Protocatechuic acid decreased telomerase reverse transcriptase (TERT) expression in DMBA-induced liver carcinogenesis mice model

Samar El-Shaheed¹⁾, Heba Sahyon²⁾, Magdy Youssef¹⁾ and Amr Negm^{1, 3)}

¹⁾ Biochemistry division, Chemistry Department, Faculty of Science, Mansoura University, Egypt

²⁾ Biochemistry division, Chemistry Department, Faculty of Science, Kafr Elsheikh University, Egypt

³⁾ Department of Chemistry, College of Science, King Faisal University, Al-Ahasa, Saudi Arabia

Abstract

Background: The protocatechuic acid (PCA) is a natural polyphenolic antioxidant commonly distributed in plants and is considered as one of the main ingredients of some traditional herbal medicine. PCA has many pharmacological effects that may be closely linked to its antioxidant activity. Herein, we newly conducted this animal experiment to evaluate the therapeutic potentials of PCA on the 7,12-Dimethylbenzanthracene (DMBA) -induced hepatocarcinogenesis in the mouse model. Additionally, the *in vitro* antioxidant activity of PCA was estimated.

Material and methods: To induce the hepatocarcinogenesis, DMBA (35 mg/kg, once per week) was orally administered for 4 weeks in the tumor test groups. After six weeks, the administration of PCA (100 mg/kg/day) was started for 4 weeks. Mice were sacrificed after ten weeks for measuring and comparing the serum hepatic enzymes level, antioxidant markers and telomerase reverse transcriptase (TERT) gene expression between the mice in DMBA with PCA (PCA group) and DMBA without PCA (non-PCA group). Also, the antioxidant activity of PCA was measured *in vitro* using two different antioxidant assays.

Results: The elevation of all liver enzymes and other hepatic biomarkers observed in DMBA group were significantly suppressed in PCA-treated group. In addition, the TERT gene expression in liver tissue was significantly decreased in PCA-treated group. Our results also showed that the PCA exhibited a high antioxidant ability *in vitro* comparable to the positive control ascorbic acid.

Conclusion: The protocatechuic acid decreased TERT expression and oxidative stress in DMBA-induced liver carcinogenesis mice model.

Keywords: Protocatechuic acid, liver carcinogenesis, antioxidant, telomerase reverse transcriptase.

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Introduction

The liver is the central organ that metabolize many potentially toxic chemicals, where they are concentrated and become activated¹⁾. Liver cancer is one of the most mortal tumors worldwide. Globally, it is the second leading cause of death in cancer related death cases²⁾.

7,12-Dimethylbenzanthracene, also known as DMBA, is a prominent genotoxic procarcinogen that is activated in the liver by detoxification enzymes to be transformed into an utmost carcinogen³⁾. DMBA exposure can cause several toxicological alterations in the liver, including oxidative damage via reactive oxygen species (ROS) release and formation of carcinogenic metabolites⁴⁾. Various studies have dealt with the toxic effect of DMBA on the liver⁵⁻⁷⁾.

Recently, herbal therapies have attracted huge attention due to their wide range of biological and therapeutic properties⁸⁾. The protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid), is a natural polyphenolic antioxidant that is found in fresh fruits, vegetables, and extra virgin olive oil as well as in processed food such as red and white grape wine and in herbal medicine^{9, 10)}. PCA has been demonstrated to have an effective anticancer activity against various cancer inducing-chemicals in different tissues, such as liver, which thought to be closely related to its antioxidant activity¹¹⁾.

Telomerase is a reverse transcriptase enzyme that synthesizes TTAGGG telomeric repeats on the ends of chromosomes to maintain the length of telomere¹²⁾. Most cancer cells have telomerase activity which supports their unlimited proliferation¹³⁾. Telomerase reverse transcriptase (TERT) is a catalytic subunit of telomerase, which, together with the telomerase RNA (TR), comprises the most important unit of the telomerase complex¹⁴⁾. It is recognized that the activity of telomerase is strongly

Correspondence to: Samar El-Shaheed, Biochemistry division, Chemistry Department, Faculty of Science, Mansoura University, Egypt. P.B. Box 33516, Tel: +201002427384; Email: samarhosny2014@yahoo.com

regulated by the level of TERT synthesis¹⁵. In the current study, our attention was directed to evaluate the *in vitro* antioxidant capacity of PCA. Additionally, we used DMBA-induced liver carcinogenesis mouse model to explore the telomerase inhibition by PCA.

Materials and methods

Allocation of intervention and control setting

Thirty-two mice were divided into 4 groups (8 animals each); normal control, PCA, DMBA, and DMBA with PCA group. Mice were given olive oil once/week for 4 weeks and daily normal saline for 4 weeks in normal control group. Mice received PCA at a dose of 100 mg/kg body weight (b.w.)/day by intraperitoneal injection until the end of the experiment in PCA group. Hepatocarcinogenesis was induced in DMBA group in which DMBA was orally administrated at a dose of 35 mg/kg b.w., dissolved in 100 μ l olive oil, once a week for four weeks as reported by the protocol of Mansour et al.¹⁶ with slight modifications. Mice received PCA (dissolved in normal saline) at a dose of 100 mg/kg b.w./day by intraperitoneal injection after six weeks of DMBA administration (starting of promotion stage) and continued for four weeks in DMBA with PCA group. The dose of PCA was selected based on the acute toxicity study achieved in our laboratory according to the Organization for Economic Co-operation and Development (OECD) (2008) guidelines¹⁷ and on the basis of other published report¹⁸. The body weights of all mice were estimated at the starting and end of the experiment.

Outcomes

Biomarkers related to liver function

Twenty-four hours after the last treatment, mice were sacrificed under ether anesthesia, blood was withdrawn directly by cardiac puncture and the sera were separated by centrifugation at 3000 rpm for 15 min. Serum alanine transaminase (ALT), aspartate transaminase (AST) and γ -glutamyl transferase (γ GT) were assayed by kinetic photometric methods using commercial kits. Total bilirubin (T.B) and albumin (Alb) were assayed by colorimetric methods using commercial kits. Alpha fetoprotein (AFP) level was estimated using ready-made Enzyme-linked immunosorbent assay kit (ELISA), according to the manufacturer's protocol. Liver from each mouse was immediately removed after dissection and weighed. The relative liver weight was also calculated.

Biomarkers related to hepatic antioxidant status

The liver tissues were precisely weighed, homogenized in cold phosphate buffer (pH 7.4). After centrifugation for 10 min. at 5,000 rpm at 4°C, the supernatants (homogenates) were utilized for antioxidant and malondialdehyde (MDA) determination. Tissue non-enzymatic reduced glutathione (GSH) was determined by using

5,5'-dithio 2-p-nitrobenzoic acid (DTNB) method as stated by Beutler et al.¹⁹. Superoxide dismutase (SOD) activity in liver homogenates was determined spectrophotometrically using commercial kit according to the method described by Nishikimi et al.²⁰. The activity of catalase (CAT) was determined spectrophotometrically using commercial kit according to the method reported by Aebi²¹ and Fossati et al.²². Determination of MDA in liver homogenates was carried out as previously described²³.

In-vitro free radical scavenging activity

The in-vitro free radical scavenging activity of PCA was examined by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity using ascorbic acid (Vitamin C) as a standard. The ABTS assay was performed following the reported procedure of Re et al.²⁴. DPPH assay was determined according to the method of Mavie et al.²⁵. The antioxidant capacity of PCA was expressed as percent inhibition of absorbance at 734 and 490 nm, respectively.

TERT gene expression

TERT gene expression was estimated by quantitative Real Time-Polymerase Chain Reaction (RT-PCR). Total ribonucleic acid (RNA) was isolated from liver tissues using RNeasy Mini Kit (Qiagen Inc., San Diego, USA) following the manufacturer's instructions. The synthesis of complementary-DNA (cDNA) was carried out using high capacity cDNA reverse transcription kit (ThermoFisher Scientific, Waltham, USA), following the manufacturer's instructions. Finally, the quantitative RT-PCR was performed using SYBR Green PCR Master Mix (ThermoFisher Scientific) on StepOnePlus RT-PCR System (ThermoFisher Scientific). The relative amount of RNA was calculated using the 2-Delta Delta CT method²⁶. The gene expression was normalized with Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and results were expressed as fold change related to normal group. The gene-specific primers used for PCR amplification are listed in Table 1.

Statistical analysis and ethical policy

The analysis of data was carried out by GraphPad Prism software (Version 6.0). The results are expressed as mean $\pm$ standard error mean (SEM). One-way analysis of variance (ANOVA) was used to determine the significance between different groups followed by Dunnett's test (multiple comparison versus control). A P-value of <0.05 was statistically significant. The ethical standard approval was taken by the institutional animal ethics committee of guidelines at Mansoura University, Egypt (cod no. Ph/chem. 19-00001), which are in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences.

Table 1 The primer sequences of the studied genes.

Gene		Sequence (5'-3')	Product length
TERT	Forward	5'- CGG AAG AGTGTGTC TGG AGC AA -3'	145
	Reverse	5'- GGA TGA AGC GGAGTC TGG A -3'	
GAPDH	Forward	5'- ACCACAGTCCATGCCATCAC -3'	452
	Reverse	5'- TCCACCACCCTGTTGCTGTA -3'	

Table 2 Changes in body weight (g) and relative liver weight/ 100 g b.w. in the control and different groups treated with PCA

Groups	Initial body weight	Final body weight	Relative liver weight (g%)
Normal control	23.38± 1.49	36.92±2.66	4.28±0.31
%		57.9%	
PCA	22.99± 2.03	36.45±2.61	4.48±0.29 ^a
%		59.17%	
DMBA	26.43± 2.8	29.29±1.47	6.38±0.66 [*]
%		10.8%	
DMBA + PCA	23.66± 1.4	36.83±3.89	4.55±0.31 [#]
%		55.7%	

Data represents the means ± SEM in each group.

% of change from initial body weight.

* Significantly different from normal group. # Significantly different from DMBA group. ^a not significant against control group.

Table 3 Effect of PCA on serum liver function and AFP level in different experimental groups

Groups	ALT (U/L)	AST (U/L)	GGT (U/L)	Alb (g/dl)	T.B(mg/dl)	AFP (pg/ml)
Normal	35.6±1.85	105.7±2.6	4.9±0.4	3.60±0.1	0.45±0.05	34.7± 2.5
PCA	38.5 ± 1.5 ^a	108.2±7.06 ^a	4.5±1.1 ^a	3.5±0.12 ^a	0.5±0.04 ^a	34.2± 1.5 ^a
DMBA	135.2 ±6.5 [*]	262.6±24.8 [*]	33.5±1.6 [*]	2.6±0.2 [*]	0.9±0.3 [*]	109.8±15.8 [*]
DMBA + PCA	47.08±1 [#]	136.3±2.8 [#]	10.4±0.4 [#]	3.32±0.1 [#]	0.52±0.06 [#]	55.3± 3.6 [#]
%	-65%	-48%	-69%	30%	-43%	-50%

Data represents the means ± SEM in each group.

% of change from carcinogen group.

* Significantly different from normal group. # Significantly different from DMBA group. ^a not significant against control group.

Drugs, measuring kits and housing of animals

PCA was obtained from, Xi'an Nate Biological Technology Co. (Wuhan, China). ABTS, DPPH, DTNB and TBA were obtained from Sigma-Aldrich Inc. (Saint Louis, USA). ALT and AST diagnostic kits were obtained from HUMAN Company (Cairo, Egypt). γGT was assayed by kinetic photometric method using kit supplied by MyBiosource, Inc. (San Diego, USA). SOD and CAT were purchased from Biodiagnostic Co. (Cairo, Egypt). AFP ELISA kit was purchased from CUSABIO Inc. (Wuhan, China).

Thirty-two adult male swiss albino mice (*Mus musculus*) weighing from 22 to 26 g, were used and housed in plastic cages. They were kept in 12/12 h light/dark cycle at 22 ± 2°C with free access to water and standard CHOW diet *ad libitum*. They were kept under observation for one week prior to starting the experiment.

Results

Clinical findings

The initial and final body weights and the relative liver weights (g liver/100 g b.w.) of the different groups are listed in Table 2. A slight elevation in final body weight was noticed in DMBA-administrated group with

10.8% change in comparison to the initial weight. The treatment of the DMBA group with PCA increased the final body weight with 55.7% change in comparison to the initial weight. The relative liver weight in DMBA group was markedly increased ($p<0.001$) to record 6.38 ± 0.66 when compared to normal control group. At the meantime, the treatment of DMBA group with PCA significantly reduced ($p<0.001$) the relative liver weight to record 4.55 ± 0.31 in comparison with DMBA administrated group but it was not significantly affected in the PCA normal treated group.

Acute toxicity

It was observed that PCA was safe up to a dose level of 2000 mg/kg. No Mortality and toxicity were induced at this level in all mice during 14-day observation period. One-tenth of the maximum dose (100 mg/ kg b.w.) of the PCA was selected for evaluation of anticancer potential.

Liver function

Results in Table 3 showed that the DMBA group exhibited a significant increase ($p<0.001$) in the activities of ALT, AST, γGT, and T. B level with a decrease in albumin level in comparison with that of normal group. The treatment with PCA after DMBA administration

Fig. 1 DPPH and ABTS radical scavenging activity of PCA

Fig. 3 The TERT expression on the liver

The relative gene expression of normal control group was defined as 1. Data represents the means $\pm$ SEM in each group. * Significantly different from control group. # Significantly different from DMBA group. ^anot significant against control group.

exhibited a significant decline in serum activities of ALT, AST, γ GT, and T. B level, with an increase in albumin level in comparison to DMBA group, but it was not significantly affected in the PCA normal treated group.

Serum level of AFP in control and treated groups was also displayed in Table 3. A significant elevation in AFP level was clearly observed in DMBA-administrated group compared to control normal group ($p < 0.001$). Meanwhile, there was a significant depletion in AFP level after administration of PCA in the DMBA group when compared to model group. Administration of PCA to normal mice resulted in a non-significant change in

Fig. 2 Antioxidant marker and MDA on the liver

(a) antioxidant markers, (b) MDA in normal and DMBA-induced liver cancer in mice. Data represents the means $\pm$ SEM in each group. * Significantly different from control group. # Significantly different from DMBA group. ^anot significant against control group.

serum AFP level in comparison to normal group.

In-vitro free radical scavenging activity and hepatic antioxidant status

The *in vitro* DPPH and ABTS-scavenging capacities of PCA were 88.1 and 118.45% at the tested concentration (1 mg/ml), respectively. Furthermore, the ascorbic acid (Vitamin C) equivalent antioxidant capacities of the PCA were 73.7 and 117% in DPPH and ABTS assays, respectively (Fig. 1). The hepatic antioxidant status in animal experiments was displayed in Fig. 2. A significant reduction in the activities of SOD and CAT along with GSH depletion was clearly noticed in DMBA-

administrated group in comparison to control healthy group ($p < 0.001$). At the same time, a prominent elevation of the MDA level ($p < 0.001$) was also noticed in the same group in comparison to normal treated group. Conversely, the treatment with PCA after DMBA administration significantly increased the antioxidant enzymes activities and cellular content of GSH with a significant decrease in MDA level when compared to carcinogen group. Administration of PCA to normal mice resulted in a non-significant change in liver antioxidant status in comparison with the normal control group.

TERT gene expression

The relative gene expression of TERT in liver tissues of the different groups was shown in Fig. 3. Profound up-regulation was recorded in TERT gene expression in the DMBA group in comparison to the normal control group. In contrast, administration of PCA to DMBA group effectively ($p < 0.001$) decreased TERT gene expression, by -75%, as compared to carcinogen group.

Discussion

Our results demonstrated that the PCA possesses antioxidant properties and it could serve as free radical inhibitors, scavenger or acting possibly as primary antioxidants. In addition, the PCA also protects liver carcinogenesis by inhibiting the antioxidant cascade and TERT expression in a mouse model administrated DMBA.

In the current study, a detectable decrease in body weight and an elevation in relative liver weight were noticed in DMBA group in comparison to normal group. This phenomena is well correlated with the proliferation of the hepatic cells upon DMBA administration and this agreed with an earlier study²⁷. The PCA treatment markedly increased body weight and decreased the mean relative liver weight in comparison to the DMBA group and these results may contribute more to effective antitumor action. Also, the current study recorded an elevation in the activities of hepatic marker enzymes; ALT, AST, γ GT and T. bilirubin level with a decrease in albumin level in the DMBA group with respect to that of control group. These results are reflecting impairment in the liver function which results in an increase in these enzymes leakage into the bloodstream²⁸. These results are consistence with those obtained previously²⁹. The increase in the activity of γ GT may be attributed to the progression of carcinogenesis, since its activity is correlated with the rate of tumor growth and differentiation³⁰.

However, the PCA-treatment significantly reversed the hepatic marker enzymes activities to their near-normal levels and this treatment finally attenuated DMBA-induced liver carcinogenesis. PCA may aid in liver cell regeneration, preserving membrane integrity, thereby suppressing the leakage of the enzymes³¹. Moreover, the PCA was reported to decrease the liver marker en-

zymes in lipopolysaccharide-induced rat hepatic damage via its anti-lipid peroxidative activity³². AFP is a tumor marker which can be developed by regenerating hepatic tumors³³. DMBA group displayed a marked increase in AFP level that can be related to the presence of premalignant liver modifications or genetic alterations that resulted from DMBA administration. The decreased level of AFP in DMBA-induced mice treated with PCA may reflect its anticancer potential in comparison with DMBA group.

The antioxidant enzymes are the first-line defense against oxidative damage and therefore offer protection against the harmful outcomes³⁴. In the current study, DMBA administration caused a marked elevation in MDA level due to excessive formation of free radicals. At the same time, there was a depletion in intracellular GSH content, associated with a decrease in the antioxidant enzymes activity (SOD and CAT). Similar results have been reported³⁵. Meanwhile, the PCA treatment after DMBA administration significantly decreased the MDA level and increased the activities of SOD and CAT, and cellular GSH content as compared to DMBA group and these may be attributed to the powerful antioxidant activity and intrinsic free radical scavenging effects of PCA that inhibit the metabolic activated carcinogens or assist the excretion of carcinogenic metabolites of DMBA³⁶.

Our results indicated that, the PCA can effectively inhibit DPPH[•] and ABTS^{•+} *in vitro* that reflect its antioxidant effect. PCA may exert its antioxidant activity by scavenging free radicals through donation of hydrogen atom (H[•]) or electron (e⁻)³⁷.

The results of RT-PCR of TERT mRNA indicated that the TERT expression was up-regulated in DMBA administrated group. This TERT up-regulation is a biological feature of preneoplastic lesions that evolve to hepatocellular carcinoma (HCC) in mice liver³⁸. TERT expression induces the growth of tumor by attuning the growth controlling genes expression and accelerating proliferation of the cell³⁹.

The TERT gene expression was decreased in response to PCA treatment without significantly affecting the expression of GAPDH, suggesting that the suppression of TERT expression by PCA is partially responsible for its anticancer activity. Approximately 90% of human cancers exhibit telomerase activity reactivation⁴⁰, therefore, telomerase is an attractive target for developing novel anticancer therapeutics selectively targeting the telomerase.

In conclusion, we showed that the PCA possesses antioxidant properties. In addition, the PCA also protects hepatocytes from carcinogenesis by inhibiting the antioxidant cascade and TERT expression in a mouse model administrated DMBA. It might be possible for PCA to use as a natural antioxidant and antitumor agent.

Conflict of Interest:

The authors declare no conflict of interests.

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