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Protective effect of green tea extract on doxorubicin induced cardiotoxicity in rats

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SUMMARY

Doxorubicin induces oxidative stress leading to cardiotoxicity causing ECG abnormalities and increases in biomarkers associated with toxicity. Green tea extract (GTE) is reported to possess antioxidant activity mainly via its polyphenolic constituent, catechins. This study was intended to determine the effect of various doses of GTE (25, 50 and 100 mg/kg/day p.o. for 30 days) on doxorubicin -induced electrocardiographic and biochemical changes in rat heart. The latter included lactate dehydrogenase (LDH), creatine kinase (CK), and glutamic oxaloacetate transaminase (GOT) in serum and superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH), as well as membrane bound enzymes like $Na^{+}K^{+}$ATPase, Ca^{2+}ATPase, Mg^{2+}ATPase and decreased lipid peroxidation (LP) in heart tissue Results demonstrated that rats which received GTE were less susceptible to such changes indicating protection afforded by GTE.

Key words: Doxorubicin; Green tea; Antioxidant; Catechins; Electrocardiogram

INTRODUCTION

It has been widely reported that doxorubicin, an anthracycline antibiotic for cancer treatment, causes cardiotoxicity, primarily due to the production of free radicals (Olson *et al.*, 1981; Doroshow and Locker, 1982; Myers, 1982; Doroshow, 1983). The clinical effectiveness of doxorubicin treatment for several cancers is affected by the dose-limiting side effect of cardiotoxicity (Lefrak *et al*, 1973). Several studies have concluded that antioxidants like α-tocopherol (αTC) (Myers *et al.*, 1977) and a-phenyl-tert-butyl-nitrone (Paracchini *et al.*, 1993) afforded protection from doxorubicin- induced myocardial injury without affecting its antineoplastic activity.

Polyphenols are plant metabolites occurring widely in foods of plant origin and possess outstanding antioxidant and free radical scavenging properties (Harbone, 1989; Scott *et al.*, 1993).Green tea is an excellent source of polyphenol antioxidants, particularly of a group known as green tea catechins (GTCs) (Zhu *et al.*, 1997). Green tea reduces iron-induced lipid peroxidation in brain homogenates as well as in cultured C6 astrocytes and lung cells (Lin *et al.*, 1998; Mazzio *et al.*, 1998). In addition, green tea has also been shown to reduce the formation of the spin-adducts of hydroxyl radicals and hydroxyl radical - DNA strand breakage *in vitro* (Hiramoto *et al.*, 1996). Green tea has been found to have inhibitory effects on chemical-induced lung tumorigenesis (Xu *et al.*, 1992). There is also considerable epidemiological evidence suggesting that the consumption of green tea lowers the risk of

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heart disease as well as several types of cancer incidences as a result of these antioxidant mechanisms (Ahmad and Mukhtar, 1999).

However, to the best of our knowledge, the effect of GTE on doxorubicin-induced cardiovascular abnormalities in rat heart have not been previously explored. Therefore, the aim of the present study was to investigate the effects of green tea extract on doxorubicin-induced cardiovascular abnormalities in a rat model.

MATERIALS AND METHODS

Chemicals

Standardized powdered, ethyl acetate extract of green tea leaves (*Camellia sinensis*) was donated by Cherain Chemicals, Baroda, India. Total polyphenolic content was 35%. Doxorubicin injection was donated by the Serum Institute of India Ltd., Pune. Epinephrine hydrochloride, super oxide dismutase (SOD), malondialdehyde and catalase, were purchased from Sigma Aldrich; USA. Reduced glutathione, 5, 5'dithiobis (-2 nitrobenzoic acid) (DTNB) and thiobarbituric acid (TBA) were purchased from Hi Media; India. All other chemicals were of analytical grade.

Animals

Adult albino rats of either sex (Wistar strain) weighing between 200 and 250 g were used for the study. The animals were fed ad libitum with standard pellet diet and had free access to water. All experiments and protocols described were approved by the Institutional Animal Ethics Committee (IAEC) of M. S. University, Baroda, India.

Experimental protocol

Chemical analysis of green tea extract

TLC fingerprint profile of the extract was established using HPTLC. For development of the TLC fingerprint, 500 mg of powdered green tea extract was extracted three times with 25 ml of methanol. Extracts were pooled, filtered and concentrated to 25 ml. Suitably diluted stock solution of methanolic extract with gallic acid standard solution and catechin were spotted on a pre-coated Silica gel G60 F254 TLC plate (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter and the plate was developed in the solvent system of toluene: ethyl acetate: formic acid (6: 6: 1). The plate was dried at room temperature and scanned using CAMAG TLC Scanner 3 at UV 254 nm and R_f values, and peak area of the resolved bands were recorded. Relative percentage area of each band was calculated from peak areas. The TLC plate was developed by spraying with 5% methanolic ferric chloride solution for the detection of phenolic compounds.

Groups and treatment schedule

Powdered green tea extract was reconstituted in distilled water. Doxorubicin injection was dissolved in sterile water for injection. The animals were divided into five groups each consisting of six rats and received following treatment

Doxorubicin- induced acute cardiotoxicity

Group I: Control group, received distilled water (3 ml/kg/day p.o. for 30 days) followed by sterile water for injection (1 ml/kg, i.v.) on 30th day.
Group II: Received distilled water (3 ml/kg/day p.o. for 30 days) followed by doxorubicin injection (10 mg/kg i.v.) on 30th day.
Groups III: Green tea extract (25 mg/kg/day p.o.for 30 days) followed by doxorubicin injection (10 mg/kg i.v.) on 30th day.
Group IV: Green tea extract (50 mg/kg/day p.o. for 30 days) followed by doxorubicin injection (10 mg/kg i.v.) on 30th day.
Group V: Green tea extract (100 mg/kg/day p.o. for 30 days) followed by doxorubicin injection (10 mg/kg i.v.) on 30th day.

After 48 hours of the injection of either doxorubicin or vehicle, electrocardiographic changes were recorded and serum markers were studied after removal of blood and the heart was excised under euthanasia

in chilled Tris buffer (10 mM pH 7.4) for measurement of tissue markers of oxidative stress.

Electrocardiography

Electrocardiograms were recorded under mild ether anesthesia through needle electrodes (Lead II) using Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA). The changes in heart rate, QT interval and ST interval were determined from the ECG.

Biochemical parameters

Serum markers: Serum levels of lactate dehydrogenase (LDH) and serum creatine kinase (CK), were determined by using standard kits of Reckon Diagnostic Ltd, India while glutamic oxaloacetate transaminase (SGOT) was estimated by using the standard kit of Span Diagnostic Pvt Ltd, India.

Biomarkers of the oxidative stress: The excised heart was then weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000 × *g* at 0°C for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assays of malondialdehyde content as indicator of lipid peroxidation (LP) (Slater and Sawyer, 1971), endogenous antioxidant enzymes, superoxide dismutase (SOD) (Misra and Fridovich, 1972), catalase (CAT) (Colowick *et al.*, 1984) and reduced glutathione (GSH) (Moron *et al.*, 1979).

Membrane bound enzymes: The sediment after centrifugation of tissue homogenate was resuspended in ice-cold Tris buffer (10 mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of different membrane bound enzymes such as Na^+K^+ATPase (Bonting, 1970), Ca^{2+}ATPase (Hjerten and Pan, 1983) and Mg^{2+}ATPase(Ohnishi *et al.*, 1982) and total proteins(Lowry *et al.*, 1975).

Statistical analysis

Results of all the above estimations have been indicated in terms of mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) followed by Tukey –Kramer multiple comparisons test with the level of significance set at $P \leq 0.05$.

RESULTS

Chemical analysis: The fingerprint chromatograms are shown in Fig. 1. Details of the fingerprint analysis are given in Table 1.

Electrocardiographic changes: The ECG changes in all the groups are summarized in Table 2. The doxorubicin administration significantly increases ST and QT interval while heart rate was significantly decreased as compared to control rats. The administration of GTE significantly restores ECG changes towards normalcy in a dose-dependent manner.

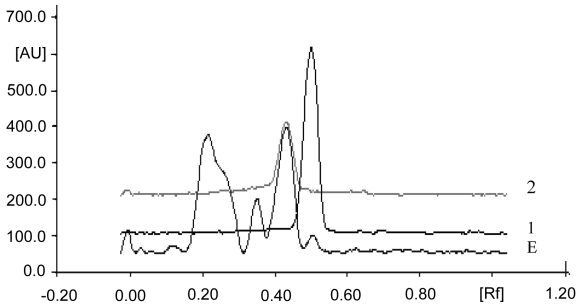

Fig. 1. TLC densitometric chromatogram of methanolic extract of Green tea with gallic acid standard and catechin standard solution. E: Extract, 1: gallic acid, 2: catechin standard solution.

Table 1. Details of fingerprint chromatograms of green tea extract after scanning at 254 nm

Extract	Solvent system	No. of spots
Methanolic extract	Toluene: Ethyl acetate: Formic acid (6 : 6 : 1).	8
Rf values	0.03, 0.12, 0.22, 0.35, 0.43, 0.50, 0.63, 0.68	
Relative %	3.30, 1.84, 33.03, 15.11, 35.09, 4.99, 1.27, 1.05	

Table 2. Effect of green tea extract (30 days) followed by acute administration of doxorubicin (10 mg/kg i.v.) on 30th day on ECG changes

Groups	ST interval (msec)	QT interval (msec)	Heart rate (bpm)
Group I	34.16 ± 2.71	70.83 ± 3.0	412.5 ± 14.32
Group II	65.83 ± 4.9***	95.83 ± 3.51***	252.33 ± 14.74***
Group III	47.5 ± 2.14**	83.33 ± 2.1**	278.5 ± 16.91 NS
Group IV	40.0 ± 1.82***	70.0 ± 2.88***	293.83 ± 15.01 NS
Group V	33.33 ± 2.78***	70.0 ± 3.16***	329.16 ± 18.72*
F value	18.98	14.88	14.98
P value	< 0.0001	< 0.0001	< 0.0001

Values are expressed as mean ± SEM. Group II was compared with Group I. Group III,IV and V were compared with Group II. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS = Non significant

Table 3. Effect of green tea extract (30 days) followed by acute administration of doxorubicin (10 mg/kg i.v.) on 30th day on the serum levels of lactate dehydrogenase, creatine kinase, and GOT

Groups	Lactate Dehydrogenage (U/L)	Creatine Kinase (U/L)	SGOT (U/ml)
Group I	169.83 ± 4.62	231.16 ± 12.68	32.33 ± 2.0
Group II	525.5 ± 10.63 ***	456.83 ± 43.71***	154.18 ± 9.68***
Group III	412.83 ± 32.53 **	425.83 ± 32.28NS	71.98 ± 8.47***
Group IV	384.5 ± 11.94***	311.66 ± 25.86*	56.1 ± 7.47***
Group V	278.0 ± 20.85 ***	269.5 ±16.04***	46.09 ± 9.91***
F value	51.88	11.94	35.72
P value	<0.0001	<0.0001	< 0.0001

Values are expressed as mean ± SEM. Group II was compared with Group I. Group III, IV and V compared with Group II. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS = Non significant

Biochemical parameters

Serum markers: The levels of serum marker enzymes in all the groups are given in Table 3. Doxorubicin administration significantly increases serum level of CK, LDH and GOT as compared to control rats. The administration of GTE significantly restores marker levels towards normalcy in a dose-dependent manner.

Biomarkers of the oxidative stress: The levels of biomarkers of oxidative stress enzymes in all the groups are presented in Table 4. Doxorubicin administration significantly increases LP while there was significant decrease in GSH, SOD and CAT levels as compared to control rats. The administration of GTE significantly improves GSH, SOD and CAT levels after doxorubicin administration while LP level changes towards normalcy in a dose-dependent manner.

Membrane bound enzymes: Doxorubicin damages cell membrane as evident from significant decrease in levels of membrane bound enzymes like $Na^{+}K^{+}$ ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase as compared to control. GTE fails to prevent damage at lower doses while significant improvement was observed at 100 mg/kg dose (Table 5).

DISCUSSION

The results indicate that doxorubicin induces pathological changes in both the ECG and biochemical markers indicative of cardiotoxicity, predominantly due to an increase in free radical production. These results were consistent with earlier studies (Neri *et al.*, 1997; Deatley *et al.*, 1999; Gewirtz, 1999). The results further suggest that administration of GTE improved the ECG and biochemical marker levels indicating decrease in

Table 4. Effect of green tea extract (30 days) followed by acute administration of doxorubicin (10 mg/kg i.v.) on 30th day on the levels of lipid peroxidation (MDA content) , reduced glutathione, superoxide dismutase and catalase in heart of rat

Groups	Lipid Peroxidation (nmoles of MDA/mg protein)	Reduced Glutathione (µg of GSH/mg protein)	Superoxide Dismutase (Units/mg protein)	Catalase (µmoles of H_2O_2 consumed/min/mg protein)
Group I	3.06 ± 0.16	9.45 ± 1.21	2.33 ± 0.36	4.02 ± 0.32
Group II	4.61 ± 0.09***	4.17 ± 0.28***	0.53 ± 0.09***	1.82 ± 0.09***
Group III	3.57 ± 0.17***	5.94 ± 0.5NS	1.23 ± 0.1 NS	1.96 ± 0.15 NS
Group IV	3.49 ± 0.05***	6.73 ± 0.15*	1.38 ± 0.11NS	2.95 ± 0.11**
Group V	3.07 ± 0.09***	7.34 ± 0.17**	2.07 ± 0.33***	4.0 ± 0.17***
F value	25.82	9.95	9.26	30.54
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III, IV and V were compared with Group II. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS = Non significant.

Table 5. Effect of green tea extract (30 days) followed by acute administration of doxorubicin (10 mg/kg i.v.) on 30th day on the levels of Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in heart of rat

Groups	Na^+K^+ATPase (µmoles of inorganic phosphrous liberated/min/mg protein	Ca^{2+}ATPase (µmoles of inorganic phosphrous liberated/min/mg protein	Mg^{2+}ATPase (µmoles of inorganic phosphrous liberated/min/mg protein
Group I	7.0 ± 0.2	3.86 ± 0.17	3.01 ± 0.17
Group II	4.04 ± 0.45**	2.09 ± 0.17***	2.04 ± 0.22*
Group III	4.23 ± 0.4 NS	2.06 ± 0.19 NS	2.39 ± 0.21 NS
Group IV	4.94 ± 0.37 NS	2.47 ± 0.12 NS	3.01 ± 0.23*
Group V	5.68 ± 0.71 NS	3.15 ± 0.13***	3.26 ± 0.21**
F value	6.87	22.49	5.68
P value	< 0.0007	< 0.0001	< 0.0021

Values are expressed as mean ± SEM. Group II was compared with Group I. Group III, IV and V were compared with Group II. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS = Non significant

oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of LP. The restoration of membrane bound enzymes like Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in GTE treated rats suggests a membrane-stabilizing protective effect of GTE. These protective effects were also supported by the restoration of serum marker enzymes.

It has been reported that catechins are important constituents of green tea that are responsible for much of the antioxidant and protective effects. We verified the catechin content of the extract using ethyl acetate as medium and found that it contains 35% catechins. In one of the study involving doxorubicin-induced fatty acid composition modification in cardiomyocytes, it was revealed that only one of the GTEs which was rich in catechin contents was able to counteract the detrimental changes and elevation of conjugated dienes (Hrelia *et al.*, 2002). It seems that antioxidant agents can protect the heart from doxorubicin-induced injury as confirmed by several studies (Quiles *et al.*, 2000). Further, it is also reported that GTE exhibits more potent antioxidant activity than other conventional antioxidants (such as vitamin E and C). At the same time, GTE also shows anti cancer action (Ahmad and Mukhtar, 1999). Thus, GTE could be a better option for ameliorating doxorubicin-induced pathological changes. We conclude that the GTE was able to prevent the electrocardiographic abnormalities and

pathological changes in biochemical markers, which were otherwise induced by doxorubicin. This protection may be due to the catechin content of GTE, which is found be a more potent antioxidant than many counterparts (Ahmad and Mukhtar, 1999).

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