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Short communication

Effect of pregnancy and tobacco smoke on the antioxidant activity of rutin in an animal model

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Abstract:
Tobacco smoke is a source of free radicals and causes oxidative stress in smokers' tissues. The aim of the current study was to evaluate the effect of rutin on the total antioxidant status (TAS) in pregnant and non-pregnant rats that were exposed to cigarette smoke. TAS in brain, lungs, liver, kidneys and plasma were measured by the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical-cation decolorization assay. In pregnant rats, a diversified distribution of endogenous antioxidants was found in comparison to the matched non-pregnant animals. In pregnant rats, TAS was higher in plasma (by 33%) and kidney (by 76%), and lower in brain (by 48%) and liver (by 50%) compared with non-pregnant rats. Generally (except liver), exposure to tobacco smoke caused an increase in the antioxidative status of pregnant compared to non-pregnant animals (by 29, 16, 18 and 87% in plasma, brain, lung and kidney, respectively). Overall, rutin had little (plasma, non-pregnant rats) or a no protective effect in the examined tissues.

Key words:
tobacco smoke, total antioxidant status, rutin, pregnant rat

Introduction

Tobacco smoke is a pervasive contaminant of the environment and public places. Tobacco smoke affects community health status, especially in the elderly, the youngest individuals and pregnant women. Education and anti-tobacco campaigns targeting pregnant women are not sufficient to raise the awareness of smoke's negative influence on the unborn offspring. Toxic effects in smokers depend largely on oxidative stress and the modulation of inflammatory reactions. Tobacco smoke is a source of free radicals (e.g., nitric oxides and the quinone/hydroquinone system). Moreover, upon inhalation into lung tissues, tobacco smoke generates a chain of reactive oxygen and nitrogen species, which are principally due to the involvement and activation of inflammatory cells and endothelial dysfunction [5]. These oxidative and nitrosative insults result in damage to lipids, proteins and nucleic acids [22]. In pregnant women and in pregnant laboratory animals, smoke impairs pregnancy outcome, fetus growth and development, and has adverse health effects in later life [11, 16].

Endogenous antioxidants in tissues and organs may counteract the damaging effects of tobacco smoke to some extent; however, chronic exposure may reduce the activity of this protective response [8].

Plant-derived alimentary products contain chemicals that exhibit various antioxidant activities. These include natural vitamins and their precursors, as well as polyphenols of diversified structures that are capable of direct radical scavenging, metal chelation, breaking oxidative chain reactions and suppression of the enzymes responsible for reactive nitrogen/oxygen species generation. Massive intake of these micronutrients has been postulated as a potential chemopreventive strategy in cardiovascular diseases, ageing and cancer [21].

However, studies examining the beneficial effects of dietary antioxidant supplementation in humans have provided inconclusive or even contradictory results [15].

A direct protective effect of multiple dietary antioxidants in the livers of smoke-exposed mice was described by Zhang et al. [24]. The authors reported a significant diminishment of lipid peroxidation and pro-inflammatory IL-6, as well a rise in vitamin E levels in animals treated with a diet supplemented with flavonoids, beta-carotene, coenzyme Q10, alpha-tocopherol, ascorbic acid, N-acetylcysteine, L-carnitine, retinol, selenium and zinc.

In our previous study, we assessed the effect of the flavonoid rutin, which is commonly consumed with plant-derived foods, on the total antioxidant status measured in non-pregnant female rats that were chronically exposed to cigarette smoke [9]. We demonstrated a tissue dependent influence of this diet supplementation on the antioxidant status measured by the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical-cation decolorization assay.

The aim of our current study was to investigate the potentially protective effect of this dietary flavonoid on the antioxidant status of pregnant rats exposed to cigarette smoke.

Materials and Methods

Animals

Female Wistar rats from the Department of Toxicology, University of Medical Sciences in Poznań, were housed in polycarbonate cages with hardwood chip bedding. A standard laboratory diet and water were available with no limitations. Throughout the entire study period, a 12/12 h light/dark cycle was maintained. After 14 days of initial acclimatization, the rats were randomized and divided into the following groups of 8 rats each: non-pregnant controls, non-pregnant smoke-exposed, non-pregnant with rutin administration exposed, and non-pregnant treated with rutin and exposed to tobacco smoke. In the pregnant rats, a similar design was utilized that included the following groups: controls, smoke-exposed, rutin-treated, and rutin administration and tobacco smoke exposed rats.

The protocol for the animal experiments was approved by the Local Ethics Commission for Animal Studies in Poznań.

Cigarette smoke exposure

Four groups of animals (smoke, pregnant/smoke, smoke/rutin and pregnant/smoke/rutin) were placed in a dynamic toxicological chamber [10] and exposed to the smoke generated from a Polish brand of cigarettes without a filter tip. The CO concentration in the chamber reflected the smoke content in the inhaled air and was continuously monitored to maintain 1,500 mg CO/m^3 of air. The level of oxygen was established at $20 \pm 0.5\%$ of the air volume. The air in the chamber was exchanged 10 times per day. Temperature and humidity were maintained at standard levels. Animals were exposed to the tobacco smoke for 6 h/day, for 3 weeks.

Rutin administration

Four groups of animals (rutin, smoke/rutin, pregnant/rutin and pregnant/smoke/rutin) received daily by gavage 2 ml of the aqueous solution of rutin at the dose of 40 mg/kg body weight. The administration of rutin began 2 weeks before the smoke exposure and was maintained over the tobacco smoke exposure period. Controls, smoke-exposed, pregnant and pregnant/smoke exposed rats were gavaged with 2 ml of distilled water.

After the last day of exposure, animals were transferred to metabolic cages so as to collect urine over the 24 h period for cotinine determination. All the animals were then scarified by decapitation. Blood was collected by heart puncture into heparin containing

tubes (10 U/ml blood) for the separation of plasma. Lungs, kidneys, brains and livers were removed, rinsed with ice-cold 0.9% NaCl, suspended in 0.5 M potassium phosphate buffer (pH 7.5) and homogenized in a Potter-Elvhjem homogenizer with a Teflon pestle. The obtained homogenates were frozen at –80°C until analysis.

Chemicals

The heparin solution of 10,000 U/ml was purchased from Polfa, Tarchomin, Poland. Rutin was a generous gift from GlaxoSmithKline Pharmaceuticals S.A., Poznań, Poland. ABTS and sodium persulfate were purchased from Sigma-Aldrich (St. Louis, USA), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Randox, Crumlin, Great Britain and all other chemicals were supplied by standard chemical suppliers.

Analytical methods

Cotinine determination in the urine was performed by a high performance liquid chromatography method as previously described [12]. Total antioxidant status was measured in the serum and tissue homogenates as trolox equivalent antioxidant capacity (TEAC), with a modification of the ABTS-cation-radical method [1].

In the plasma and tissue samples, protein quantification was determined according to the Lowry method [18].

Statistical analysis

All statistical calculations were carried out with the STATISTICA 6.0 computer program. For statistical analyses, the data obtained from the cotinine determination was compared with a one way-ANOVA. However, for analyzing the TAS data, a multivariate analysis with a Tukey *post-hoc* test was applied.

Results and Discussion

In our current study, an animal exposed to 1,500 mg CO/m^3 of air was considered receiving a heavy rate of exposure, and was monitored by a carboxyhemoglobin determination in the tested animal (25% of total hemoglobin content) [14]. Also, the cotinine concentration in the urine (8.5–13.2 μg/ml) of smoke-exposed rats confirmed the rate of exposure; however, no effect of pregnancy or rutin administration was demonstrated (Tab. 1).

Tab. 1. Cotinine concentration in the rat's urine collected 24 h after exposure to tobacco smoke

Subjects treatment	Smoke-exposed rats	Pregnant /smoke -exposed rats	Rutin /smoke-exposed rats	Pregnant /rutin/smoke-exposed rats
Cotinine (μg/ml)	13.2 ± 5.7	8.5 ± 3.6	12.8 ± 3.1	9.2 ± 0.9

Results are presented as the mean ± SD of 8 rats/group. One way ANOVA did not show statistical significant differences between the groups

The rutin used in our experiment for the long-term dietary antioxidant supplementation is a flavonoid abundantly present in plant foods. Structurally, it is a glycoside composed of aglycone quercetin and disaccharide rutinose. Upon ingestion, the sugar moiety of rutin is processed *via* intestinal flora and is converted into quercetin, which the liver conjugates with glucuronide and/or sulfate. These conjugates can be found circulating in rat plasma, and their further enzymatic hydrolysis yields isorhamnetin (a methylated form of quercetin) and quercetin itself [18]. In view of the presented metabolic pathway of rutin in animals treated with this phytochemical, the biological action of quercetin should be considered.

However, due to the confined bioavailability of plasma levels of quercetin, conjugates are found in low levels and are not very likely to exert a direct measurable effect on antioxidant status *in vivo* [20].

Beside antioxidant effects *in vivo*, the pro-oxidant activity of quercetin has been described and its potential health impact goes beyond the modulation of oxidative stress.

In our study, the assessment of plasma antioxidant capacity based on the ABTS radical cation reduction depended mainly on albumin, uric acid, ascorbic acid, α-tocopherol and bilirubin [3].

Uric acid is responsible for approximately 1/5–1/3 of the plasma antioxidant potential [3], but rats are also capable of metabolizing uric acid into alantoin and this regular metabolic pathway may contribute to the low antioxidant capacity of rat plasma. Rats produce ascorbic acid; however, this hydrophilic antioxi-

Tab. 2. Effect of rutin administration on total antioxidant status (TAS) measured in tissues of non-pregnant and pregnant rats exposed to tobacco smoke

	TAS [μmol trolox equivalent antioxidant capacity/mg protein]							
	Non-pregnant rats				Pregnant rats			
	Controls	Rutin	Smoke-exposed	Rutin/smoke-exposed	Controls	Rutin	Smoke-exposed	Rutin/smoke-exposed
Lungs	33,0 ± 4.38	32.0 ± 0.39	44.0 ± 0.32[a]	37.0 ± 2.76	33.0 ± 0.60	41.0 ± 1.45[b,c]	51.0 ± 0.04[b,c]	39.0 ± 8.49
Liver	70.0 ± 3.54	49.0 ± 6.16	51.0 ± 1.91[a]	28.0 ± 0.71[a]	46.0 ± 2.55[c]	59.0 ± 9.69	50,0 ± 1.52	54.0 ± 1.17[c]
Brain	77.0 ± 4.88	50.0 ± 12[a]	51.0 ± 1.52[a]	45.0 ± 0.88[a]	52.0 ± 2.55[c]	66.0 ± 21.50	62.0 ± 3.89[b,c]	53.0 ± 0.35[c]
Kidneys	50.0 ± 1.66	60.0 ± 1.68[a]	31.0 ± 1.45[a]	28.0 ± 0.39[a]	88.0 ± 1.31[c]	56.0 ± 1.63[b]	58.0 ± 3.15[b,c]	55.0 ± 7.96[b,c]
Plasma	24.0 ± 0.57	23.0 ± 0.46	24.0 ± 1.13	32.0 ± 0.46[a]	32.0 ± 0.48[c]	23.0 ± 11.31[b]	31.0 ± 3.04[c]	28.0 ± 2.62

Results are expressed as the mean ± SD of 8 rats/group. Multivariate analysis revealed $F(2, 24) = 0.8235$; $p = 0.4509$ for lung, $F(2, 23) = 5.1310$; $p = 0.0143$ for liver, $F(2, 24) = 7.4995$; $p = 0.0033$ for brain, $F(2, 24) = 11.5715$; $p = 0.0003$ for kidney, $F(2, 24) = 2.0890$; $p = 0.1458$ for plasma. [a] $p < 0.05$ *vs.* the appropriate control for non-pregnant rats, [b] $p < 0.05$ *vs.* appropriate control of pregnant rats, [c] $p < 0.05$ *vs.* the appropriate treated for non-pregnant rats (Tukey *post-hoc* test)

dant provides little contribution to the reduction of the ABTS radical cation [3].

In our earlier paper, we reported a lack of clear differences in the vitamin C content in control and smoke-exposed rats. Also, the plasma content of vitamin A in rats exposed to tobacco smoke was not changed in comparison to the matched controls. Meanwhile, a significant vitamin A depletion in tissues was found [13].

The results of antioxidant status measurements obtained in the current study from the non-pregnant and pregnant rats are presented in Table 2. In all tissues tested from the pregnant females, values were similar to the pattern of changes presented in our previous report examining non-pregnant rats treated with rutin and exposed to tobacco smoke [9].

The plasma value of TAS in the non-pregnant rats did not differ from controls, smoke or rutin alone groups; however, the plasma of the rutin/smoke treated animals exhibited an antioxidant power that was significantly elevated (by 33%). TAS in the plasma of pregnant controls was 33% higher than in their non-pregnant counterparts, and was 29% higher in smoke-exposed pregnant animals than in non-pregnant rats. No changes in TAS levels were found in rutin/smoke exposed pregnant and non-pregnant animals.

Benito et al. [2] showed a higher plasma antioxidant status in rats fed for 10 days on a diet supplemented with quercetin (0.3% w/w) as compared to controls. The discrepancy with our results was mainly due to the variability in the experimental protocol. As these authors reported, the daily food intake per rat was approximately 30 g and the estimated total dose of ingested quercetin (an aglicone of rutin) was around 900 mg/rat, which exceeded the amount of quercetin calculated for each experiment (200 mg/rat).

Exposure of the non-pregnant rats to the tobacco smoke resulted in a significant rise (by 33%) of antioxidant capacity in the lungs. In contrast, in the other tested tissues (liver, brain, kidney), there was a marked (27–38%) decrease of the antioxidant capacity. In the lungs of pregnant females, the exposure to smoke and rutin alone caused increases in the antioxidant capacity by 54 and 24%, respectively, in comparison to the non-treated controls. In addition, these values from smoke and rutin exposed animals were higher (by 16 and 28%, respectively) than in their non-pregnant counterparts (Tab. 2).

Lungs are the organs of primary contact with the toxic constituents of smoke and the observed increase of their TAS may correspond to the supposed mobilization of antioxidant defense mechanisms. The action of smoke free radicals on lung tissue is strengthened by reactive oxygen and nitrogen species, which are generated and released from phagocytic cells that are present in lung. Smoke enhances the recruitment of

a large number of phagocytes to the insulted area, as well as their pro-inflammatory activation [17].

Numerous published reports have indicated that dietary flavonoids, including quercetin, are capable of lowering NADPH oxidase, which is a major source of reactive oxygen species (ROS) in phagocytes and/or affect other mechanisms modulating antioxidant responses and redox-dependent reactions [6].

Several reports have presented data on the oxidative stress obtained from cigarette smoke-challenged rats; however, variations in the exposure regimes and in the choice of measured parameters has led to some unconvincing conclusions. Würzel et al. has described increases in the lung content of the α-tocopheryl quinine, yet no changes were observed in the lung levels of vitamins C or E, levels of glutathione, or in levels of markers of oxidative damage such as malondialdehyde and protein carbonyls [23]. In the review of Chow [4], it was suggested that an increase in superoxide dismutase activity and vitamin E concentration in the lungs of rats chronically exposed to tobacco smoke would help to counteract oxidative stress and to resist further damage to smoke exposure. Accumulation in the lungs of the lipophilic antioxidant tocopherol, which is incorporated into the phospholipids bilayers of membranes, is probably linked to the defense mechanisms and metabolic adaptations to conditions of oxidative and toxic stress and/or to inflammation. In our experiments, this hypothesis might be reflected by the increased antioxidant capacity of the lungs in non-pregnant (by 33%) and pregnant (by 54%) rats exposed to smoke compared to controls. A decreased antioxidant capacity was found in brain (by 50%), liver (by 37%) and kidneys (by 60%) of the non-pregnant smoke-exposed animals. In their respective pregnant counterparts, antioxidant depletion was measured only in kidneys (by 50%); however, some elevation was found in brain (by 18%).

Comparisons of the antioxidant capacities of tissues from non-pregnant and pregnant animals has revealed differential changes, whereby only values obtained in the lungs of pregnant rats were, to some extent, parallel with the results found in their non-pregnant counterparts. Generally (except in liver), the increase in the antioxidative status of rats exposed to tobacco smoke was elevated by pregnancy (Tab. 2). During pregnancy in mammals, an increase in ROS generation appears. This increase is reflected by a higher tendency for oxidative damage, which is mainly due to the mild deprivation of vitamin E [7]. In the current study, this trend might be reflected by lower TAS values in the brains and livers of pregnant controls as compared to non-pregnant females.

In conclusion, we can state that the total antioxidant status of the rat is modulated by exposure to tobacco smoke in non-pregnant and pregnant females, and a diet supplemented with the antioxidant rutin has little or no protective effect on changes effected by smoke exposure in the examined tissues. However, pregnancy elevated the antioxidative status of rats exposed to tobacco smoke in most of the tissues examined.

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