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Naringin, a Grape Fruit Bioflavonoid Protects Mice Bone Marrow Cells against the Doxorubicin-Induced Oxidative Stress

Ganesh Chandra Jagetia*[1] and C. Lalrinengi[1]

[1]*Department of Zoology, Mizoram University, Aizawl-796 004, India*

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***Corresponding author:** Dr. Ganesh Chandra Jagetia, Professor, Department of Zoology, Mizoram University, Tanhril, Aizawl-796 004, Mizoram, India, Tel: 011-389-2330724/2330227; Email: gc.jagetia@gmail.com*

Abstract

Doxorubicin an anthracylcine group of antibiotics is under frequent clinical use to treat numerous neoplastic disorders and it kills cancer cells by inducing different reactive oxygen species that increase oxidative stress in the neoplastic cells. Since there is no preferential accumulation of doxorubicin it is also taken up by normal cells which also suffer from the oxidative stress induced by it leading to their killing or development of second malignancies. The doxorubicin is known to kill bone marrow stem cells leading to its suppression. Therefore, present study was designed to study the effect of naringin treatment on doxorubicin-induced oxidative stress. The mice were administered with 0, 1, 5 or 10 mg/kg body weight of doxorubicin or the animals were given 10 mg/kg body weight naringin either before one hour or after one hour of doxorubicin treatment. The bone marrow was extracted at 0.5, 1, 2 and 4 h post-drug treatment and was processed for the estimation of glutathione (GSH), glutathione-s-transferase (GST), catalase and superoxide dismutase (SOD) and lipid peroxidation (LOO). Treatment of mice with doxorubicin alone led to a significant decline in the GSH concentration, and activities of GST, catalase and SOD accompanied by an elevation in the lipid peroxidation in the bone marrow cells of mice. Treatment of mice with naringin before and after doxorubicin administration resulted in a significant elevation in all antioxidants at all times, except lipid peroxidation that did not reveal any significant alteration. The GSH showed a maximum decline at half hour whereas GST and SOD continued to decline until 4 h post-treatment. However, a maximum decline in catalase activity was recorded at 1 h post treatment. In contrast LOO increased at 1 h post treatment and remained almost unaltered up to 4 h post-treatment in all the three groups. The pretreatment of naringin was more effective than the post-treatment. Our study demonstrates that naringin alleviated the doxorubicin-induced oxidative stress by raising the antioxidant status and marginally reducing lipid peroxidation.

Keywords: Mice, Bone marrow, Doxorubicin, Naringin, Glutathione, Glutathione-S-Transferase, Catalase, Lipid peroxidation.

Introduction

Doxorubicin (Adriamycin; also known as hydroxydaunorubicin) is a photosensitive antibiotic isolated from *Streptomyces peucetius*, a soil bacteria in the 1960s [1]. Doxorubicin belongs to an anthracycline group of antibiotics, and is closely related to the natural product daunomycin. Doxorubicin is one of the highly effective anthracyline groups of cancer chemotherapeutic drugs, which acts on the specific phase of the cell cycle, especially the S phase of cell division [2]. The doxorubicin has been introduced in the clinics to treat various malignant diseases in the early 1970s and remains an integral part of various modern chemotherapeutic regimens where it is used to treat different malignant neoplasia including Hodgkin's and non-Hodgkin's lymphomas, myeloblastic leukemias, breast cancer, small cell lung cancer, ovarian cancer, childhood solid tumors, hepatocarcinomas, soft tissue sarcomas, Kaposi's sarcoma, and bone tumors [3-7].

Though the mechanism of action of doxorubicin remains a bit controversial, it is well established that the doxorubicin kills neoplastic cells by binding to nucleic acids and by inhibiting topoisomerase enzymes, presumably by specific intercalation of the planar anthracycline nucleus with the DNA double helix [8,9]. The recent reports indicate that doxorubicin induces oxidative stress by free radical production and mitochondrial dysfunction [10]. The presence of iron in mitochondria helps to increase free radical production leading to cardiotoxicity, because heart tissue has only a limited defense system and cannot cope up with the free radical induced toxicity of doxorubicin [10-14]. Another adverse effect of DNA intercalating agents is production of second malignancies in the long-term survivors [15]. Apart from cardiotoxicity the use of doxorubicin is also associated with bone marrow and hematological toxicities. Since doxorubicin is highly effective anticancer agent its full potential can only be realized if its adverse effects are considerably reduced. Therefore, it is necessary to screen natural products that can alleviate the doxorubicin induced adverse side effects.

The grapefruit (*Citrus paradisii*), arose in Barbados as an accidental cross between an orange (*C. sinensis*) and a shaddock or pomelo (*C. maxima*), both of which were introduced from Asia in the seventeenth century, and was first introduced into Florida in the 1820s. The three major types of grapefruits that exist today are white, pink/red and ruby/rio red varieties. Grapefruit juice combines the sweet and tangy flavour of the orange and shaddock and also provides up to 69% of the recommended dietary allowance (RDA) for vitamin C. The grapefruit juice

contains flavonoids in the form of glycosides. Naringin is the most abundant flavonoid present in the juice, flower, and rind of grapefruit and constitutes up to 10% of the dry weight. It is relatively soluble in water and grapefruit juice contains up to 100 to 867 mg/L of naringin [16]. Upon ingestion, the naringin is converted into aglycone form known as naringenin and sugars by the action of intestinal flora [17]. Naringin has a wide range of biological actions including cholesterol-lowering, antiatherogenic and anti-inflammatory [18,19]. It has been reported to act as a cardioprotective, radioprotective, neuroprotective and antimutagenic agent [20-27]. Naringin has been found to reduce belomycin and doxorubicin induced genotoxicity in mice bone marrow, inhibit chemical carcinogenesis in mice, protect against iron-induced oxidative stress, lung fibrosis, osteoporosis, lipodystrophy and dyslipidemia in rats [28-34]. The administration of 16 g/kg b. wt. naringin to rats in acute toxicity studies has been reported to be nontoxic and its daily administration for 13 weeks at a dose of 1250 mg/kg did not induce any adverse side effects in rats indicating its safety [35]. Apart from cardiotoxicity doxorubicin also causes bone marrow depression that hampers utilization of its full potential in cancer treatment regimens. Therefore, it was desired to study the protective effect of naringin on the doxorubicin induced oxidative stress in the bone marrow of mice [36].

Materials and Methods

Chemicals and reagents

Naringin ((2S)-7-[(2S,3R,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-2-yl]oxy-5-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-chromen-4-one), was procured from Acros Organics Ltd., Geel (Belgium), whereas the doxorubicin was supplied by Getwell Pharmaceuticals, Gurgaon, India. The glutathione, 2-thiobarbituric acid (TBA), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), phenazine methosulphate, ethylenediaminetertaacetic acid (EDTA), diethylenetriaminopenta-acetic acid (DETAPAC) nitroblue tetrazolium (NBT), 5-thio-(2-nitrobenzoic acid)] (TNB), 1-chloro-2,4-dinitrobenzene (CDNB), nitroblue tetrazolium, tetraethoxypropane, nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemicals Co. St. Louis, USA. Other routine chemicals were procured from Merck India, Mumbai.

Animals care and Handling

The animal care and handling were done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Eight to ten weeks old male Swiss albino mice weighing 25-30g were selected from an inbred colony maintained under the controlled conditions of temperature (23±2°C), humidity (50±5%) and light (12 of light and dark, respectively). The animals had ready access to sterile food and water throughout the study. The animals were kept in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was cleared by the Animal Ethics Committee, of the Mizoram University, Aizawl, India.

Preparation of the drug and mode of administration

Naringin and doxorubicin was dissolved in sterile double distilled water (DDW) freshly each time immediately before use. The naringin (NIN) or doxorubicin (DOX) was administered intraperitoneally

Experimental

The animals were divided into the following groups:

1. DOX group:- The animals of this group were intraperitoneally administered with 0, 1, 5 or 10 mg/kg b. wt. of doxorubicin alone.

2. **NIN+DOX:-** This group of animals was intraperitoneally administered with 10mg/kg b. wt. Naringin one hour before administration of 0, 1, 5 or 10 mg/kg b. wt. doxorubicin. The 10 mg/kg naringin was selected based on our earlier study where 10 mg/kg b. wt. of naringin was found to provide maximum protection against the DOX-induced DNA damage, when compared to other doses [30].

3. DOX+NIN:- The animals of this group were given 10 mg/kg b. wt. naringin intraperitoneally one hour after the administration of 0, 1, 5 or 10 mg/kg b. wt. doxorubicin.

The animals of all groups were killed by cervical dislocation at 0.5, 1, 2 and 4 h posttreatment for determination of glutathione (GSH), glutathione-s-transferase (GST), catalase, superoxide dismutase (SOD) and lipid peroxidation (LOO) in the bone marrow of mice. Usually five animals were used for each dose of DOX in each group at each time point and a total of 240 animals were used for the whole experiment. The bone marrow cells were extracted by removing the femora of each animal at the above specified posttreatment times and placed onto a wet Whatman filter paper. The femora were freed from muscles and other tissues, cleaned and the bone marrow was flushed using PBS into individual tubes.

Preparation of homogenate

The tubes were centrifuged resuspended in PBS and one million cells were homogenized for the estimation of GSH, GST, catalase, SOD and LOO.

Total Proteins

The protein contents were determined according to the modified method of Lowry's [37].

Glutathione

Glutathione contents were measured by the method of Moron et al., with minor modifications. Briefly, the proteins in 1 ml supernatant were precipitated by 0.5 ml ice cold 10% 5-sulfosalicylic acid [38]. The tubes were kept on ice for 10 min, centrifuged (Sorvall Instruments RC5C, DuPont, Minnesota, USA) at 15,000 rpm at 4°C for 15 min and the supernatant free of

proteins was collected. The entire supernatant was immediately mixed with 0.5 ml of NADPH (4 mg of reduced form was dissolved in 100 ml of 0.5% $NaHCO_3$), 0.5 ml of glutathione reductase (6 units/ml in 0.1 M phosphate buffer, pH 7.0) and 1 ml of 0.6 M DTNB [prepared in 0.2 M phosphate buffer (pH 8)]. The formation of TNB was read against blank at 412 nm in a UV-Visible double beam spectrophotometer (Shimadzu Corporation, Tokyo, Japan). A sample blank lacking GSH was used to determine the background rate and the resulting background rate of product formation was subtracted from the sample values prior to GSH quantification. The GSH concentration has been expressed as µmol/million cells. Standard curve was prepared from a stock solution of 10 mM GSH (30.7 mg GSH/10 ml) in 5% 5-sulfosalicylic acid diluted to 1-10 nM GSH/ml.

Glutathione-S-Transferase

Glutathione-S-transferase was estimated by the method of Habig and Pabst [39]. Briefly, the bone marrow cell homogenate was mixed with 0.1 M potassium phosphate buffer, CDNB and 10 mM GSH, and incubated for 10 min at 37°C. The absorbance was read against the blank at 340 nm using a double beam UV-VIS spectrophotometer. The absorbance of the samples was read at 1min intervals. GST activity has been expressed as nmol/mg of protein.

GST activity = Absorbance of sample – Absorbance of blank× 1000/9.6 × Vol of sample

Catalase

The catalase activity was estimated by the catalytic reduction of hydrogen peroxide [40]. Briefly, hydrogen peroxide was added to the tissue homogenate and incubated at 37°C. The decomposition of hydrogen peroxide was monitored at specific time intervals by recording the absorbance against the blank at 240 nm using a double beam UV-VIS spectrophotometer at the intervals of 0.5 and 10s intervals up to 30s. The average difference in absorbance in 30s was calculated.

Superoxide Dismutase

The SOD was estimated by the method as described earlier [41]. Briefly, the cell homogenate was mixed with reagent consisting of phenazine methosulphate, nitroblue tetrazolium and NADH and incubated for 90 sec. at 30°C and the reaction was stopped by adding acetic acid and n-butanol. Blank was prepared by adding the reagent without the sample and incubated for 90 sec. at 30°C and the reaction was stopped by adding acetic acid and n-butanol. The absorbance of sample was measured against the blank at 560 nm in a UV-VIS spectrophotometer.

Lipid Peroxidation

The level of various thiobarbituric acid reactive substances (TBARS) including malondialdehyde, lipid hydroperoxides and aldehydes in the cell homogenates was measured by the method of Ohkawa *et al.,* [42]. The method is based on the spectroscopic estimation of malondialdehyde and thiobarbituric acid 1:2 adduct formation in the reaction mixture. Briefly, 1 ml supernatant was heated with thiobarbituric acid (0.8%), sodium dodecyl sulphate (0.1%) and acetic acid (20%) in a boiling water bath for 30 min (lipoproteins get precipitated). The resultant mixture was cooled, extracted with n-butanol-pyridine, and the absorbance of the butanol layer was recorded at 532 nm using UV-Visible double beam spectrophotometer. The resulting concentration of TBA reactive substances is expressed as nmol/million cells obtained from a standard curve of tetraethoxypropane.

Statistical analyses

The statistical significance among all the groups was determined by Students "t" test and analysis of variance (ANOVA). The results are the average of three individual experiments. The data of each experiment did not differ significantly from one another and hence, all the data are combined and means calculated. A p value of < 0.05 was considered statistically significant.

Results

The results are expressed as mean ± SEM (standard error of the mean) in Table 1-5.

Table 1: The effect of 10 mg/kg body weight naringin on the doxorubicin-induced depletion in the glutathione concentration (µmol/million cells) in the bone marrow of mice at various post treatment times

Post treatment time (h)	Doxorubicin(mg/kg body weight)									
	0	1			5			10		
	Control	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN
0.5	1.80±0.03	0.36±0.01‡	0.53±0.013*	0.43±0.013	0.18±0.018‡	0.29±0.018	0.23±0.019	0.12±0.003‡	0.20±0.006	0.18±0.009
1	1.80±0.03	0.42±0.02‡	0.57±0.016*	0.50±0.022	0.23±0.035‡	0.35±0.037	0.29±0.037	0.13±0.022‡	0.18±0.073	0.16±0.015
2	1.80±0.03	0.48±0.05‡	0.65±0.05*	0.55±0.05	0.30±0.024‡†	0.42±0.022†	0.36±0.023	0.14±0.011‡	0.19±0.011	0.16±0.011
4	1.80±0.09	0.51±0.01‡†	0.68±0.018*	0.58±0.015†	0.31±0.017‡†	0.43±0.018†	0.30±0.075	0.11±0.011‡	0.17±0.011	0.13±0.016

P < 0.05, No symbol=Non-significant

‡- statistical significance was calculated against control with the positive control i.e DOX treatment alone.

*- statistical significance was calculated against DOX alone treatment in their respective treatment hours.

#- statistical significance was calculated against DOX+NIN combination treatment in their respective treatment hours.

†- statistical significance was calculated against their corresponding 0.5h treatment combinations.

Table 2: The effect of 10 mg/kg body weight naringin on the doxorubicin-induced alleviation in the glutathione–s–transferase activity (nmol/million cells) in the bone marrow of mice at different post treatment times

Post treatment times (h)	Doxorubicin(mg/kg body weight)									
	0	1			5			10		
	Control	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN
0.5	4.25±0.01	3.99±0.19	4.70±0.10*	4.61±0.057	3.42±0.26‡	4.11±0.06*	3.99±0.11	2.76±0.02‡	3.28±0.18†	3.23±0.01
1	4.25±0.01	4.07±0.13	4.52±0.18	4.30±0.33	3.36±0.14‡	3.82±0.23	3.75±0.12	2.35±0.22‡	3.01±0.19*	3.07±0.2*
2	4.25±0.01	3.67±0.01	4.29±0.05*	3.97±0.12*	3.12±0.15‡	3.56±0.06	3.55±0.10	2.31±0.04‡	2.81±0.02	2.81±0.02
4	4.25±0.01	3.56±0.14	4.20±0.11†	3.85±0.02	2.98±0.12‡†	3.53±0.02	3.46±0.13	2.33±0.10‡	2.71±0.12	2.65±0.11

P < 0.05, No symbol=Non-significant

‡- statistical significance was calculated against control with the positive control i.e DOX treatment alone.

*- statistical significance was calculated against DOX alone treatment in their respective treatment hours.

#- statistical significance was calculated against DOX+NIN combination treatment in their respective treatment hours.

†- statistical significance was calculated against their corresponding 0.5h treatment combinations.

Table 3: The effect of 10 mg/kg body weight naringin on the doxorubicin-induced depletion in the catalase activity (nmol/million cells)in the bone marrow of mice at various post treatment times

Post-treatment times (h)	Doxorubicin (mg/kg body weight)									
	0	1			5			10		
	Control	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN
0.5	29.17±0.03	11.63±1.21‡	19.49±0.70*#	18.49±1.16*	10.63±0.60‡	14.63±1.91*#	12.73±0.69*	8.71±1.30‡	13.86±0.31*	11.85±0.51*
1	29.17±0.03	8.54±0.62‡†	15.79±0.66*†	15.14±0.15*†	7.87±0.23‡†	10.95±1.92*†	9.91±0.42*†	7.54±0.27‡†	8.97±0.37*†	8.23±0.40*†
2	29.17±0.03	10.07±0.57‡†	16.28±1.02*#†	14.52±1.14*†	8.67±1.39‡	10.54±0.71*†	10.03±1.40†	7.38±0.85‡†	8.91±1.27*	8.22±1.36*†
4	29.17±0.03	9.83±0.53‡†	16.29±0.41*#†	15.18±0.87*†	8.99±0.43‡†	10.50±0.45*#†	9.29±0.47*†	5.52±0.49‡	9.94±0.82*†	8.85±0.56*†

P < 0.05, No symbol=Non-significant

‡- statistical significance was calculated against control with the positive control i.e DOX treatment alone.

*- statistical significance was calculated against DOX alone treatment in their respective treatment hours.

#- statistical significance was calculated against DOX+NIN combination treatment in their respective treatment hours.

†- statistical significance was calculated against their corresponding 0.5h treatment combinations.

Table 4: The effect of 10 mg/kg body weight naringin on the doxorubicin-induced decline in the superoxide dismutase activity(Units/million cells)in the bone marrow of mice at different post treatment times.

Post treatment times (h)	Doxorubicin(mg/kg body weight)									
	0	1			5			10		
	Control	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN
0.5	5.12±0.03	3.03±0.07‡	4.55±0.08*	4.43±0.13*	2.78±0.09‡	3.95±0.09*	3.62±0.10*	1.83±0.11‡	3.12±0.10*	2.90±0.12*
1	5.12±0.03	2.61±0.06‡†	4.13±0.07*	4.01±0.12*	2.36±0.08‡	3.53±0.08*	3.20±0.09*	1.41±0.10‡	2.70±0.09*	2.48±0.11*
2	5.12±0.03	2.30±0.08‡†	3.81±0.09*	3.69±0.14	2.04±0.10‡	3.21±0.10*	2.88±0.11	1.09±0.12‡†	2.38±0.10*	2.16±0.13*

4	5.12±0.03	2.21±0.07‡†	4.02±0.08*	3.78±0.13*	1.96±0.09‡†	3.42±0.09*	2.97±0.10*	1.01±0.11‡†	2.59±0.10*	2.25±0.12*

P < 0.05, No symbol=Non-significant

‡- statistical significance was calculated against control with the positive control i.e DOX treatment alone.

*- statistical significance was calculated against DOX alone treatment in their respective treatment hours.

#- statistical significance was calculated against DOX+NIN combination treatment in their respective treatment hours.

†- statistical significance was calculated against their corresponding 0.5h treatment combinations.

Table 5: The effect of 10 mg/kg body weight naringin on the doxorubicin-induced increase inlipid peroxidation(TBARs (nmol/million cells)in the bone marrow of mice at various post treatment times.

Post treatment times (h)	Doxorubicin (mg/kg body weight)									
	0	1			5			10		
	Control	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN	DOX	NIN+DOX	DOX+NIN
0.5	0.09±0.002	0.12±0.002‡	0.12±0.008	0.10±0.01	0.17±0.005‡	0.14±0.001	0.13±0.007	0.20±0.005‡	0.195±0.01	0.194±0.01
1	0.09±0.002	0.13±0.003‡	0.13±0.001	0.12±0.003	0.16±0.007‡	0.162±0.01	0.161±0.004	0.24±0.02‡	0.23±0.02	0.24±0.004
2	0.09±0.002	0.14±0.01‡	0.14±0.006	0.13±0.004	0.19±0.015‡	0.18±0.00	0.18±0.01	0.24±0.02‡	0.23±0.02	0.22±0.02
4	0.09±0.002	0.13±0.006‡	0.12±0.01	0.11±0.008	0.18±0.003‡	0.18±0.01	0.17±0.01	0.23±0.005‡	0.22±0.007	0.22±0.01

P < 0.05, No symbol=Non-significant

‡- statistical significance was calculated against control with the positive control i.e DOX treatment alone.

*- statistical significance was calculated against DOX alone treatment in their respective treatment hours.

#- statistical significance was calculated against DOX+NIN combination treatment in their respective treatment hours.

†- statistical significance was calculated against their corresponding 0.5h treatment combinations.

Glutathione

The spontaneous glutathione concentration in the non-drug treated control Swiss albino bone marrow cells is 1.80 ± 0.03 nmol/million cells (Table 1). Administration of naringin did not alter the GSH concentration significantly in mice bone marrow cells as compared to control (data not shown). When the mice were given different concentrations of doxorubicin it caused a dose dependent decline in the GSH concentration and a maximum depletion in GSH concentration was observed for 10 mg/kg b. wt. DOX (Table 1). GSH concentration showed highest decrement at 1/2 h post-treatment that continued to elevate until 4 h posttreatment without restoration to normal level (Table 1). Administration of naringin 1 h before DOX treatment resulted in a significant rise in the GSH concentration as early as 0.5 h post treatment, which continued to rise up to 4 h posttreatment where the GSH concentration was greatest. The trend in GSH rise was almost similar in the bone marrow cells of mice that received naringin 1 h after DOX treatment however; the amount was lesser than the pretreatment group (Table 1). The increasing dose of DOX tapered this effect and when 10 mg/kg DOX was given the elevation in GSH concentration was not significantly greater in both the NIN+DOX and DOX+ NIN groups (Table 1).

Glutathione-S-transferase

The glutathione-s-transferase activity in the bone marrow cells of non-drug treated control mice is 4.25±0.01 nmol/million cells and naringin administration alone did not alter the glutathione-s-transferase activity significantly as compared to control (data not shown) (Table 2). Treatment of mice with different doses of DOX reduced GST activity significantly at 5 and 10 mg/kg DOX at all the posttreatment times (Table2). The activity of GST declined continuously with time and the lowest GST activity was observed at 4 hours posttreatment (Table 2). Treatment of naringin before and after DOX administration resulted in an elevation in the GST activity that depended on the assay time and DOX dose (Table 2). A maximum elevation in GST activity was recorded for NIN+DOX group at 0.5 h posttreatment in NIN+DOX as well as DOX+NIN group The pattern of decline in the GST activity was similar to that of DOX treatment (Table 2).

Catalase

The catalase activity in the bone marrow of normal untreated mice is 29.17nmol/million cells, which remained unchanged after naringin administration (data not shown) (Table 3). The treatment of mice with different doses of DOX reduced the catalase activity in a dose dependent manner and the greatest reduction in the catalase activity was observed for 10 mg/kg DOX (Table 3). The DOX treatment also caused a time dependent attrition in the catalase activity and maximum reduction was observed at 1 h post treatment with an attempt of recovery thereafter (Table 3). Despite the signs of recovery the catalase activity was far from normal (Table 3). The catalase activity continued to decline in the bone marrow of mice treated with 10 mg/kg DOX until 4 h posttreatment where it was lowest in these animals (Table 3). This reduction in catalase activity by DOX was 3, 3.25 and 5.3 fold at 4 h posttreatment for 1, 5 and 10 mg/kg DOX, respectively (Table 3). The administration of naringin before and after DOX treatment significantly elevated the catalase activity and maximum elevation was observed at 0.5 h post treatment (Table 3). Thereafter catalase activity declined at 2 h post-treatment and increased thereafter (Table 3).

Superoxide dismutase

The base line SOD activity in the mice bone marrow cells is 5.12 ± 0.03 Units/million cells and naringin treatment did alter the spontaneous SOD activity significantly (data not shown) (Table 4). The administration of different doses of doxorubicin in mice resulted in a dose dependent alleviation in the SOD activity at all post treatment assay times as compared to control (Table 4). The lowest SOD activity was observed for 10 mg/kg DOX, which was almost five fold lower at 4 h post treatment (Table 4). The SOD activity showed a drastic decline at 0.5 h and continued to decline until 4 h post-treatment where a nadir was reached (Table 4). Naringin treatment before DOX administration did not alter the pattern of decline in the SOD activity in NIN+DOX group, except that the SOD activity was significantly enhanced (Table 4). The SOD activity was 2 fold greater at 0.5 h, whereas this elevation was approximately 3.5 fold at 1 and 2 h post-DOX-treatment, respectively (Table 1). Treatment of naringin before or after DOX administration caused a significant elevation in the SOD activity at all post treatment times (Table 4). At lower DOX doses the elevation was greater when compared to the higher dose of DOX (Table 4). Despite this rise in the SOD activity by naringin it was far from normal (Table 4).

Lipid peroxidation

The rate of lipid peroxidation in the bone marrow cells of Swiss albino mice is 0.09±0.002 nmol/million cells (Table 5). Naringin treatment did not significantly alter the spontaneous lipid peroxidation in mice bone marrow (data not shown). The doxorubicin administration resulted in a dose dependent but significant rise in the lipid peroxidation when compared to the control, and a highest lipid peroxidation was observed in the bone marrow of mice receiving 10 mg/kg DOX (Table 5). The analysis of lipid peroxidation with time did not show any significant alteration as the differences were not statistically significant (Table 5). Administration of naringin before and after DOX treatment did not significantly reduce lipid peroxidation although it was marginally lesser than the DOX alone treated groups (Table 5).

Discussion

Doxorubicin is widely used anthracycline group of antibiotics in clinical condition to treat different type of neoplastic disorders. It is used alone or in combination with other chemotherapeutic drugs to treat difficult neoplasia. However, its use is associated with severe bone marrow depression and life threatening cardiomyopathy [10, 36]. One of the important mechanisms by which DOX induces oxidative stress is production of free radicals therefore it is desirable to reduce the oxidative stress by alleviating free radical production. The interaction of DOX free radicals in the presence of iron further aggravates the oxidative stress and naringin has been reported to suppress free radicals and chelate iron [24,31]. The first line of antioxidants in cellular milieu is glutathione reductase, catalase, superoxide dismutase and trace elements like selenium, copper, zinc etc. The second line of defence includes cellular antioxidants like glutathione and third defence include a set of complex enzymes that carry out the repair of damages of macromolecules vital for cell survival [43]. Therefore present study was undertaken to study the modulatory effect of naringin given before and after DOX treatment on the glutathione, glutathione-s-transferase, catalase, superoxide dismutase and lipid peroxidation in mice bone marrow.

The GSH (γ-glutamylcysteinylglycine), a low molecular, water soluble non-protein thiol is synthesized in all living cells from three amino acids including glutamic acid, cysteine and glycine, which play a crucial role in combating oxidative stress in the cells by donating one electron and donation of two electrons by GSH results in the formation of GSSG, which is converted back to GSH by glutathione reductase [44-47]. The doxorubicin treatment has increased the oxidative stress in the bone marrow cells of mice by alleviating the GSH contents in a dose dependent manner. A similar effect has been observed earlier in the liver of mouse and rats treated with DOX [25,48-50]. Treatment of mice with naringin before and after DOX administration raised the GSH contents and the effect was better in the naringin pretreated group than the naringin post-treatment. Earlier investigations conform to our observations where a similar effect has been observed in mice heart and rat livers pretreated with naringin however, reports on post-treatment studies are lacking [25,26,50,51]. The other agents like *Agele marmelos* and glutamine, an amino acid have been reported to increase the GSH concentration in mice and rats treated with DOX [48,49]. The reduction in oxidative stress by GSH is the outcome of its reaction with free radicals directly or its participation through other enzymatic reaction [52]. It also conjugates with NO radical to form S-nitrosoglutathione adduct. This product is cleaved by thioredoxin system to generate GSH and NO back [47]. The other functions of GSH include its conjugation with electrophiles and physiological metabolites, which are essential in cell physiology [53].

The observation that DOX inhibited the GST activity in mouse bone marrow is in accordance with earlier findings where DOX has been reported to reduce GST activity in mouse heart and mice and rat liver [25, 51]. The treatment of naringin before and after DOX treatment elevated the GST activity and the effect of both pre and post naringin treatment was almost similar. The earlier studies have shown that naringin pretreatment elevated the GST activity in mouse heart and liver and rat liver [25, 50]. DOX-induced GST alleviation may have played a significant role in inducing oxidative stress as GSTs are essential in protecting against the toxic insult and DNA damage [54]. GSTs catalyze conjugation of a variety of substances with GSH causing detoxification and it also modulates cell proliferation and death [55]. The reduction of GSTs by DOX may be crucial in suppressing cell death and enhanced cell killing. Their overexpression in cancer cells have been considered as a cause of chemotherapy resistance [56].

Catalase or oxidoreducatase is a tetrameric intra cellular enzyme present in animals, plants and microorganisms. The accumulation of hydrogen peroxide in cellular milieu is

detrimental to the health of cells and catalase mainly cleaves hydrogen peroxide into non-toxic products like water and molecular hydrogen, neutralizing the toxic effect of hydrogen peroxide [57]. Treatment of mice with different doses of DOX resulted in a dose dependent reduction in the catalase activity in their bone marrow cells, which could be one of the reasons of myelosuppression in the patients. The earlier studies have reported a reduction in the catalase activity after DOX treatment in the mice and rat heart and liver [25,26,49,50]. The administration of mice with naringin before and after DOX treatment elevated the catalase activity, where both the pre- and post-treatment were equally effective especially at higher DOX doses. Likewise, early studies have indicated an increase in the catalase activity in the mouse and rat heart and liver [25,26,50,51]. The extract of *Aegle marmelos* has been found to reduce the DOX induced catalase activity in mouse heart earlier [49]. This increase in catalase activity by naringin may have played an important role in reducing the oxidative stress triggered by DOX in the present study.

The superoxide ions are generated during respiration, which are converted into the less harmful product hydrogen peroxide, which is neutralized by glutathione peroxidase and catalase [58-60]. However, DOX enhances the generation of these radicals and presence of iron converts the superoxide radicals into the more reactive, toxic and damaging species of OH radicals [10]. The DOX treatment has been found to deplete SOD activity in a dose dependent manner in the bone marrow of mice in the present study. A similar effect has been reported earlier in the mice heart and liver and rat liver [25,49,50]. The naringin treatment before and after DOX administration has been reported to retard DOX-induced attrition in the SOD activity. Earlier naringin treatment has been reported to protect against the DOX-induced depletion in the SOD activity in mice and rats [25,26,50,51]. The elevated level of SOD may have protected mice bone marrow cells from DOX-induced oxidative stress.

Lipid peroxidation is produced as a result of membrane damage as the lipids of the cell membrane interact with free radicals produced and undergo lipid peroxidation [61]. The production of lipid peroxidation has been considered as one of the hallmarks of oxidative stress. The DOX increased lipid peroxidation in the mouse bone marrow which is in conformation to earlier studies where DOX has been found to increase lipid peroxidation in the mouse and rat heart and liver [25,26,48-51,62]. Earlier studies have shown that naringin pretreatment resulted in the reduction of DOX induced lipid peroxidation in vitro and in mouse and rat heart and liver [23,26, 29,31,50,51]. Similarly, naringin alleviated the bleomycin-induced lipid peroxidation in rat lung earlier [33]. However, naringin treatment was not very effective in arresting the lipid peroxidation in bone marrow cells in the present study.

The exact mechanism of reduction of oxidative stress by naringin in the bone marrow cells of mice is not clearly understood. The DOX generates free radicals by the activation of NADPH oxidase system and naringin has been reported to inhibit the activation of NADPH oxidase leading to the attrition of free radical formation [63,64]. Therefore suppression of DOX-induced free radical generation by naringin may be one of the important mechanisms that may have helped to keep the activity of GST, catalase and SOD higher accompanied by more availability of glutathione. The presence of naringin may have neutralized free radicals immediately after their production since it has been reported to scavenge free radicals earlier [24]. It is well established that DOX generates higher amount of free radicals in the presence of iron and chelation of iron by naringin may have arrested the DOX induced free radicals reducing oxidative stress. Naringin has been reported to chelate iron [31]. DOX has been reported to transcriptionally activate NF-κB and COX-II in cardiomyocytes which is responsible for increased oxidative stress and inhibition of NF-κB and COX-II by naringin may have reduced DOX-induced oxidative stress [65-67]. Naringin has been reported to arrest transcriptional activation of of NF-κB and COX-II in vitro [64,68]. Nrf2 is essential in the expression of various antioxidant genes and its inhibition by DOX may have contributed to raise the oxidative stress in mice bone marrow cells leading to decline in all these antioxidants. The activation of Nrf2 elements by naringin may have been responsible for increased activities of GST, catalase and SOD along with raised GSH. The naringin has been reported to activate Nrf2 signalling pathway earlier [69].

Conclusions

The present study demonstrates that DOX has been able to increase the oxidative stress in mice bone marrow cells by increasing lipid peroxidation and reducing the activities of GST, catalase and SOD accompanied by a decreased concentration of GSH, whereas naringin pre- and post-DOX-treatment increased the activities of GST, catalase and SOD and also elevated GSH contents in the bone marrow of mice reducing the oxidative stress. The inhibition of the activation of NADPH oxidase, NF-κB, and COX-II may have contributed to the protective effect exerted by naringin along with activation of Nrf2 signaling at molecular level.

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