

The role of catechin against doxorubicin – induced cardiotoxicity in Ehrlich Ascites Carcinoma Cells (EAC) bearing mice

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Abstract : Doxorubicin (Dox) is a chemotherapy drug used for treatment of wide variety of cancers. It known that, Dox may cause cardiotoxicity by producing free radicals and oxidative stress along the period of treatment. Catechin is considered one of the flavonoids which has powerful antioxidant properties and free radicals scavenger. The present work was designed to investigate the protective role of catechin on doxorubicin – induced cardiotoxicity in Ehrlich Ascites Carcinoma (EAC) bearing- mice and to test whether catechin has an effect on the antitumor properties of the Dox. Mice were divided into five groups as follows: (G1): Control group, (G2) Mice were injected with Ehrlich Ascites Carcinoma (EAC) cells (2.5×10^6 EAC/ml) to form a solid tumor , (G3) Mice were inoculated with (2.5×10^6 EAC/ml) and injected (i.p.) with Doxorubicin (15 mg/kg), (G4) Mice were inoculated with (EAC) at the same dose and were injected (i.p.) with (200mg/kg) Catechin , Group5 (G5) Mice were injected (i.p.) with Doxorubicin 15mg/kg of and 200mg/kg of Catechin in addition to the inoculation with EAC (2.5×10^6 EAC/ml). Dox (15mg/kg) and /or Catechin (200mg/kg) were administrated after 10 days in EAC bearing- mice through a period of 2 weeks in six equal injections. Results showed that, EAC -bearing mice treated with Dox plus Catechin recorded decrease in the mean tumor weight and significant increase in the cumulative mean survival time as compared to the other treated groups. Biochemical studies of EAC inoculation showed decline in serum total protein and lactate dehydrogenase activities, while serum total lipid has significantly increased. The treatment of tumor-bearing mice with Dox plus Catechin (G5) improved these levels. Significant increase in cardiac lipid peroxidation and glutathione contents for both tumor-bearing mice (G2) and doxorubicin groups (G3) were recorded. Combined treatment of Dox and Catechin (G5) caused amelioration in these contents. Glutathione peroxidase and superoxide dismutase activities showed highly significant increase in all treated groups. Administration of Dox plus Catechin (G5) modulate these activities. In conclusion, the present study suggested that Catechin treatment may significantly reduce cardiotoxicity induced by doxorubicin in Ehrlich Carcinoma - bearing mice by the induction of the cardiac antioxidant enzymes and blocking lipid peroxidation. Also, Catechin enhances the antitumor properties of doxorubicin by increasing its inhibitory effect on tumor growth. [Journal of American Science 2010;6(4):146-152]. (ISSN: 1545-1003).

Keywords : Ehrlich Ascites Carcinoma (EAC) ; Doxorubicin ; Catechin ; Antioxidant enzymes ; Lipid peroxidation ; Heart ; Mice.

1. Introduction

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives every year (Abdullaev et al., 2000). An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or combination) to block the development of cancer in humans (Gupta et al., 2004). Anthracyclines constitute a major class of cytotoxic agents for the treatment of cancer. Among these, doxorubicin possesses a broad spectrum of antitumor activity, exhibits a wide activity against leukemia's breast, lung, thyroid and ovarian carcinomas (Lonnie 2002 and Aleisa et al., 2007).

Doxorubicin caused cardiotoxic effect in animals (Kalender et al., 2002) and in patients (Bast et al., 2007). It is widely accepted that oxidative stress and the production of free radicals are involved in doxorubicin both in terms of antitumor effects and cardiotoxicity (Singal et al., 2000). Olson et al. (1988) suggested that this toxicity could involve

doxorubicinol, the primary circulating metabolite of doxorubicin. Doxorubicinol could accumulate in the heart and contribute significantly to the chronic cumulative cardiotoxicity of doxorubicin therapy. Hence, different treatment schedules were used to decrease drug levels in plasma and myocardium and it has also been proposed free radical scavengers and flavonoids might be effective in lessening the pathological changes observed after anthracycline treatment (Kalender et al., 2002). Antioxidants protect cells and tissues against free radical which caused oxidative damage and injury (Nakagawa and Yokozawa et al., 2002). Green tea's antioxidant effects seem to be dependent upon the polyphenol (Catechin) fraction (Kumamoto, 1998). Several epidemiological and in vitro studies suggest that Catechins have beneficial effects on human health, serving to protect against congestive heart failure, cancer, myoglobinuric acute renal failure, reduce the incidence of myocardial ischemia and the risk of ischemic heart disease

mortality, due to their antioxidant activities (Du and Lou 2008 ; Korish and Arafah 2008).

So, the present work was designed to investigate the protective role of Catechin against doxorubicin – induced cardiotoxicity in Ehrlich carcinoma cells (EAC) bearing- female mice and also to test whether Catechin has an effect on the antitumor properties of doxorubicin.

2. Materials and Methods

2.1 Tumor cell line:

Because Ehrlich Ascites Carcinoma cells were reported to show greater initial growth and total cell count in female mice than male mice (Vincent and Nicholls, 1967), the present study used female mice as experimental animals. The initial inoculation of Ehrlich Ascites Carcinoma (EAC) cells was kindly provided by the National Cancer Institute (Cairo University, Egypt). The tumor line was maintained in female mice by weekly intra-peritoneal injection of 2.5×10^6 cells/mouse according to the method recommended by the Egyptian National Cancer Institute, Cairo University. Such developed tumor is characterized by its moderate rapid growth which could not kill the animal due to the accumulation of ascites before about 14 days after transplantation. Cells were counted before injection using the bright line haemocytometer and dilutions made by physiological saline. The desired number of cells was injected in a volume of 0.5 ml/mouse. Solid Ehrlich carcinoma was induced by inoculation of 2.5×10^6 cells in the back between the thighs of each animal (Fahim et al., 1997).

2.2 Animals:

Adult female Swiss albino mice, weighing 22-25 gm were obtained from the animal house of Theodore Bilharis Institute (Cairo, Egypt). The local committee approved the design of the experiments, and the protocol conforms to the guidelines of the National Institutes of Health (NIH). The animals were maintained on a standard pellet diet and tap water *ad libitum*.

2.3 Chemical compounds:

Doxorubicin used in the present work was manufactured by Pharmacia Italia S.P.A., with molecular weight 543.5262 and chemical formula: $C_{27}H_{29}NO_{11}$. CAS Number is 29042-30-6. Doxorubicin was administered intraperitoneally in six equal injections (each containing 2.5 mg/kg) to animals over a period of 2 weeks for accumulative dose of 15 mg/kg body wt. according to (Timao and Singal, 2000). All mice were treated for two weeks with doxorubicin after bearing 9-day –old solid Ehrlich carcinoma.

(+)-Catechin was purchased from Fluka Chemicals (Ronkonkama, NY). Catechin chemical formula is $C_{15}H_{14}O_6$ with molecular weight 290.272 and CAS Number 7295-85-4. Catechin is soluble in water and was administered at dose (200 mg/kg body wt) in mice in six intraperitoneal (i.p.) injections alone

or 30 min after doxorubicin (2.5 mg/kg body wt) administration according to (Kalender et al., 2005).

2.4 Groups of Experimental Animals:

A total number of 125 female Swiss albino mice were used in the experimental groups. Female mice used in the present work according to many previous studies (Ali, 2005 and Fouda, 2005). Animals were divided into five groups, each containing 25 mice as follows: **Group1:** Control group (G1): Mice of this group maintained on a standard pellet diet and water applied *ad libitum* were used as a normal or negative control group. **Group2:** Ehrlich tumor group (G2): Mice of this group were inoculated in the back between the thighs of each animal with Ehrlich Ascites Carcinoma (EAC) cells. Each mouse was injected subcutaneously with 2.5×10^6 EAC/ml to form a solid tumor (Fahim et al., 1997) this group known as tumor or positive control group. **Group3:** Tumor and Doxorubicin treated group (G3): Mice of this group were inoculated with 2.5×10^6 of (EAC) in the back between the thighs of each animal and injected (i.p.) with Doxorubicin (15 mg/kg). **Group4:** Tumor and catechin treated group (G4): Mice of this group were inoculated with Ehrlich Ascites Carcinoma (EAC) in the back between the thighs of each animal with the above dose. Also these mice were injected intraperitoneally (i.p.) with (200mg/kg) catechin according to (Kalender et al., 2005). **Group5:** Tumor, doxorubicin and catechin treated group (G5): Mice of this group were injected (i.p.) with doxorubicin 15mg/kg of and 200mg/kg of catechin additional to the inoculation of EAC in the back between the thighs of each animal.

2.5 Anti-tumor studies

The anti-tumor activities of doxorubicin and/or catechin were assessed on 168 mice bearing solid Ehrlich Carcinoma that were classified into 4 equally sized groups (Gs.2-5) each group containing 42 mice. Ten days after inoculating the EAC cells (when the tumor became papale), the animals in Gs (3-5) were i.p. injected with the Doxorubicin (15 mg/kg) in six equal injections (each containing 2.5 mg/kg) over a period of 2 weeks and/or Catechin (200 mg/kg) was administered in mice in six injections (i.p.) 30 min after Doxorubicin administration, then left untreated until the end of the experiment. The animals in G (2) received no treatment and served as a control group. The weight of the solid tumor was determined by killed 3 animals every week and the mean weight of the tumors, the cumulative mean survival time (CMST) of the animals and the tumor growth inhibition ratio (T/C %) were recorded according to Fahim et al. (2003). Where: The cumulative mean survival time (CMST) is the days which animals were leaved, Increase of life span (ILS %) = Mean survival time of test / Mean survival time of control $\times 100$ and the tumor growth inhibition ratio (T/C %) = Mean

tumor weight of control - Mean tumor weight of test / Mean tumor weight of control $\times 100$.

2.6 Blood sampling:

At the end of the experimental period, mice were over night fasted, anaesthetized under diethyl ether (Sigma Co. USA) and blood was collected by cardiac puncture where blood was collected in dry clean tubes. The blood percolates along the wall of the centrifuge tube to prevent risk of hemolysis. The blood tubes were left 30 minutes then centrifuged at 3000 rpm for 30 minutes. After centrifugation the serum was separated at once, divided into aliquots and stored at -70°C until used in the biochemical analysis.

2.7. Biochemical measurement:

2.7.1. Serum analysis:

Total protein in serum was determined by the method of (Doumas, 1975). Using a commercial kit purchased from Stanbio chemicals (USA) through Gamma Trade Company. Total lipids in serum were determined by the method of Zollner (1962) using a commercial kit purchased from Bio-diagnostic Company (Egypt). LDH kit for the quantitative determination of Lactate Dehydrogenase in serum was determined by the method of Kachmar (1976). This kit was purchased from Stanbio chemicals (USA) through Gamma Trade Company.

2.7.2. Tissue analysis:

Heart were excised immediately from animals (mice) after dissection and carefully washed with cold saline, dried and weighed. Fresh heart usually 0.1gm was used for tissues analysis. Estimation of lipid peroxidation (MDA) carries out according to the method of Ohkawa et al. (1979). This method is based on the measurement of malondialdehyde (MDA) as one of the main end products of lipid peroxidation by the thiobarbituric acid test. Determination of glutathione (GSH) content carries out according to the method of Beutler et al. (1963). The method upon determination of a yellow color that developed 5,5dithiol-bis (2-nitrobenzoic acid) (DTNB) is added to sulfhydryl compounds. Glutathione peroxidase (GSH-PX) was measured colorimetrically according to the method of Gross et al. (1967). Estimation of super oxide dismutase (SOD) carries out according to the method of Minami and Yoshikawa (1979). This method is based on the generation of superoxide anions by pyrogallol autoxidation.

2.8 Statistical analysis:

Data were analyzed using Students "t" test according to the method of Hin and Wetherill (1975). Analysis of variance (ANOVA) and Fisher's significant difference test (Winer, 1971) were also

used. P values of 0.05 or less were considered statistically significant.

3. Results

3.1 Anti-tumor studies:

Treatment of tumor-bearing animals with the doxorubicin (G3) and doxorubicin plus catechin (G5) recorded an increase in the life span (ILS %), which reached to 112.5% and 131.25%, respectively, compared to tumor-bearing controls (G2). On day 60, at which all animals of both (G 2) and (G 4) were dead, no marked difference in the mean tumor weight were recorded ($8.21 \pm 0.57g$) and ($8.91 \pm 0.68g$) respectively, (Table 1). These results were markedly reduced to ($5.12 \pm 0.29g$) in G (3) and ($5.28 \pm 0.38g$) in G (5). Also, the most elevation of the tumor growth inhibition ratio (T/C %) was recorded in the doxorubicin treated group (G3) as compared to G (4) and G (5).

3.2 Biochemical studies:

The sera levels of total protein in the groups of (2), (3) and (4) were significantly decreased ($P < 0.001$) compared to the normal control group (Table 2). On contrast, G 5 showed no difference ($P > 0.05$) compared to G1. Tumor bearing mice (G2) and catechin treated (G4) exhibited a very highly significant increase of serum total lipid concentration, the mean total lipid were recorded (595.38 ± 15.37 mg/l) and (591.32 ± 10.06 mg/l), respectively as compared to G1 (159.73 ± 4.55 mg/l). While G3 and G5 showed a highly significant improvement in serum total lipid level as compared to G2 recording (366.11 ± 10.35 mg/l) and (170.11 ± 12.28 mg/l), respectively. A very highly significant decrease of serum lactate dehydrogenase was observed in G2 (460.47 ± 12.99 U/L) in relation to G1 (770.61 ± 10.45 U/L). The treatment of tumor bearing mice with doxorubicin (G3) or catechin (G4) recorded a very highly significant elevation of serum LDH as compared to G1 and G2. The cardiac MDA levels of all groups except G5 showed marked elevation. A very highly significant increase in the GSH level was observed in G2 (28.42 ± 2.01 mg/g tissue) compared to G1 (21.73 ± 1.26 mg/g tissue). On the other hand, the rest of other groups showed no statistical difference when compared to G1. Cardiac GPx activity was significantly increased in G2, G3 and G4 where recorded (1072.64 ± 27.62 U/mg), (923.98 ± 23.79 U/mg) and (945.74 ± 16.50 U/mg) in compared to G1 (583.92 ± 16.00), while the GPx level in G5 showed no statistical difference (617.59 ± 20.43 U/mg). The SOD activity of all groups recoded a highly significant increased. This elevation was strongly observed in G3, where it recorded (1.96 ± 0.13 U/g) when compared to G1.

Table (1): Anti-tumor activities of Doxorubicin and/or catechin on Ehrlich carcinoma compared to tumor-bearing controls.

PTI (days)	G2		G3		G4		G5	
	M	MTW±SE	M	MTW±SE	M	MTW±SE	M	MTW±SE
10	0/8	1.10±0.11	0/8	1.12±0.21	0/8	1.20±0.13	0/8	1.11±0.12
17	0/8	2.60±0.26	0/8	1.49±0.15	0/8	2.52±0.28	0/8	1.44±0.16
24	0/8	4.23±0.35	0/8	1.41±0.42	0/8	4.32±0.18	0/8	1.38±0.18
30	0/8	4.54±0.28	0/8	1.71±0.26	0/8	4.61±0.34	0/8	1.72±0.26
36	0/8	5.21±0.45	0/8	2.97±0.17	0/8	5.42±0.31	0/8	2.88±0.43
42	1/8	5.98±0.51	0/8	3.30±0.25	0/8	6.13±0.62	0/8	3.48±0.68
48	2/8	6.52±0.38	1/8	3.84±0.36	3/8	6.82±0.32	0/8	3.93±0.34
54	4/8	7.82±0.44	2/8	4.53±0.18	4/8	7.93±0.71	0/8	4.89±0.29
60	8/8	8.21±0.57	6/8	5.12±0.29	8/8	8.91±0.68	2/8	5.28±0.38
66			8/8	5.68±0.81			5/8	6.24±0.52
72							8/8	6.53±0.67
MTW	5.13		3.12		5.32		3.53	
MST days	48		54		46.50		63	
ILS%			112.50		96.88		131.25	
T/C%			39.4		3.58		31.19	

PTI = Post tumor inoculation.

M = Mortality.

MTW = Mean tumor weight (g)

MST (days) = mean survival time.

ILS= Increase of life span %

T/C= Tumor inhibition ratio

Table 2: The effect of doxorubicin and/or Catechin on heart tissue parameters in Ehrlich carcinoma bearing mice

Groups	Total protein g/dl	Total lipid mg/L	LDH U/L	MDA n mol/gm	GSH mg/g	GPx U/mg	SOD U/g
G1	7.30±0.08	159.73±4.55	770.61±10.45	3.25±0.17	21.73±1.26	583.92±16.00	1.07±0.05
G2	5.68±0.21 -22.19%	595.38±15.3 272.73%	460.47±12.99 -40.18%	5.48±0.22 68.63%	28.42±2.01 30.80%	1072.64±27.62 83.69%	1.50±0.08 40.17%
	a ***	a ***	a ***	a ***	a **	a ***	a ***
G3	6.63±0.13 -9.18%	366.11±10.35 129.20%	975.71±31.04 26.61%	8.05±0.25 147.52%	24.87±1.60 14.45%	923.98±23.79 58.24%	1.96±0.13 83.33%
	a ***	a ***	a ***	a ***	N.S.	a ***	a ***
G4	5.55±0.13 -23.97%	591.32±10.06 270.19%	1219.60±16.31 58.26%	5.28±0.15 62.44%	26.01±2.01 19.72%	945.74±16.50 61.97%	1.47±0.07 38.02%
	a ***	a ***	a ***	a ***	N.S.	a ***	a ***
G5	7.23±0.07 -0.96%	170.11±12.28 6.49%	891.69±21.43 15.71%	3.00±0.18 -7.60%	24.25±1.60 11.59%	617.59±20.43 5.77%	1.56±0.15 45.79%
	N.S.	N.S.	a ***	N.S.	N.S.	a ***	a ***
ANOVA F(4.45)	P<0.001 39.15	P<0.001 372.72	P<0.001 197.53	P<0.001 106.10	P<0.001 2.03	P<0.001 103.69	P<0.001 9.80

Values are presented as: mean±S.E.

% = Percentage of change from normal control (G1)

a *** = highly significant (P<0.001) from control (G1)

a ** = highly significant (P<0.01) from control (G1)

N.S. Non significant

4. Discussion

4.1. Anti-tumor studies:

In the present study, treatment of tumor-bearing mice with doxorubicin only and with both doxorubicin plus catechin exerted a marked effect in the retardation of tumor growth. This is demonstrated by marked increase in the percentage of survivors, the cumulative mean survival time on day 40 and the marked decrease in the mean tumor weight as compared to tumor-bearing mice group. In addition, the present data indicate that the combined treatment of doxorubicin and catechin caused a remarkable increase in mean survival time (63 days) as compared to single doxorubicin treatment (54 days). It merits note that a more pronounced effect was observed in the combined treatment represented by higher increases in the life span of animals (131.25%) and the tumor inhibition ratio (31.19%), compared to tumor-bearing animals treated with doxorubicin which recorded (112.50% and 39.4%), respectively. These observations are consistent with the previous findings by Balasubashini et al. (2006) and Majumder et al. (2006). It has been shown by Gewirtz (1999) that the capacity of doxorubicin to inhibit DNA synthesis has been proposed as a mechanism of action of doxorubicin on cancer cells and may be also due to the effect of Catechin which increased concentration of doxorubicin in cancer cells and may have anticarcinogenic and antiproliferative properties (Ahmed and Mukhtar, 1999; Crespy and Williamson, 2004). Moreover, Sadzuka et al. (1998) found that green tea components, such as caffeine, theanine, (-)-epigallocatechin gallate (EGCG) and flavonoids have inhibitory effects on the doxorubicin efflux from Ehrlich ascites carcinoma cells. Thus, it is suggested that EGCG and flavonoids may enhance DOX induced antitumor activity and increase the DOX concentrations in tumors through the inhibition of DOX efflux. However, the present findings are in line with the main concept of cancer research that evaluation of any tested substance depends on extension of the survival time of cancer patients and that an increase in the life span of drug-treated tumor bearing mice $\geq 125\%$ is considered indicative of presumptive drug activity (Fahim et al., 2003).

4.2 Biochemical studies:

The inhibitory effect of tumor inoculation on protein content may be explained based on that the tumor cells tend to concentrate amino acids for its protein synthesis and growth while normal host suffered from this inhibition (Wiseman and Ghadially, 1958). The inhibition in total protein content in doxorubicin treated group may be due to the oxidative stress and reduction of protein synthesis in the tissue (Swift et al., 2002). In doxorubicin and Catechin treated group, protein level back to normal as a result of the antioxidant properties of Catechin and its ability to scavenge ROS induced by doxorubicin

administration (Kalender, 2005). The increments in total lipid level of tumor group and doxorubicin treated group may be due to the deamination of protein in tissue, reduction in ATP production due to swelling and vacuolization of mitochondria and disturbance in glutathione synthesis using NADPH as a cofactor (Naidu et al., 2002). In doxorubicin plus Catechin treatment total lipid level back to normal because that Catechin increase the glutathione-S-transferase and decrease glucose-6-phosphate enzyme which consider to be important coenzyme in bio-synthesis of fatty acids. Also, Catechin inhibits the absorption of cholesterol and promotes cholesterol excretion (Miura et al., 1994). LDH activity was inhibited in tumor group due to increase in inflammatory cells which caused reduction in protein level (Saad-Hossne et al., 2003). The increase in cardiac enzyme in doxorubicin treated group could to an increase in its release following doxorubicin-induced lipid peroxidation in cardiac membranes (Deepa and Varalakshmi, 2003). Treatment with doxorubicin plus Catechin caused improvement in LDH activity which related to the free radical scavenging activity, so Catechin protect cardiac membrane from free radicals and decrease lipid peroxidation level, as a result of that LDH prevented from releasing outside the cell (Schmidt *et al.*, 2005). The significant increase in cardiac MDA in these groups may be due to that cancer cells induced excessive production of free radicals leading to damage lipids and can induced lipid peroxidation (Fouda, 2005) This elevation in MDA level in doxorubicin treated group related to the metabolism of doxorubicin which lead to the release of iron ions that react with cellular components specially the poly unsaturated fatty acids in cell membrane and increase the MDA level (Kalender *et al.*, 2002 and 2005). The significant decrease in GSH level in tumor group may be due to lack of amino acids which used in making of GSH (Deepa and Varalakshmi, 2003). The inhibition of cardiac GSH level in the doxorubicin group compared to tumor bearing group explicable by an increased rate of transformation of GSH to oxidized glutathione (GSSG) as a result of GSH consumption to get ride of H₂O₂ (Fouda, 2005). The significant elevation in cardiac GSH-PX and SOD in tumor group may be correlated to the oxidative stress on the host in response to the continual generation of free radicals by the increasing tumor load (Fahim *et al.* 2003). The level of GSH and GSH-Px and SOD activities back to normal in doxorubicin plus Catechin group due to normalization of lipid peroxidation and Catechin scavenges hydroxyl radicals which may initiate lipid peroxidation (Zhao et al., 2001 and Mai and Altucci 2009).

5. Conclusions

From the results of the present work can conclude that: Catechin supplementation might be significantly reducing doxorubicin- induced cardiotoxicity in Ehrlich ascites carcinoma cells- bearing mice. This protection

of Catechin was demonstrated by the induction of the antioxidant enzymes systems to increase the disposal of overproduction reactive free oxygen radicals after doxorubicin treatment and by blocking the lipid peroxidation in heart tissue. In addition to Catechin enhances the antitumor properties of doxorubicin by increase the inhibitory effect of doxorubicin on tumor growth.

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