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Subacute effect of cigarette smoke exposure in rats: Protection by pot marigold (*Calendula officinalis* L.) extract

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Abstract

This study was carried out to determine the preventive effect of *Calendula officinalis* L. (pot marigold) on rats exposed to cigarette smoke (CS). Rats were divided into three groups as control, CS and CS + pot marigold (PM). The rats in the CS and CS + PM groups were subjected to CS for 1 h twice a day for 23 days. PM (100 mg/kg body weight) was given to rats in the CS + PM group by gavage, 1 h before each administration period. While malondialdehyde, protein carbonyl contents and reduced glutathione level of the CS group increased, their levels diminished by PM administration. In addition, glutathione peroxidase (GPx), superoxide dismutase activities and β-carotene, vitamins A and C levels decreased in the CS group compared to control, however activities of these enzymes and concentration of vitamins were elevated by PM supplementation. This investigation showed that administration of PM supplied relative protection against subacute CS-induced cell injury.

Keywords

Cigarette smoke, pot marigold, oxidative stress, antioxidant, cell injury

Introduction

Cigarette smoke (CS), with its over 4700 different substances (Genbacev-Krtolica, 2005; Rahman and MacNee, 1996), most of which are described as toxic, mutagenic and carcinogenic (Czekaj et al., 2002; Florek et al., 1999), is involved in the development of various chronic pulmonary, cerebrovascular and cardiovascular diseases, cancers and several others (Diana, 1993; Kyerematen and Vesell, 1991; Pryor, 1997). Harmful influences of CS can be mainly associated with its ingredients like nicotine, cadmium, benzopyrene, oxidants, acetone, mercury and inducers of reactive oxygen species (ROS) like nitric oxide (NO), nitrogen dioxide (NO_2), peroxynitrite and nitrosamines.

The number of ROS, especially O_2^-, hydroxyl radical. (OH) and hydrogen peroxide (H_2O_2) increases after CS exposure in cells (Rahman, 2005). Reactive oxygen species have a considerable role in smoking-related diseases (Church and Pryor, 1985; Rahman and MacNee, 1996) by initiating lipid peroxidation (LPO) and protein oxidation (Frei et al., 1991; Reznick et al., 1992) as well as causing DNA strand breaking (Kodama et al., 1997; Nakayama et al., 1985). One puff of CS includes 10^{15} oxidant radicals (McCusker and Hoidal, 1990).

Reactive oxygen species are suppressed or scavenged by cellular antioxidant defense systems consisting of enzymatic mechanisms such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) as well as non-enzymatic mechanisms such as reduced glutathione (GSH), vitamins A, C and E. Preventive role of antioxidants was reported in modifying the major diseases related to CS (Anbarasi et al., 2005;

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Tiwari, 2004). When ROS increased excessively, antioxidant deficiency occurs. This inadequacy may be enhanced by usually insufficient intake of both supplementary and dietary antioxidants (Zondervan et al., 1996). One of the materials being used as antioxidant source to eliminate free radicals in CS are plant extracts (Chang et al., 2010).

Calendula officinalis L. known as pot marigold (PM) is an annual herb belonging to family Asteraceae. It grows in Mediterranean region and is cultivated in Europe and America for therapeutical (Von and Die, 1992) and other goals. Especially, its flowers have long been used in folk medicine (Duke et al., 2002). PM has been reported to have many pharmacological features including antioxidant (Preethi et al., 2006), anti-inflammatory (Della Logia et al., 1994), antifungal (Kasiram et al., 2000), antibacterial (Dumenil et al., 1980), antitumoral (Jiménez-Medina et al., 2006) and antiviral (Barbour et al., 2004). The primary chemical constituents of PM include steroids, terpenoids, free and esterified triterpenic alcohols, phenolic acids, flavonoids (quercetin, rutin, narcissin, isorhamnetin and kaempferol) and other compounds (Re et al., 2009). Among these compounds, flavonoids and polyphenols were defined as possessing antioxidant properties (Atoui et al., 2005).

The aim of current study is to determine the preventive mechanism of PM flower extract on oxidative damage in the lung, heart, brain and serum of rats exposed to CS by measuring the oxidative markers, enzymatic and non-enzymatic antioxidants.

Materials and methods

Plant material and extraction

Samples of *Calendula officinalis* L. was obtained from herbal store in Sanliurfa, Turkey. The plant sample was authenticated by Assist. Prof. Esat ÇETİN, PhD. The extraction protocol was performed according to modified method of Chandran and Kuttan (2008). Flowers of PM (400 g) were air-dried at room temperature and powdered coarsely. It was extracted with 350 ml ethyl alcohol at 25°C for 5 days after maceration in 225 ml distilled water. It was placed in a dark chamber at room temperature and shaken every day. Afterwards the blend was filtered and stored in deep freeze for 24 h. The PM extract was concentrated under reduced pressure and lyophilized (Tel Star Cryodos) to get the dry residue. Finally, the extract was mixed with distilled water before administration. The concentration of PM dose (100 mg/kg body weight) used in this trial was adjusted according to the study reported by Chandran and Kuttan (2008).

Experimental animals

Experiments were performed on 21 Sprague-Dawley male albino rats (240–260 g) that were housed in plastic cages (seven rats per cage) in a temperature-controlled room (22°C) and given free access to laboratory chow with water. The 12-h light/dark cycle was from 7 a.m. to 7 p.m.

Experimental protocol

The animals were divided into three groups, each composed of seven animals. (1) Control group: inhaled fresh air and received 1 ml physiological water by intragastric administration for 23 days before the killing process. (2) CS group: exposed to CS for 1 h twice a day and received 1 ml physiological water by intragastric administration for 23 days. (3) CS + PM group: exposed to CS for 1 h twice a day and received 1 ml PM (100 mg/kg bw) by intragastric administration during the trial period. All animals received humane care in compliance with the European Community Guidelines on the care and use of laboratory animals.

Rats were exposed to CS in a 44 × 44 × 100 cm glass box which was used for passive smoke exposure. A rubber tube, with one tip connected to blower, was placed into the box. Two lit cigarettes were mounted to the tip of tube in box and they were pumped by the blower for 60 min twice a day. There was a hole for air evacuation in the opposite wall of glass box. Moreover, fresh air was circulated regularly into the box from a further gap. Commercial filter-tipped cigarettes were used in current study. Food was not given to rats during the inhalation in box.

Preparation of lung, heart, brain and serum samples

At the end of the treatments, rats were anesthetized with ketamine (100 mg/kg ip; Enaida et al., 2002). Blood samples were taken from the animals into tubes with K_3EDTA by injectors from the heart under light anesthesia and then the rats were euthanized with inhalation of CO_2. The serum samples were obtained by centrifuging the blood samples at 3000 rpm for 15 min at 4°C. Lung, heart and brain samples were carefully removed and kept in Petri dishes for washing

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with physiological saline (0.9% NaCl). These samples were kept at –80°C until analysis. Tissues were homogenized in ice-cold phosphate buffer solution for 5 min using both an ultrasonic (Bandelin, UW 2070) and a mechanic (Heidolf, silent crusher M) homogenizers and then centrifuged at 7000 g-force for 15 min. All processes were carried out at 4°C. While protein concentration and oxidant/antioxidant parameters were determined in lung, heart and brain supernatants, the β-carotene and vitamins A and C levels were measured in serum.

Determination of GPx (EC 1.11.1.9) and SOD (EC 1.15.1.1) activities

Glutathione peroxidase activity was determined by using the Cayman Chemical Glutathione Peroxidase Assay Kit (Cat. no 703102) that measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Activity of SOD was measured using assay kit (Cayman, Cat. no 706002) that utilizes tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. The SOD assay measures all the three types of SOD (Cu/Zn, Mn and FeSOD).

GSH determination

GSH concentration was assayed by reacting with O-phthaldialdehyde (OPT, 10 mg/10 ml methanol) according to the modified method of Lee and Chung (1999). Pure reduced GSH was used as standard for calibration. GSH levels were measured by using spectrofluorimetry (Jasco 6000, USA), with excitation at 345 nm and emission at 425 nm.

Antioxidant vitamin determination

The levels of β-carotene at 425 nm and vitamin A (retinol) at 325 nm were detected after the reaction of serum: ethanol: hexane at the ratio of 1:1:3, respectively, by Suzuki and Katoh (1990). Serum vitamin C level was determined after derivatization with 2,4-dinitrophenylhydrazine by Omaye et al. (1979).

MDA determination

The LPO product malondialdehyde (MDA) was estimated using the modified thiobarbituric acid-reactive substance (TBARS) method by Hegde et al. (2003). MDA was measured by spectrofluorimetry, with excitation at 520 nm and emission at 555 nm. Calculations were performed using a linear regression from tetraethoxypropane for the MDA standard curve.

Protein carbonyl determination

Protein oxidation was measured by Cayman's protein carbonyl (PC) Assay Kit (Cat. no 10005020) as carbonyl content in the samples. This kit bases on the principle which utilizes the 2,4-*Dinitrophenylhydrazine* (*DNPH*, Brady's reagent) reaction to measure the PC content in homogenate (Levine et al., 1990). The amount of protein-hydrozone produced is quantified spectrophotometrically at an absorbance 360 nm by 96-well plate reader (Spectra Max M5). The carbonyl content was standardized to protein concentration.

Protein determination

The protein content in the samples was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Statistical analysis

The results were analyzed using ANOVA test. The level of statistical significance was set at $p < 0.05$. Results were presented as means ± standard deviation (SD).

Results

During passive smoke exposure, the effect of PM administration on the rats were monitored by measuring MDA, PC, GSH, vitamin levels and GPx, SOD activities. Results are shown in Tables 1 and 2. While MDA and PC levels of CS group increased significantly nearly in all tissues compared to control, their levels diminished markedly in lung and brain tissues by PM administration. MDA level of CS + PM group was significantly higher in brain tissue and PC content of this group elevated markedly in lung compared with control. In CS group, GSH concentration significantly augmented in all tissues comparing with control. Its level attenuated markedly in lung and brain tissues of CS + PM group compared to CS group. Glutathione peroxidase and SOD activities decreased markedly in lung, heart and brain tissues of CS group compared to control; however, their activities were elevated significantly by PM supplementation in all of these tissues except brain. With regard to vitamins, there was a significant elevation of β-carotene and vitamin A levels in CS group compared to control group.

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Table 1. The protective effect of PM on CS induced oxidative stress in some tissues of rats

Tissue	Parameters	Control	CS	CS + PM
Lung	MDA (nmol/g)	29.03 ± 2.02	40.05 ± 2.70[a]	32.71 ± 3.06[b]
	PC (nmol/mg prot.)	1.18 ± 0.23	3.36 ± 1.04[a]	1.84 ± 0.25[a,b]
	GSH (μmol/g)	2.08 ± 0.84	3.20 ± 0.26[a]	2.63 ± 0.44[b]
	GPx (nmol/min/g)	128.72 ± 12.94	80.13 ± 17.76[a]	102.94 ± 9.94[a,b]
	SOD (U/mg protein)	2.16 ± 0.06	1.62 ± 0.23[a]	2.36 ± 0.10[a,b]
Heart	MDA (nmol/g)	25.05 ± 4.91	35.45 ± 10.01	28.58 ± 4.60
	PC (nmol/mg prot.)	3.10 ± 0.63	5.79 ± 1.52[a]	4.07 ± 0.72
	GSH (μmol/g)	2.00 ± 0.39	3.30 ± 0.45[a]	2.54 ± 0.55
	GPx (nmol/min/g)	105.88 ± 13.53	77.83 ± 13.51[a]	131.05 ± 17.25[a,b]
	SOD (U/mg protein)	1.59 ± 0.10	1.19 ± 0.22[a]	1.77 ± 0.38[b]
Brain	MDA (nmol/g)	101.51 ± 7.27	149.20 ± 9.26[a]	122.35 ± 15.68[a,b]
	PC (nmol/mg prot.)	2.23 ± 0.36	3.30 ± 0.72[a]	2.61 ± 0.65
	GSH (μmol/g)	2.96 ± 0.41	3.82 ± 0.71[a]	2.60 ± 0.33[b]
	GPx (nmol/min/g)	76.19 ± 6.61	46.18 ± 8.85[a]	56.06 ± 8.25[a]
	SOD (U/mg protein)	1.80 ± 0.12	1.31 ± 0.18[a]	1.55 ± 0.16[a,b]

Abbreviations: CS: cigarette smoke, PM: pot marigold, MDA: malondialdehyde, PC: protein carbonyl, GSH: reduced glutathione, GPX: glutathione peroxidase, SOD: superoxide dismutase.
Note: Values are given as the mean ± SD.
[a]Control versus treated $p < 0.05$ level.
[b]CS versus treated $p < 0.05$ level.

Table 2. The protective effect of PM on antioxidant vitamin levels of CS exposed rats

Parameters	Control	CS	CS + PM
β-Carotene (μg/dl)	27.52 ± 3.88	21.32 ± 2.94[a]	23.45 ± 2.06
Vitamin A (μg/dl)	126.31 ± 8.05	114.93 ± 7.61[a]	119.65 ± 1.57
Vitamin C (mg/dl)	0.67 ± 0.15	0.58 ± 0.09	0.65 ± 0.15

Abbreviations: CS: cigarette smoke, PM: pot marigold.
Values are given as the mean ± SD.
[a]Control versus treated $p < 0.05$ level.

Discussion

CS was determined as a major risk factor for diseases of many organs including liver, heart and brain. In this study, we investigated the alterations in oxidant/antioxidant status of these organs after CS exposure. In addition, the concentration of serum antioxidant vitamins (β-carotene and vitamins A and C) were detected. To the best of our knowledge, protective effect of PM against CS-induced oxidative damage was assessed in current study for the first time.

CS includes many oxidants and pro-oxidants that generate free radicals and increase the oxidative stress in tissues (Kiyosawa et al., 1990; Park et al., 1998; Reznick et al., 1992; Smith and Hodges, 1987). Thus, CS is a constant oxidant stress on the respiratory tract. A cell could protect itself against oxidative stress by the use of endogenous and exogenous antioxidants. Depending on this data, smokers are usually directed to use antioxidants (Smith and Hodges, 1987). Current study was realized to determine whether PM would be an effective antioxidant against smoking-mediated oxidative damage.

Carbon- and oxygen-centered free radicals are known to cause damage by LPO and protein oxidation in membranes of cells (Halliwell, 1987). In our study, MDA and PC concentrations were measured to assess the levels of this damage in lipid and proteins, respectively. MDA and PC contents of CS group increased significantly almost in all tissues compared to control group. PM administration decreased their levels slightly in heart and markedly in lung and brain tissues. Elevated MDA and PC levels in CS group were compatible with the results of previous studies (Altuntas et al., 2002; Baskaran et al., 1999; Luchese et al., 2007; Ozan et al., 2001; Panda et al., 2001;

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Valenca et al., 2008). These results demonstrated that PM can decrease the toxicity of CS-mediated free radicals and sustained normal cell integrity.

GSH is a vital non-protein thiol, which plays a leading role via coordinating the antioxidant defense process in cells. In current study, GSH level is significantly higher in CS group compared to control in all tissues. However, its content in lung and brain tissues decreased markedly in CS + PM group compared to CS group. The fact that elevated GSH levels in CS group supports the data obtained from some earlier studies (Khanduja et al., 1985; Ozan et al., 2007). It is likely that the highest level of GSH in CS group was stimulated non-enzymatically against the free radicals-mediated damage induced by CS. Its level might be decreased in CS + PM group due to free radical scavenging activity of PM.

While GPx and SOD activities of CS group were significantly lower compared with control group, their activities were markedly higher in CS + PM group than CS group almost in all tissues. Moreover, activities of these enzymes fluctuated in CS + PM group compared with control. The lowest GPx and SOD activities in CS group determined in this study show similar results with previous investigations (Anbarasi et al., 2006; Luchese et al., 2009; Ramesh et al., 2007, 2008). Decrease of these enzyme activities might be attributed to the adaptive response to counteract LPO and protein oxidation or oxidants present in CS. Increased activity of these enzymes by PM supplementation may be related to the antioxidant feature of PM (Preethi et al., 2006).

There was statistically significant elevation of β-carotene and vitamin A levels in CS group compared to control group. Increased oxidant status induced by CS may lead to their depletion. This result is in accordance with some other studies (Kayan et al., 2009; Li et al., 2003).

In conclusion, this study demonstrated that CS exposure damages the antioxidant defense system in different tissues of rats. Intragastric administration of PM relatively ameliorated these alterations with its antioxidant properties. Thus, we consider that PM supplement might have some useful influences in smoke-induced oxidative stress.

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