (PCEs) and 1000 normochromatic erythrocytes (NCEs) were examined for the presence of micronuclei (MN) under ×1000× magnification using a Nikon microscope. In addition, the number of PCEs among 1000 NCE per animal was recorded to evaluate bone marrow suppression and mitotic activity was calculated as %PCE = [PCE/(PCE + NCE)] × 100.

Detection of apoptosis

To study the impact of quercetin on the cisplatin-induced apoptosis in mouse bone arrow cells, four groups consisting of 10 mice each were used. Animal treatment was the same as in groups 1, 3, 7 and 9. Five animals from each group were killed by cervical dislocation at 24 h after cisplatin treatment and the rest were sacrificed at 48 h after cisplatin injection. The bone marrow cells were collected in tubes containing 1.5 ml foetal calf serum then centrifuged and washed with ice-cold phosphate-buffered saline (PBS) (pH 7.4). DNA content was quantified with PI according to the method described by Nicoletti *et al.* (22) with some modifications. Briefly, bone marrow cells (1×10^6) were incubated in 1 ml of PBS containing PI (50 μg/ml), RNase A (0.5 mg/ml), sodium citrate (0.1%) and Triton X-100 (0.1%) at 37°C for 30 min and then analysed by the FACSCalibur flow cytometry. Cells containing hypodiploid DNA were considered as apoptotic.

Annexin V–PI staining assay was employed to further classify bone marrow cells in early apoptosis and late apoptosis/necrosis stages. Apoptosis was judged by translocation of phosphatidylserine to the cell surface using a Vybrant™ Apoptosis Assay Kit#2 (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, 5 μl of Alexa Fluor 488 Annexin V solution and 1 μl of PI (100 μg/ml) were added to 0.1 ml of the bone marrow cells (1×10^6 cells) suspended in 1× binding buffer, mixed well and incubated at room temperature for 15 min in the dark. After incubation, 400 μl of 1× binding buffer was added, mixed gently and the samples were kept in ice. The stained cells were then analysed by flow cytometry using a FACSCalibur cytometer (BD Biosciences). Cells which are in the early apoptotic process are stained with Annexin V-Alexa Fluor 488 alone. Live cells showed no staining by either PI or Annexin V-Alexa Fluor 488. Late apoptotic/necrotic cells were stained by both PI and Annexin V-Alexa Fluor 488. Cells that were damaged during the isolation procedure were stained only with PI. A total of at least 10 000 cells were analysed per sample. The amount of early apoptosis and late apoptosis/necrosis was calculated using a computer system CellQuest (BD Biosciences).

Measurement of oxidative stress markers

Animal treatment was the same as in the measurement of apoptosis study. The animals were killed by cervical dislocation at 24 or 48 h after cisplatin treatment. Bone marrow cells were collected in tubes containing saline for estimation of malonodialdehyde (MDA) and reduced glutathione (GSH) levels. MDA generated by lipid peroxidation was quantified in the bone marrow cells according to the method of Ohkawa *et al.* (23), based on thiobarbituric acid reactivity. The MDA levels of the samples were calculated from the standard curve using the 1,1,3,3-tetramethoxypropane as the standard and expressed as micromole per gram protein. GSH was assayed with 5,5′-dithiobis(2-nitrobenzoic acid) according to the protocol described by Ellman (24). The concentration of GSH was calculated from a standard curve that was obtained from freshly prepared standard solution of GSH and expressed as micromole per gram protein. Estimation of protein was carried out according to the procedure of Lowry *et al.* (25) using bovine serum albumin as the standard.

Data analysis

Data were expressed as the mean ± standard deviation (SD) of the means. The analysed parameters were tested for homogeneity of variance and normality and were found to be normally distributed. The data were, therefore, analysed by employing non-parametric tests, Mann–Whitney *U* test or Kruskal–Wallis test followed by Dunn's multiple comparisons test. Data on oxidative stress parameters were analysed using analysis of variance, followed by Tukey–Kramer for multiple comparisons. Results were considered significantly different if the *P*-value was <0.05.

Results

Chromosome analysis

The results revealed that cisplatin when given at a single dose of 4 or 8 mg/kg cause high incidences of chromosomal aberrations and abnormal metaphases in the bone marrow cells of mice. The mitotic index at metaphase stage was also significantly decreased after treatment with 8 mg/kg of cisplatin compared to the corresponding solvent control group, indicating bone marrow suppression (Table II). On the other hand, quercetin alone failed to induce chromosomal aberrations confirming its non-clastogenicity. The quercetin was also not found to be cytotoxic, as there were no significant changes in the mitotic index over the corresponding control. Moreover, when pretreatment of different doses of quercetin was given prior to cisplatin treatment, decreased rates of clastogenic changes were observed and the higher dose of quercetin gave the more effective reduction in the total chromosomal aberrations and abnormal metaphases. In addition, reduction in cisplatin-induced clastogenicity by quercetin was evident at 24 h and to a much greater extent at 48 h of cell cycle. All types of chromosomal aberrations induced by cisplatin, including breaks, fragments, rings and other damages, were found to be reduced by quercetin.

Micronucleus test

The results obtained from the micronucleus study are presented in Table III. Treatment of mice with quercetin did not induce any significant variation in the incidence of micronucleated polychromatic erythrocyte (MNPCE) as compared to the control value. In addition, quercetin was not cytotoxic to the bone marrow (i.e. no statistically significant decrease in the %PCE) at the tested dose levels. Cisplatin treatment caused significant increases in the frequencies of MNPCE. Moreover, the mitotic index at the interphase stage was significantly decreased after treatment with 8 mg/kg of cisplatin compared to the corresponding solvent control group. With regard to the animals treated with quercetin before cisplatin, a weak protection was observed with 50 mg/kg of quercetin. With 100 mg/kg pretreatment, however, quercetin produced a clear significant inhibitory effect on the MNPCE induced by cisplatin in comparison to the cisplatin alone. The frequencies of micronucleated normochromatic erythrocyte (MNNCE) in all groups were not significantly different in comparison with the solvent controls. The reduction of mitotic index induced by 8 mg/kg of cisplatin at the interphase stage was found to be restored by quercetin pretreatment.

Apoptosis

The results obtained with Annexin V–PI double staining are represented in Table IV. Bone marrow spontaneous apoptosis level did not show significant variation in quercetin-treated animals compared to the solvent controls. At the 24-h sampling time, the early apoptotic and late apoptotic/necrotic cells level observed in cisplatin-treated animals were significantly increased compared to the solvent control group. However, by 48 h, the number of early and late apoptotic/necrotic cells was similar to that in the control group. Animals pretreated with

Table II. Distribution of the different types of chromosomal aberrations, total structural aberrations, numerical aberrations, abnormal metaphases and mitotic index in bone marrow of mice treated with quercetin and/or cisplatin (means ± SDs)

Groups and chemicals (mg/kg)	Types and numbers of structural aberrations						Total structural aberrations (%)	Numerical aberrations (% of polyploidy)	Abnormal metaphases (%)	Mitotic index (%)
	G′	B′	G″	B″	F	R				
24-h sampling regimen										
Control	3	3	0	0	2	0	1.00 ± 0.0	0.20 ± 0.4	1.00 ± 0.0	4.26 ± 1.2
Quercetin 50	2	3	1	0	1	1	1.00 ± 0.7	0.20 ± 0.4	0.80 ± 0.4	4.54 ± 1.0
Quercetin 100	2	5	2	0	1	0	1.20 ± 0.4	0.20 ± 0.4	1.20 ± 0.4	4.06 ± 1.4
Cisplatin 4	16	149	8	13	13	8	36.6 ± 6.5*	1.60 ± 0.8$^{\#}$	24.4 ± 4.0*	3.30 ± 1.4
Quercetin 50 and cisplatin 4	14	108	4	7	8	5	25.6 ± 2.7 ^{b}	1.00 ± 1.2	17.6 ± 2.7^{a}	3.78 ± 1.2
Quercetin 100 and cisplatin 4	8	70	3	4	8	2	16.8 ± 2.1^{b}	0.80 ± 1.3	11.6 ± 3.3^{b}	4.00 ± 1.1
Cisplatin 8	24	198	10	20	16	10	48.8 ± 5.3**	1.80 ± 0.8$^{\#}$	31.2 ± 3.5**	2.30 ± 0.9$^{\#}$
Quercetin 50 and cisplatin 8	8	164	3	14	9	5	38.4 ± 6.4^{a}	0.60 ± 0.5	21.2 ± 4.7^{a}	3.14 ± 0.8
Quercetin 100 and cisplatin 8	7	106	3	12	4	4	25.2 ± 5.4 ^{b}	0.40 ± 0.5	10.8 ± 2.0^{b}	3.64 ± 1.5
48-h sampling regimen										
Control	1	3	1	2	2	0	1.40 ± 0.5	0.00 ± 0.0	1.20 ± 0.4	4.20 ± 0.9
Quercetin 50	2	2	0	1	1	0	0.80 ± 0.4	0.00 ± 0.0	0.80 ± 0.4	4.16 ± 0.9
Quercetin 100	2	4	1	1	2	0	1.40 ± 0.5	0.20 ± 0.4	1.00 ± 0.0	4.26 ± 0.8
Cisplatin 4	6	48	3	7	13	3	14.2 ± 2.9*	0.20 ± 0.4	9.80 ± 1.1*	3.70 ± 1.0
Quercetin 50 and cisplatin 4	4	24	2	3	3	2	6.40 ± 1.8^{b}	0.20 ± 0.4	4.20 ± 1.1^{b}	3.46 ± 0.9
Quercetin 100 and cisplatin 4	3	13	1	2	4	1	4.00 ± 1.2^{b}	0.20 ± 0.4	2.80 ± 1.1^{b}	4.10 ± 1.1
Cisplatin 8	9	77	5	6	24	6	22.6 ± 2.1**	0.80 ± 0.4	19.2 ± 2.9**	2.96 ± 0.4$^{\#}$
Quercetin 50 and cisplatin 8	5	51	2	2	6	2	12.2 ± 4.0^{b}	0.40 ± 0.5	8.60 ± 3.2^{b}	3.34 ± 0.7
Quercetin 100 and cisplatin 8	2	24	2	1	4	1	6.00 ± 1.0^{b}	0.20 ± 0.4	3.20 ± 1.4^{b}	3.76 ± 1.1

One hundred metaphases were scored for chromosomal aberrations per mouse, for a total of 500 metaphases per treatment. Cells with gaps were not included in the total structural aberrations or abnormal metaphases. The mitotic activity per animal was evaluated by calculating the number of dividing cells in a population of 1000 cells. *$P < 0.05$ and **$P < 0.01$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test); $^{\#}P < 0.05$ versus control; $^{a}P < 0.05$ and $^{b}P < 0.01$ versus the corresponding cisplatin alone (Mann–Whitney *U* test). G′, chromatid gaps; B′, chromatid breaks; G″, chromosome gaps; B″, chromosome breaks; F, fragments; R, rings.

100 mg/kg quercetin showed significant decrease in the percentage of early and late apoptotic/necrotic cells induced by cisplatin at 24-h sampling time. However, at 48-h sampling time, the percentage of early and late apoptotic/necrotic cells in cisplatin-treated group decreased to close to that in the control and quercetin groups.

The results of counting of nuclei with a hypodiploid DNA content after staining with PI are presented in Figure 1. The results obtained by this method were similar to those obtained with the Annexin V–PI double-staining procedure. As observed with Annexin V–PI double staining, the percentage of apoptosis level did not show significant variation in quercetin-treated animals compared to the solvent controls. Compared to the control groups, the percentage of apoptotic cells was significantly increased by cisplatin treatment only at the 24-h sampling time. Animals pretreated with 100 mg/kg quercetin showed significant decrease in the percentage of apoptotic cells in comparison to those treated with cisplatin alone.

Table III. Frequencies of MNPCE, MNNCE and mitotic activity (% PCE) in bone marrow of mice treated with quercetin and/or cisplatin (means ± SDs)

Groups and chemicals (mg/kg)	MNPCE (%)	MNNCE (%)	PCE (%)
24-h sampling regimen			
Control	0.28 ± 0.08	0.02 ± 0.04	48.0 ± 2.1
Quercetin 50	0.24 ± 0.05	0.02 ± 0.04	48.0 ± 1.8
Quercetin 100	0.30 ± 0.07	0.04 ± 0.05	49.0 ± 1.7
Cisplatin 4	2.72 ± 0.46*	0.12 ± 0.08	45.8 ± 2.7
Quercetin 50 and cisplatin 4	1.66 ± 0.28[b]	0.04 ± 0.08	46.2 ± 3.1
Quercetin 100 and cisplatin 4	0.78 ± 0.14[b]	0.06 ± 0.08	47.6 ± 2.6
Cisplatin 8	3.16 ± 0.45**	0.20 ± 0.15	41.6 ± 3.4[#]
Quercetin 50 and cisplatin 8	3.00 ± 0.47*	0.12 ± 0.10	43.0 ± 4.1
Quercetin 100 and cisplatin 8	2.16 ± 0.25[b]	0.08 ± 0.13	46.6 ± 3.2
48-h sampling regimen			
Control	0.28 ± 0.10	0.04 ± 0.05	48.0 ± 1.5
Quercetin 50	0.28 ± 0.08	0.02 ± 0.04	47.4 ± 2.6
Quercetin 100	0.26 ± 0.11	0.06 ± 0.08	48.4 ± 1.8
Cisplatin 4	0.70 ± 0.15[#]	0.10 ± 0.07	45.4 ± 1.5
Quercetin 50 and cisplatin 4	0.44 ± 0.18	0.06 ± 0.05	45.8 ± 2.7
Quercetin 100 and cisplatin 4	0.38 ± 0.08[a]	0.04 ± 0.05	48.2 ± 1.7
Cisplatin 8	1.38 ± 0.44**	0.14 ± 0.11	44.2 ± 2.5[#]
Quercetin 50 and cisplatin 8	0.86 ± 0.21	0.08 ± 0.04	45.6 ± 1.6
Quercetin 100 and cisplatin 8	0.58 ± 0.21[a]	0.06 ± 0.08	48.0 ± 1.8[a]

The mitotic activity at interphase stage was calculated as %PCE = [PCE/(PCE + NCE)] × 100. *$P < 0.05$ and **$P < 0.01$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test); #$P < 0.01$ versus corresponding control; [a]$P < 0.05$ and [b]$P < 0.01$ versus the corresponding cisplatin alone (Mann–Whitney U test).

Oxidative stress markers

The effect of quercetin on cisplatin-induced oxidative stress in mice was assessed by measuring bone marrow GSH and MDA levels. GSH level did not show significant variation after treatment of mice with quercetin as compared to the solvent controls. The GSH level observed in cisplatin-treated animals was significantly decreased in comparison to the solvent controls at both sampling time. Animals pretreated with quercetin showed significant increases in GSH level over the cisplatin alone (Figure 2). No significant change in MDA content was observed in bone marrow cells after quercetin treatment in a dose of 100 mg/kg compared to the controls at both sampling time. The MDA content in mice treated with cisplatin was significantly increased at both sampling time. The cisplatin-induced MDA formation was abrogated by quercetin and decreased to the level not significantly different from the level of MDA in the solvent controls (Figure 3).

Discussion

The influence of quercetin on cisplatin-induced clastogenesis and apoptosis in non-tumour cells *in vivo* has not been reported yet. In this article, the impact of quercetin on clastogenesis and apoptosis of the mouse bone marrow cells were examined. The current study demonstrates that quercetin was neither clastogenic nor apoptogenic at the doses tested. Moreover, it is able

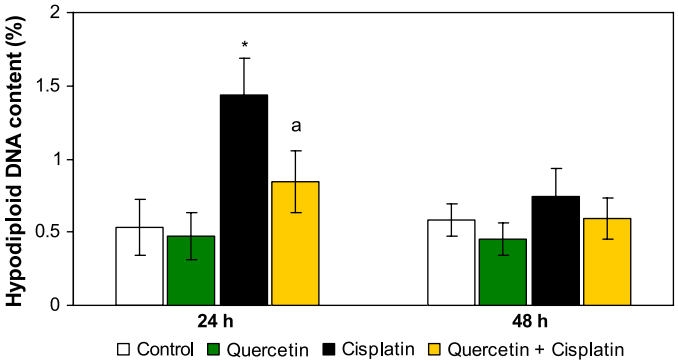

Fig. 1. The percentage of apoptotic cells in bone marrow of mice 24 or 48 h after treatment with quercetin (100 mg/kg) and/or cisplatin (8 mg/kg). % Apoptotic cells denote the percentage of cells with hypodiploid DNA content. Data are presented as mean ± SD of percent of PI-stained cells of three independent experiments. *$P < 0.05$ versus the corresponding control (Kruskal–Wallis test followed by Dunn's multiple comparisons test); [a]$P < 0.01$ versus the corresponding cisplatin alone (Mann–Whitney U test).

Table IV. The percentage of early and late apoptosis/necrosis in bone marrow of mice treated with quercetin and/or cisplatin

Groups and chemicals (mg/kg)	Live cells (%)	Early apoptotic cells (%)	Late apoptotic and necrotic cells (%)	Damaged cells (%)
24-h sampling regimen				
Control	97.28 ± 0.53	0.87 ± 0.29	0.57 ± 0.25	1.27 ± 0.41
Quercetin 100	97.47 ± 0.54	0.70 ± 0.14	0.73 ± 0.15	1.09 ± 0.39
Cisplatin 8	89.01 ± 0.71**	4.99 ± 0.92*	3.39 ± 1.02**	2.59 ± 0.71
Quercetin 100 and cisplatin 8	94.87 ± 1.27[b]	1.99 ± 0.65[b]	1.99 ± 0.50[a]	1.13 ± 0.53
48-h sampling regimen				
Control	97.05 ± 0.72	0.59 ± 0.11	0.61 ± 0.16	1.73 ± 0.55
Quercetin 100	97.52 ± 0.61	0.62 ± 0.19	0.61 ± 0.18	1.23 ± 0.43
Cisplatin 8	96.84 ± 0.49	0.97 ± 0.17	0.83 ± 0.33	1.34 ± 0.33
Quercetin 100 and cisplatin 8	97.59 ± 0.48	0.63 ± 0.17	0.73 ± 0.29	1.03 ± 0.39

The results are presented as mean values ± SDs of percent of Annexin V–PI double-stained cells from three independent experiments. *$P < 0.05$ and **$P < 0.01$ versus the corresponding control (Kruskal–Wallis test followed by Dunn's multiple comparisons test); [a]$P < 0.05$ and [b]$P < 0.01$ versus the corresponding cisplatin alone (Mann–Whitney U test).

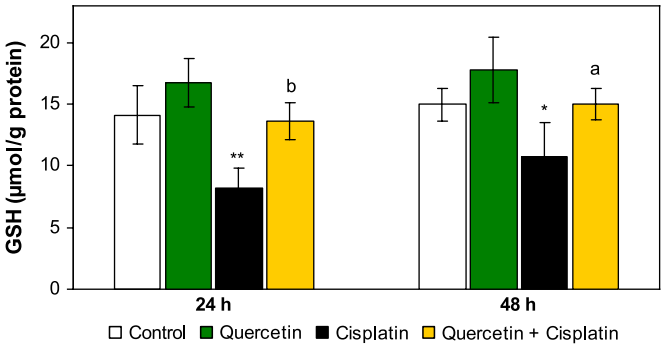

Fig. 2. Reduced GSH contents in bone marrow of mice 24 or 48 h after treatment with quercetin (100 mg/kg) and/or cisplatin (8 mg/kg) (mean ± SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the corresponding control; $^{a}P < 0.05$ and $^{b}P < 0.01$ versus the corresponding cisplatin alone (one-way analysis of variance followed by Tukey–Kramer multiple comparisons test).

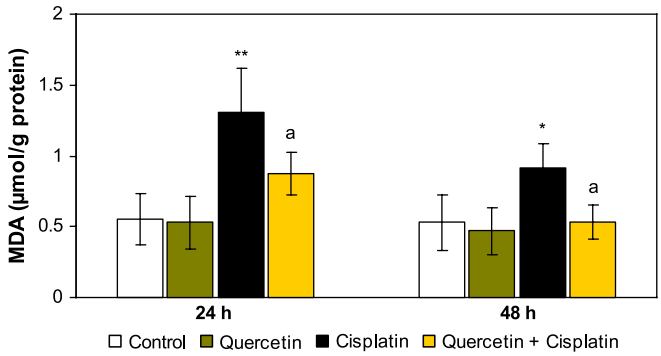

Fig. 3. Lipid peroxidation levels (MDA) in bone marrow of mice 24 or 48 h after treatment with quercetin (100 mg/kg) and/or cisplatin (8 mg/kg) (mean ± SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the corresponding control; $^{a}P < 0.05$ versus the corresponding cisplatin alone (one-way analysis of variance followed by Tukey–Kramer multiple comparisons test).

to protect mouse bone marrow cells against the cisplatin-induced clastogenicity and apoptosis. These results corroborate earlier studies, where oral administration of quercetin did not cause DNA damage or apoptosis in the bone marrow cells (26–30). *In vivo*, quercetin displays rather a protective activity against DNA damage. *In vitro* experiments indicated that quercetin used at high concentrations may be mutagenic, but when given in low concentrations, it provided protection against the DNA damage induced by H_2O_2 (31). These results have not been confirmed, however, by any *in vivo* experiments with oral administration of quercetin. The lack of clastogenicity of quercetin *in vivo* can be explained by its low bioavailability and by degradation of its aglicone form caused by intestinal bacterial flora or *O*-methylation, glucuronidation and sulphatation in the gastrointestinal tract (32). Importantly, quercetin *in vivo* is swiftly metabolized to non-clastogenic forms, i.e. 3′-*O*-methylquercetin and 4′-*O*-methylquercetin (33).

Developments of chromosomal aberrations and micronucleus have been commonly used as sensitive biological indicator in the clastogenic bioassays of a drug (34). In present study, the development of these clastogenic parameters was seen after cisplatin treatment and it supports earlier findings of its clastogenic properties (19,35,36). The clastogenicity of cisplatin in bone marrow cells was well investigated by Edelweiss *et al*. (37) and Choudhury *et al*. (38), who observed that the most impressive effect of a single dose of cisplatin was an increase in the frequency of chromosome aberrations and in the number of abnormal metaphases obtained after cisplatin treatment. Cisplatin applied intraperitoneally induced clastogenesis in bone marrow cells and persisted for 48 h after treatment. This damage, particularly in the population of undifferentiated cells that constitutes bone marrow, is dangerous because it can lead to mutations and DNA rearrangements. If such cells survive and proliferate, the risk of secondary acute myeloid leukaemia and other drug-related cancers can increase (4–6).

In the current study, the chromosomal aberration pattern revealed that chromatid breaks and gaps occurred more frequently. The total numbers of structural and numerical chromosomal aberrations as well as aberrant metaphases were noticed to be maximum at 24 h of treatment, which decreased during the second phase of cell cycle (48-h sampling time). It has been reported that chemicals in general produce the highest frequency of aberrations in rodents 24 h after single exposure, which roughly coincides with the normal length (22–24 h) of the cell cycle (21,39). However, decrease in aberrations at 48 h after treatment could be due to various possible reasons, such as clearance of drug from the body, post-replication repair process etc. In fact, an involvement of post-replication repair process in cisplatin-induced DNA damage has been established (40).

The data of the micronucleus test have shown that cisplatin significantly elevated the MN in the mouse erythropoietic system. The comparative analysis of the frequency of MN in the mice treated with cisplatin alone and quercetin plus cisplatin also showed similar trend as noted for chromosomal aberrations analysis; however, the occurrence of comparatively lower frequency of MN than chromosomal aberrations could be due to the fact that the fate of chromosomal fragments is uncertain so far their segregation in MN is concerned. Apart from that, all fragments do not necessarily form visible MN (41). The results of these clastogenic parameters in the present study showing the significant reduction in cisplatin-induced clastogenic effect in presence of quercetin clearly suggest the protective role of quercetin on cisplatin's clastogenic potentials. Moreover, the protection afforded by quercetin revealed that quercetin could exert dose- and time-dependent anti-clastogenic effect during both phases of cell cycle. High suppressive effects were observed at 48-h sampling time indicative of greater detoxification ability in later stages. In fact, the ability of quercetin to confer marked protection against many toxic chemical agents has been described. Quercetin mediated inhibition of bacterial mutagenicity induced by different mutagen (42,43), and rat clastogenicity induced by etoposide (26,27) has been reported. Quercetin protective ability against the genotoxicity of mitomycin C and bleomycin (44) or benzo[*a*]pyrene and 2-amino-3-methylimidazol[4,5-*f*] quinoline (45) was also demonstrated. In addition, the inverse relationship between urinary anti-mutagenicity and level of tobacco-related DNA adducts in the urothelium of smokers strongly suggests that bladder mucosal cells of smokers are partially protected by ingesting dietary flavonoids (e.g. quercetin), against the harmful effects of tobacco-derived bladder carcinogens (46).

In order to study the impact of quercetin on cisplatin-induced apoptosis in the bone marrow cells of mice, the exposure of phosphatidylserine on the surface of early apoptotic cells caused by the test chemicals was assessed using the Annexin V–PI staining and the counting of nuclei with a hypodiploid DNA content was quantified after staining with PI. Whereas

cisplatin-induced apoptosis in mouse bone marrow cells was detectable at 8 mg/kg, quercetin given at 100 mg/kg did not cause apoptosis. The present study thus confirms the previous *in vivo* experiments and indicates the non-apoptogenic effect of quercetin in bone marrow cells (30). It is worthwhile to note that the proportion of apoptotic cells induced by cisplatin reached a peak after 24 h and returned to the control level after 48 h of cisplatin treatment. This result is similar to the one obtained by the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling method of cisplatin-induced apoptosis in the mouse bone marrow cells (47). A possible reason is that the apoptotic cells in the bone marrow may be rapidly phagocytosed.

The current study showed that quercetin markedly decreased the degree of apoptosis of cisplatin-treated animals. Quercetin reduced the percentage of both early and late apoptotic/necrotic cells and fragmentation of nuclear DNA induced by cisplatin as detected by the exposure of phosphatidylserine on the surface of apoptotic cells and the appearance of nuclei with hypodiploid DNA content, respectively. Similar results were also obtained with isolated mouse thymocytes, proving that quercetin did not exert cytotoxic activity but protected those cells against glucose oxidase-mediated apoptosis (48). Other study revealed that quercetin in a dose of 150 mg/kg/day for five consecutive days inhibited the proliferation and apoptosis of renal glomerular cells in rats with experimental glomerulonephritis (49). Cytoprotective and anti-apoptotic effects of quercetin may be associated with upregulation of haem oxygenase-1 mRNA. High level of haem oxygenase-1 expression usually correlates with resistance to apoptosis and provides strong protection against oxidative stress to many cancer as well as normal cells (50,51), whereas inhibition of haem oxygenase-1 expression enhances sensitivity of cells to apoptotic stimuli (50).

Cisplatin is a heavy metal complex with two labile chlorides groups which upon hydrolysis in aqueous solution form various reactive species, which are recognized as one of the pathogenic intermediates following chemotherapy (52). Accumulation of these reactive species results in cellular oxidative stress and if not corrected can lead to the damage of important biomolecules such as membrane lipids, proteins and DNA. To determine whether the observed anti-clastogenic and anti-apoptotic effects of quercetin were due to an enhancement of the scavenger of free radicals generated by cisplatin, oxidative stress markers such as lipid peroxidation and GSH were measured after the animals were treated with cisplatin and/or quercetin. Cisplatin is able to generate reactive oxygen species, which cause damage to cellular genome and also the cell membrane leading to lipid peroxidation (52,53). MDA, the product of lipid peroxidation, also interacts with DNA causing strand breaks that in turn develop into chromosomal breaks. These chromosome breaks may appear as MN in the daughter cell after the first cell division. Quercetin is known as a potent-free radical scavenger and a metal chelator, capable of inhibiting lipid peroxidation in *in vitro* and *in vivo* systems (30,54). Behling *et al.* (55) demonstrated that quercetin is beneficial in preventing the accumulation of MDA, tubular damage and renal dysfunction through scavenging the hydroxyl radicals in cisplatin-induced nephrotoxicity in adult rats. In agreement with the above-cited reports, the present experiment showed that cisplatin treatment caused significant increases in lipid peroxidation levels and quercetin pretreatment reduced the cisplatin-induced lipid peroxidation significantly.

It has been reported that cisplatin induces a decrease in plasma concentrations of antioxidants in patients treated for solid tumours (56). This can induce clastogenicity and apoptosis through the failure of the antioxidant defence mechanisms since antioxidants are able to protect non-tumour cells acting as anti-clastogens without compromising the anti-neoplastic effects. Because GSH has been reported to be implicated in the metabolism of cisplatin (57), the GSH contents were measured in bone marrow of mice and the results from this part of experiment suggest the possible involvement of cellular GSH as a mechanistic step in quercetin-mediated protection against cisplatin-induced clastogenicity and apoptosis. It was seen that cisplatin treatment resulted a significant decrease in bone marrow GSH with the development of clastogenic effects. A decrease in bone marrow GSH noted after cisplatin treatment could lead to less protective mechanism in bone marrow cells and thereby developing more cisplatin-induced bone marrow toxicity. In fact, induction of metallothionein in bone marrow cells of mice effectively prevents clastogenic effects of cisplatin (58). However, in the group of mice pretreated with quercetin showing decreased clastogenic and apoptotic effects and a significant increase in bone marrow GSH suggests the definite significance of GSH also. It could be the elevated level of GSH to protect the cells against cisplatin-induced clastogenicity and apoptosis.

In summary, a critical point of this study is the possibility that there may be a therapeutic window for the use of cisplatin in combination with quercetin so that its harmful side effects in normal cells are minimized. The deleterious effects of cisplatin might be, at least in part, mediated by an oxidative stress mechanism that may be prevented or reduced by radical scavengers. As mentioned in the Introduction, cisplatin has a direct effect on nucleophilic bases in DNA, an important component of its anti-tumour activity, and this will be unchanged by any manipulations that alter the redox reaction. Bone marrow suppression and apoptogenic effects of cisplatin could contribute to the anaemia that follows therapy with cisplatin. The improvement in mitotic activity of bone marrow cells of animals pretreated with quercetin in cisplatin toxicity may focus attention on the beneficial effect of quercetin to overcome one of the most serious problems in cancer chemotherapy, which is the bone marrow suppression and related immunosuppression. Quercetin was effective in reducing clastogenesis and apoptosis induced by cisplatin in bone marrow cells and may possibly decrease the risk of secondary tumours in cells that were not originally neoplastic. The protective effect of quercetin could be possibly ascribed to its radical scavenger effect that modulated the changes induced by cisplatin. Based on the data presented here, strategies can be developed to decrease the deleterious effects of cisplatin in normal cells by using quercetin.

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