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Modulation of irinotecan-induced genomic DNA damage by theanine

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ABSTRACT

The possible chemoprotective activity of theanine against irinotecan-induced genomic DNA damage towards mouse bone marrow cells was investigated. Chromosomal aberrations, DNA damage, micronuclei formation and mitotic activity were studied in the current study as markers of genomic damage. Oxidative DNA stress markers such as 8-hydroxydeoxyguanosine, lipid peroxidation, reduced and oxidized glutathione levels were assessed as a possible mechanism underlying this amelioration. Theanine was neither genotoxic nor cytotoxic in mice at doses equivalent to 30 or 60 mg/kg for 12 days. Pretreatment of mice with theanine significantly reduced irinotecan-induced genomic damage in the bone marrow cells and these effects were dose dependent. Irinotecan induced marked biochemical alterations characteristic of oxidative DNA stress, including increased 8-hydroxydeoxyguanosine, enhanced lipid peroxidation and reduction in the reduced/oxidized glutathione ratio. Prior administration of theanine ahead of irinotecan challenge ameliorated these oxidative DNA stress markers. Overall, this study provides for the first time that theanine has a protective role in the abatement of irinotecan-induced genomic damage in the bone marrow cells of mice that resides, at least in part, on its ability to modulate the cellular antioxidant levels and consequently protect bone marrow from irinotecan genotoxicity.

1. Introduction

Irinotecan, a semisynthetic analog of camptothecin, is a potent inhibitor of topoisomerase-I activity (Mathijssen et al., 2002). Irinotecan has received FDA approval as a component of first-line combination chemotherapy of patients with metastatic colorectal and ovarian cancer (Punt and Koopman, 2008; Oostendorp et al., 2010). Studies focused on this drug have demonstrated that the cytotoxic potency of irinotecan is due to the stability of the DNA-topoisomerase I cleavable complexes and prevents religation of the DNA strand, which leads to double-strand DNA breakage and cell death (Liu et al., 2000). Like other DNA topoisomerase inhibitors, irinotecan can initiate genotoxic stress, leading to apoptosis and anti-tumor therapy, but on the other hand can give rise to chromosome instability and mutagenesis that may lead to the development of secondary tumors from cells that were not originally neoplastic (Attia, 2008a). Important toxicities, however, are also commonly noted, including hematologic and intestinal toxicities (Rothenberg et al., 1993).

Known as L-theanine, this unique dietary supplement has already proved valuable in other areas of health. Theanine was shown to have different effects between normal tissues and tumors. Some exciting preliminary research involves the synergistic use of theanine combined with anti-tumor drugs (Sugiyama and Sadzuka, 2003). It increases the concentration of these drugs in the tumor without altering the concentration in other tissues and also reduces their toxicity to other tissues (Sugiyama and Sadzuka, 2003, 2004). Drugs with which theanine has been found to be synergistic include irinotecan, doxorubicin, idarubicin and pirarubicin (Sugiyama and Sadzuka, 2003; Liu et al., 2009). Theanine may work in this respect by inhibiting the glutamate transporter, which increases the concentration of the drug in the tumor cells (Sugiyama and Sadzuka, 2003). However, the subtypes of the glutamate transporter expressed in the tumor probably differ from those in normal cells; therefore the efficacy of theanine may be distinct in the tumor and normal tissues.

In tumor-bearing mice, irinotecan slightly reduced the tumor weight and theanine significantly enhanced the inhibition of tumor growth induced by irinotecan. Moreover, the increase in concentrations of irinotecan was never observed in normal tissues (Sugiyama and Sadzuka, 2003, 2004). Thus, the intention of the present study was to investigate the potential effects of theanine on protecting the cellular genome against the genotoxicity of irinotecan which may be proved of importance for cancer patients receiving chemotherapy with such drug.

2. Materials and methods

2.1. 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

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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 experiments on animals were carried out according to the Guidelines of the Animal Care and Use Committee at College of Pharmacy, King Saud University. The total number of animals in this study was 90 treated mice and 10 vehicle control.

2.2. Drugs and chemicals

Irinotecan (Developmental Therapeutics Program, NCI, Bethesda, USA), theanine and cyclophosphamide (Sigma–Aldrich St. Louis, MO, USA) were dissolved in sterile dH_2O. All other chemicals will be of the finest analytical grade. Theanine (30 and 60 mg/kg/day) was administered by gavage for 12 days. Theanine is an *umami* (the fifth taste sensation)-component of tea and is contained 2–3% in tea leaves (Goto et al., 1996). Upon conversion of animal dose to the equivalent human dose [human dose (mg/kg) = mouse dose (mg/kg) × (3/37)], a dose of 60 mg/kg theanine in mice corresponded to 4.86 mg/kg in humans. Accordingly, for an average person weighing 60 kg, 291 mg theanine would be needed. While not reasonably achievable through consumption of plants, this concentration may be provided through a daily oral supplement. Irinotecan was injected intraperitoneally at doses of 30 and 60 mg/kg on days 1, 4, 8 and 12. The injected volume was 0.01 ml per 1 g body weight. Bone marrow cells were sampled 24 h after the last treatment of irinotecan. The multiple treatments scheme and sampling time for irinotecan were selected upon the positive results of our previous study (Hardman et al., 2002) and the selected doses are within the dose range used for human chemotherapy.

2.3. Experimental protocol

Mice were divided into ten groups of 10 mice each as follows: Group 1: mice served as a control group and treated daily with the sterile dH_2O for 12 consecutive days. Groups 2 and 3: mice were treated orally with theanine in doses of 30 and 60 mg/kg/day, respectively, once a day, for 12 consecutive days. Group 4: mice were injected with irinotecan alone at a dose of 30 mg/kg on days 1, 4, 8 and 12. Groups 5 and 6: mice were treated with theanine at doses of 30 and 60 mg/kg/day, respectively, once a day, for 12 consecutive days and 30 mg/kg of irinotecan was administrated on the days 1, 4, 8 and 12, 1 h after regular theanine exposure. Group 7: mice were injected with irinotecan alone at a dose of 60 mg/kg on days 1, 4, 8 and 12. Groups 8 and 9: mice were treated with theanine at doses of 30 and 60 mg/kg/day, respectively, once a day, for 12 consecutive days and 60 mg/kg of irinotecan was administrated on the days 1, 4, 8 and 12, 1 h after regular theanine exposure. Group 10: mice were intraperitoneally injected with cyclophosphamide in a single dose of 25 mg/kg as a positive control mutagen. The animals were sacrificed by cervical dislocation 24 h after treatment with cyclophosphamide or 24 h after the last dose of irinotecan.

2.4. Detection of metaphase chromosomal aberrations

For metaphase chromosomal aberrations study, at 22 h post-treatment with cyclophosphamide or 22 h post-treatment with the last doses of irinotecan, five mice from each group were caged separately and colchicinized intraperitoneally at the rate of 4 mg/kg. After an interval of 2 h of colchicine treatment, femurs bone marrow cells were collected from the mice killed by cervical dislocation. The slides were prepared and stained as mentioned earlier (Adler, 1984). All slides were coded and scored under 1000× magnification using a Nikon microscope. One-hundred well-spread metaphase plates per mouse were scored for both structural and numerical chromosomal aberrations in bone marrow cells as described previously (Attia, 2010a). 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.

2.5. Detection of DNA damage

The remaining five animals from each group were sacrificed and bone marrow cells from one femur were collected for detection of DNA damage. Single and double DNA strand breaks were assessed by alkaline single cell gel electrophoresis (alkaline comet assay) as previously descript (Attia et al., 2010). Strictly speaking, 'breaks' in the context of the alkaline comet assay include apurinic/apyrimidinic sites, which are alkali labile. The slides were stained with ethidium bromide (20 μg/ml) and studied using a fluorescent microscope (Nikon, Japan) equipped with appropriate filters. Hundred individual cells were selected for calculations for each analysis; all experiments were carried out at least three times, each with two parallel slides per data point. Single cells were analyzed with TriTek CometScore version 1.5 software. The extent of DNA damage can be quantified by the use of this sensitive technique, with the most frequently reported measure being the olive tail moment [Olive Tail Moment = (Tail mean–Head mean) × Tail% DNA/100].

2.6. Detection of interphase micronucleus formation

The remaining femora from the same animals used for the alkaline comet assay was used for estimation of micronuclei (MN) frequencies and mitotic activity. Bone marrow smears were done and the slides were stained with May-Gruenwald/Giemsa solutions as described earlier (Adler, 1984; Attia, 2007). Per animal, 2000 polychromatic erythrocytes (PCE) were blindly scored microscopically for the presence of MN. In addition the number of PCEs among 1000 normochromatic erythrocytes (NCE) per animal was recorded to evaluate bone marrow suppression (Attia et al., 2005). Mitotic activity was calculated as% PCE = [PCE/(PCE + NCE)] × 100.

2.7. Measurement of DNA oxidative stress markers

To study the effect of theanine on the oxidative DNA damage induced by irinotecan, peripheral blood samples from the same groups used for the comet assay and micronucleus test were collected from the heart for estimation of serum 8-hydroxydeoxyguanosine (8-OHdG), lipid peroxidation, reduced glutathione (GSH) and oxidized glutathione (GSSG) levels. The amount of 8-OHdG in serum was determined using the Bioxytech 8-OHdG-ELISA Kit (OXIS Health Products, Portland, OR, USA) according to the manufacturer's instructions. Briefly, a 50-μl serum sample or standards and 50 μl of reconstituted primary antibody were added to each well of 8-OHdG-coated microtitre plates and incubated at 37 °C for 1 h. Plates were washed three times with phosphate-buffered saline, and horseradish peroxidase-conjugated secondary antibody was added. After incubation at 37 °C for 1 h, unbound secondary antibody was removed, and the plate was washed again three times. The amount of antibody bound to the plate was determined by the development of color intensity after the addition of a substrate containing 3,3′,5,5′-tetramethylbenzidine. The reaction was terminated by the addition of phosphoric acid, and the absorbance was measured using a FLUOstar OMEGA microplate reader (BMG LABTECH Ltd., Germany) at a wavelength of 450 nm (Attia, 2012). The concentration of 8-OHdG was interpolated from a standard curve drawn with the assistance of logarithmic transformation. Calibration curves were generated with standardized samples and the data were fitted to a four-variable logistic function. Reported values are the average of triplicate determinations and serum levels of 8-OHdG are expressed as ng/ml.

The extent of lipid peroxidation was assayed serum by measuring one of the end products of this process, the thiobarbituric acid-reactive substances (TBARS) by the method of Ohkawa et al. (1979) as described previously (Attia, 2008b). The concentration of TBARS was calculated using standard curves of increasing 1,1,3,3-tetramethoxypropane concentrations, and expressed as nmol/ml. Serum GSH was assayed with 5,5′-dithiobis(2-nitrobenzoic acid) according to the protocol described by Ellman (1959) as described previously (Attia, 2008b). GSSG was assayed with DTNB, glutathione reductase and NADPH as described by Anderson (1985). The concentrations of GSH and GSSG were calculated from standard curves that were obtained from freshly prepared standard solutions of GSH and GSSG, respectively, and expressed as μg/ml. The value obtained for GSH was divided by the GSSG value to get the GSH/GSSG ratio.

2.8. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of the means. The data were analyzed by Mann–Whitney *U*-test and Kruskal–Wallis test followed by Dunn's multiple comparisons test or unpaired *t* test and analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons. Results were considered significantly different if the *P*-value was <0.05.

3. Results

3.1. Effect of theanine on irinotecan-induced metaphase chromosomal aberrations

As shown in Table 1, animals treated with the positive control cyclophosphamide showed a high frequency of total chromosomal aberrations in bone marrow cells after treatment in comparison with the negative control ($P < 0.01$). Moreover, drastic inhibition in the metaphase mitotic activity was recorded following cyclophosphamide administration ($P < 0.05$). Theanine treatment did not exhibit any significant differences in the frequency of total chromosomal aberrations or mitotic activity compared to the solvent control at the tested doses. Significant increase in the frequency of chromosomal aberrations was observed in animals treated with 30 or 60 mg/kg irinotecan and the higher dose of irinotecan gave more chromosomal aberrations. Theanine pre-treatment reduced the frequency of chromosomal aberrations in irinotecan treated

Table 1
Distribution of the different types of chromosomal aberrations, total aberrations, abnormal metaphases and mitotic index in bone marrow of mice treated with theanine and/or irinotecan or cyclophosphamide (means ± SD).

Treatment groups (mg/kg)	Number and types of structural aberrations						Total structural aberrations (%)	Numerical aberrations (percent of polyploidy)	Abnormal metaphases (%)	Mitotic index (%)
	G′	B′	G″	B″	F	R				
Control	3	3	1	0	2	1	1.20 ± 0.44	0.20 ± 0.08	1.20 ± 0.44	4.22 ± 0.43
Theanine 30	2	3	1	1	2	1	1.40 ± 0.54	0.00 ± 0.00	1.20 ± 0.44	4.34 ± 0.30
Theanine 60	1	2	1	1	1	0	0.80 ± 0.44	0.00 ± 0.00	0.80 ± 0.44	4.24 ± 0.59
Irinotecan 30	9	54	4	4	5	4	13.4 ± 2.7**	0.80 ± 0.08	8.20 ± 1.5**	2.44 ± 0.3**
Theanine 30 + Irinotecan 30	5	30	2	2	2	2	7.20 ± 1.7[b]	0.40 ± 0.10	4.60 ± 0.89[a]	3.32 ± 0.14[b]
Theanine 60 + Irinotecan 30	3	6	3	3	2	2	2.60 ± 0.54[b]	0.20 ± 0.08	1.40 ± 0.89[b]	3.62 ± 0.48[b]
Irinotecan 60	15	78	7	8	8	7	20.2 ± 2.2**	1.40 ± 0.1*	13.8 ± 3.5**	2.14 ± 0.2**
Theanine 30 + Irinotecan 60	9	52	6	8	6	4	14.0 ± 4.44	0.60 ± 0.10	5.40 ± 1.67[b]	3.04 ± 0.45[a]
Theanine 60 + Irinotecan 60	7	25	3	3	4	2	6.80 ± 1.30[b]	0.40 ± 010[a]	4.20 ± 1.0[b]	3.44 ± 0.6[b]
Cyclophosphamide 25	7	54	4	13	6	6	15.8 ± 2.86[##]	0.60 ± 0.10	8.80 ± 2.16[##]	3.20 ± 0.56[#]

Each treatment group and control group consisted of five animals. One hundred metaphases were scored for chromosomal aberrations per mouse, for a total of 500 metaphases per group. G′ = Chromatid gaps, B′ = Chromatid breaks, G″ = Chromosome gaps, B″ = Chromosome breaks, F = Fragments and R = Rings. 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<.05 versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test).

** $P < 0.01$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test)

[a] $P < 0.05$ versus the corresponding irinotecan alone.

[b] $P < 0.01$ versus the corresponding irinotecan alone.

[#] $P < 0.05$ versus control (Mann–Whitney U test).

[##] $P < 0.01$ versus control (Mann–Whitney U test).

animals in comparison to those treated with irinotecan alone. Moreover, treatment with irinotecan induced significant decreases in the mitotic index of bone marrow cells at both doses, indicating bone marrow suppression. Pre-treatment of mice with theanine significantly elevated the reduced mitotic indices to nearly normal level especially in animals pre-treated with 60 mg/kg theanine and 30 mg/kg irinotecan.

3.2. Effect of theanine on irinotecan-induced DNA damage

The results of alkaline comet assay are shown in Fig. 1. Theanine treatment did not exhibit any significant difference in the level of olive tail moment compared to the solvent control at either dose tested. The positive control cyclophosphamide significantly increases the level of olive tail moment compared to the control group ($P < 0.05$). The results revealed that irinotecan when given at multiple doses of 30 and 60 mg/kg cause significant increase in the level of olive tail moment in comparison to those of the solvent control group and the higher dose of irinotecan gave the more DNA damage. However, when pre-treatment of different doses of theanine was given prior to irinotecan treatment, decreased rates of DNA damage were observed and the higher dose of theanine gave the more effective reduction of olive tail moment in animals treated with irinotecan ($P < 0.01$).

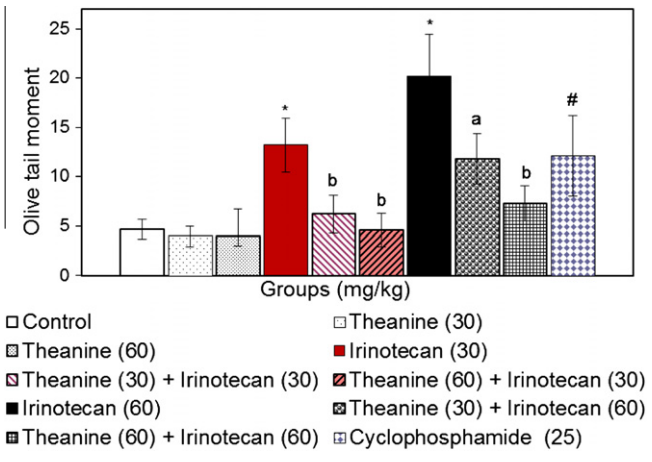

Fig. 1. DNA damage in bone marrow of mice 24 h after the last treatment with indicated doses of theanine and/or irinotecan or cyclophosphamide (mean ± SD). Olive Tail Moment = (Tail mean–Head mean) × Tail% DNA/100. *$P < 0.05$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test). [a]$P < 0.05$ and [b]$P < 0.01$ versus the corresponding irinotecan alone; [#]$P < 0.05$ versus control (Mann–Whitney U test).

3.3. Effect of theanine on irinotecan-induced MNPCE and bone marrow suppression

The results of the micronucleus test are presented in Table 2. The frequency of micronucleated polychromatic erythrocytes (MNPCE) in the positive control mutagen cyclophosphamide was significantly higher when compared to the solvent control group ($P < 0.01$). Irinotecan only at 60 mg/kg significantly increased the

Table 2
Frequency of MNPCE and mitotic activity (% PCE) in bone marrow of mice treated with indicated doses of theanine and/or irinotecan or cyclophosphamide (mean ± SD).

Treatment groups (mg/kg)	% MNPCE (mean ± SD)	% PCE (mean ± SD)
Control	0.30 ± 0.07	49.0 ± 2.23
Theanine 30	0.32 ± 0.08	48.6 ± 1.51
Theanine 60	0.28 ± 0.08	49.2 ± 1.78
Irinotecan 30	0.44 ± 0.09	45.2 ± 1.30*
Theanine 30 + Irinotecan 30	0.42 ± 0.08	48.2 ± 1.64[a]
Theanine 60 + Irinotecan 30	0.38 ± 0.08	48.6 ± 1.67[b]
Irinotecan 60	0.96 ± 0.11**	42.4 ± 3.43*
Theanine 30 + Irinotecan 60	0.50 ± 0.02[a]	46.8 ± 2.04[a]
Theanine 60 + Irinotecan 60	0.42 ± 0.19[b]	48.4 ± 1.67[b]
Cyclophosphamide 25	1.18 ± 0.14[##]	44.8 ± 3.11[#]

Each treatment group and control group consisted of five animals. MNPCE, micronucleated polychromatic erythrocyte. The mitotic activity at interphase stage was calculated as% PCE = [PCE/(PCE + NCE)] × 100.

* $P < 0.05$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test).

** $P < 0.01$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test).

[a] $P < 0.05$ versus the corresponding irinotecan alone.

[b] $P < 0.01$ versus the corresponding irinotecan alone.

[#] $P < 0.05$ versus control (Mann–Whitney U test).

[##] $P < 0.01$ versus control (Mann–Whitney U test).

frequency of MNPCE compared to the solvent control ($P < 0.01$). Furthermore, in presence of irinotecan at both doses the percentage of PCE decreased significantly, which indicates a suppression of bone marrow proliferation. Theanine treatment did not exhibit any significant difference in the frequency of MNPCE compared to the solvent control at both tested doses. In addition, theanine was not cytotoxic to the bone marrow at the tested doses level. With regard to the animals treated with theanine before irinotecan, both does of theanine produced clear significant inhibitory effects on the MNPCE induced by irinotecan in comparison to the irinotecan alone. The reduction of mitotic index induced by 60 mg/kg of irinotecan at the interphase stage was found to be restored by theanine pretreatment.

3.4. Effect of theanine on irinotecan-induced DNA oxidative stress

The effect of theanine on the irinotecan-induced DNA oxidative stress was assessed by measuring serum 8-OHdG, TBARS, GSH and GSSG levels. The results of serum estimation of 8-OHdG, which is excised from oxidized DNA are shown in Fig. 2A. There was no significant difference in the 8-OHdG levels between theanine treated animals and control group at the tested doses level. Significant increases of serum 8-OHdG was observed after 30 and 60 mg/kg irinotecan treatment as compared to the control group. With regard to the animals treated with theanine before irinotecan, a weak protection was observed with 30 mg/kg of theanine. With 60 mg/kg pre-treatment, however, theanine produced a clear significant suppressive effect on the serum 8-OHdG induced by irinotecan in comparison to the irinotecan alone ($P < 0.01$).

As shown also in Fig. 2B, no significant change in TBARS level was observed in serum after theanine treatment compared to the control. The TBARS level in mice treated with 60 mg/kg irinotecan was significantly increased compared to the control ($P < 0.01$). The irinotecan-induced TBARS formation was completely abrogated by theanine pre-treatment and decreased to a level nearly similar to the control value. As shown in Table 3, serum GSSG and GSH levels did not show any significant variation in theanine-treated animals compared to the solvent control. The GSH level observed in irinotecan-treated animals was significantly decreased, together with increase in GSSG level as compared to the control group. So that GSH/GSSG ratio significantly decreased, indicating increased oxidative stress ($P < 0.01$). Animals pre-treated with theanine showed a significant increase in GSH level over irinotecan-treated group and increased to the level significantly different from the level of GSH in the irinotecan treated alone. The GSSG level was also significantly decreased in theanine pre-treated animals compared to irinotecan-treated group. Consequently, the GSH/GSSG ratio was increased in theanine treated animals and was statistically significant when compared to the irinotecan-treated mice. The higher dose of theanine gave the more effective ameliorations in these DNA oxidative stress markers.

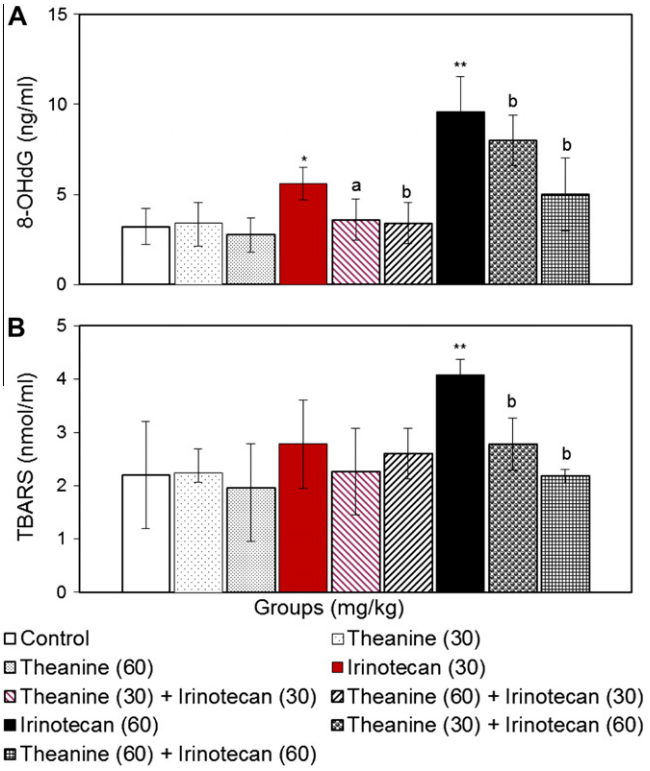

Fig. 2. Levels of 8-hydroxydeoxyguanosine (8-OHdG) (A) and lipid peroxidation (TBARS) (B) in serum of mice 24 h after the last treatment with indicated doses of theanine and/or irinotecan (mean ± SD). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (One way ANOVA and post hoc Tukey–Kramer multiple comparison test). $^{a}P < 0.05$, $^{b}P < 0.01$ versus the corresponding irinotecan alone (Unpaired t test).

4. Discussion

In the present study, the hypothesis that theanine may protect bone marrow cells against the genomic damage induced by the anticancer topoisomerase-I inhibitor, irinotecan was tested. The current study demonstrated for the first time that theanine was neither genotoxic nor cytotoxic in mouse bone marrow cells with the doses tested. Moreover, it is able to protect mouse bone marrow cells against the irinotecan-induced genomic damage and decline in the cell proliferation as observed by reduction in the chromosomal aberrations, DNA damage, MN formations and increases in mitotic activities, respectively. This effect, however, is dose dependent, been detected at high dose of theanine. The positive control mutagen cyclophosphamide was used in this study and the results of this compound were in the same range as those of the earlier studies (Attia et al., 2009a,c; Bakheet et al., 2011). These data confirmed the sensitivity of the experimental protocol followed in the detection of genomic damaging effects.

It was concluded that irinotecan is a somatic cell genotoxic agent. Because of its clastogenic potential, irinotecan is able to induce DNA damage and formation of MN *in vitro* (Kasuba et al., 2010) and *in vivo* (Hardman et al., 2002; Attia et al., 2009b; Vrdoljak et al., 2009). In addition, in previous *in vitro* study carried out by Cunha et al. (2002), irinotecan was genotoxic and recombinogenic agent in the Drosophila wing spot test. In agreement with these reports, the present experiment showed that exposure to irinotecan caused significant increase in the bone marrow chromosomal aberrations, DNA damage and MN formations as compared to the values obtained after treatment with the solvent control. Prior administration of theanine ahead of irinotecan challenge ameliorated these genotoxic markers and clearly suggests the protective role of theanine on irinotecan's genotoxic potentials. The genotoxic protection was also directly correlated with mitotic activity as an obvious protection was noted with theanine pretreated animals when bone marrow suppression was examined at both metaphase and interphase stages where, reductions in irinotecan-induced myelosuppression in mice pre-treated with theanine were observed. Moreover, the protection afforded by theanine revealed that theanine could exert dose dependent anti-genotoxic effects.

The present metaphase chromosomal analysis and comet assay showed that exposure to 30 mg/kg irinotecan yielded 11.12- and 2.8-fold increases in total structural aberrations and DNA damage, respectively. However, in the MN test, it was found that the lowest

Table 3
Levels of reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH/GSSG ratio in serum of mice 24 h after the last treatment with indicated doses of theanine and/or irinotecan (mean ± SD).

Treatment groups (mg/kg)	GSH (μg/ml)	GSSG (μg/ml)	GSH/GSSG ratio (%)
Control	3.80 ± 0.44	0.53 ± 0.11	7.34 ± 1.25
Theanine 30	3.76 ± 0.82	0.44 ± 0.11	8.80 ± 2.19
Theanine 60	4.60 ± 0.89	0.39 ± 0.08	12.3 ± 3.3**
Irinotecan 30	2.98 ± 0.66*	0.98 ± 0.08**	3.06 ± 0.8**
Theanine 30 + Irinotecan 30	3.40 ± 0.54	0.66 ± 0.23 [a]	5.58 ± 1.68[a]
Theanine 60 + Irinotecan 30	3.60 ± 0.54	0.42 ± 0.10 [b]	8.83 ± 1.62[b]
Irinotecan 60	2.42 ± 0.47*	1.22 ± 0.38**	2.09 ± 0.6**
Theanine 30 + Irinotecan 60	2.60 ± 0.54	0.96 ± 0.39	3.22 ± 1.77
Theanine 60 + Irinotecan 60	3.60 ± 0.89[a]	0.66 ± 0.23[a]	5.97 ± 2.46[b]

Each treatment group and control group consisted of five animals.
* $P < 0.05$ versus control (One way ANOVA and post hoc Tukey–Kramer multiple comparison test).
** $P < 0.01$ versus control (One way ANOVA and post hoc Tukey–Kramer multiple comparison test).
[a] $P < 0.05$ versus the corresponding irinotecan alone (Unpaired t test).
[b] $P < 0.01$ versus the corresponding irinotecan alone (Unpaired t test).

genotoxic dose, which caused MN formation, was 60 mg/kg of irinotecan. These observations suggest that chromosomal aberrations and DNA damage are induced at lower doses of irinotecan than MN formation hence; the metaphase chromosomal analysis and comet assay are more sensitive assays than MN test. It must of course be noted that the assays measure different end-points. True chromatid discontinuities and gaps are detected in the metaphase chromosomal analysis assay, DNA strand breaks and alkali-labile sites are measured in the comet assay and chromosome loss or damage are measured in MN test. Therefore, the present data confirm the general idea, that the genotoxicity of chemicals should be tested by different methods.

Limited data have been published on oxidative stress associated with irinotecan. Current evidence points to camptothecins as the agents that generate high levels of reactive oxygen species (Conklin, 2004). Accumulation of these reactive oxygen species in normal cells during cancer chemotherapy can lead to the damage of important biomolecules such as membrane lipids, proteins and DNA, causing DNA damage that in turn develop into chromosomal aberrations (Attia, 2010b). To determine whether the observed protective effects of theanine were due to an enhancement of the scavenger of reactive oxygen species generated by irinotecan, oxidative stress markers such as serum 8-OHdG, lipid peroxidation, GSH and GSSG levels were measured after the animals were treated with irinotecan and/or theanine. As a commonly used oxidative DNA damage marker, 8-OHdG is one of the major oxidatively modified DNA base products *in vivo* and is also the most mutagenic and carcinogenic, resulting in GC-to-TA transversion unless repaired before DNA replication (Floyd, 1990). TBARS, the product of lipid peroxidation also interacts with DNA causing strand breaks that in turn develop into chromosomal breaks. These chromosomal breaks may also appear as MN in the daughter cell after the first cell division. The present study demonstrates that significant increases of serum 8-OHdG and TBARS were observed after irinotecan treatment. The irinotecan-induced increases in 8-OHdG and TBARS were markedly reduced in theanine pre-treated animals. By reducing oxidative DNA damage, theanine might generate a beneficial effect on the defence against genomic damage in normal cells.

The current study also demonstrated that irinotecan induces a decrease in the GSH/GSSG levels in the mouse serum. This can induce genomic damage through the failure of the antioxidant defence mechanisms, since antioxidants such as GSH are able to protect normal cells. The increased GSH and GSH/GSSG levels suggest that protection by theanine may be mediated through the modulation of cellular antioxidant levels. These observations confirm earlier studies in which theanine was reported to elevates GSH, glutathione peroxidise and to reduce lipid peroxidation (Sadzuka et al., 1996; Sugiyama and Sadzuka, 2004). In the liver, theanine can be easily taken up and immediately metabolized to glutamate, and then converted to GSH (Sugiyama and Sadzuka, 2004). This increment in GSH concentration may result in both reduced 8-OHdG and lipid peroxidation levels induced by irinotecan in normal cells. Consequently theanine protects tissues from irinotecan toxicity. A decrease in serum GSH/GSSG ratio noted after irinotecan treatment could lead to less protective mechanism and thereby developing more irinotecan induced toxicity. However, in the group of mice pre-treated with theanine showing decreased cyto-genotoxic effects and significant increased in the serum GSH/GSSG ratio suggests the definite significance of GSH also. It could be the elevated level of GSH to protect the cells against irinotecan induced genomic damage.

5. Conclusions

Prior administration of theanine ahead of irinotecan challenge to male mice ameliorated all the cytogenetic parameters altered by irinotecan. The improvement in mitotic activity of bone marrow cells of animals pre-treated with theanine in irinotecan toxicity may focus attention on the beneficial effect of theanine to overcome the haematological toxicity induced by irinotecan. The genotoxic effects of irinotecan might be, at least in part, mediated by an oxidative stress mechanism that may be prevented or reduced by theanine. Although theanine itself does not have antioxidant properties (Sadzuka et al., 1996), it is predicted that intake of theanine accompanied by the increase in effective GSH on the oxidative stress. It is, however, worth noting that combination therapy with antioxidants and irinotecan provided protection against the irinotecan's genotoxicity without interfering with its anticancer efficacy (Kontek et al., 2010; Frömberg et al., 2011). These results might be explained if it is considered that the anticancer activity of irinotecan is due to inhibition of DNA topoisomerase-I activity, which is the result of the stabilization of the DNA-topoisomerase I cleavable complexes, and not by free radical production, which can be inhibited by free radical scavengers. Moreover, theanine also possesses anti-tumor activity (Sugiyama and Sadzuka, 2003; Liu et al., 2009). Thus, theanine might be a good alternative to reduce genotoxic risks associated with irinotecan therapy without diminishing its anti-tumor activity.

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7. Conflict of Interest

The author declares that there are no conflict of interest.

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