EXPERIMENTAL STUDY

Dietary intake of flavonoids and hyperoxia-induced oxidative stress related cough in guinea pigs

Brozmanova M[1], Bartos V[2], Plank L[2], Plevkova J[1], Tatar M[1]

Department of Pathophysiology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia.
brozmanova@jfmed.uniba.sk

Abstract

There is many evidence that inhalation of high oxygen concentration has a toxic influence on pulmonary function and structures. Hyperoxia-induced oxidative stress is well characterized in rodents and has been used as a valuable model of human respiratory distress syndrome. We have previously shown that hyperoxic exposure of guinea pigs is associated with suppression of cough reflex. The goal of this study was to determine the effects of dietary intake of antioxidant flavonoids (Flavin7, Vita Crystal Slovakia Ltd., 2 ml/kg b.w.) on hyperoxia-induced oxidative stress in lung tissue directed on cough reflex. The experimental group (n=8) was pretreated with Flavin7 as a single daily dose for 14 days and subsequently exposed to 100 % O2 for 60 h. Hyperoxic group (n=8) inhaled 100 % O_2 only. Control group (n=8) was exposed to normoxia. Cough was induced by inhalation of citric acid aerosol at time before and after exposure to hyperoxia. Cough was also induced by mechanical stimulation of airways in anaesthetized animals just after the end of oxygen exposition. When to compare animal groups before and after hyperoxia, our results have shown a significant decrease 2 (1–6) vs 6 (4–6) p=0.041 in citric acid-induced cough in hyperoxic animals and no significant changes 8 (5.5–8.5) vs 5 (4–6.5) p=0.055 in animals with antioxidant therapy. Mechanically-induced cough after hyperoxia was not influenced by substitution with flavonoids. In conclusion, our results indicate that flavonoids attenuated hyperoxia-induced down-regulation especially of chemically-induced cough *(Tab. 2, Fig. 2, Ref. 30)*. Full Text (Free, PDF) *www.bmj.sk*.

Key words: hyperoxia, antioxidants, flavonoids, citric acid-induced cough, mechanically-induced cough.

Numerous studies have shown that pulmonary oxidant stress plays an important pathogenetic role in disease conditions including acute lung injury, adult respiratory distress syndrome, hyperoxia, ischemia-reperfusion, sepsis, radiation injury, COPD, and inflammation (Christofidou-Solomidou and Muzykantov, 2006). Hyperoxia-induced lung injury is well characterized in rodents and has been used as a valuable model of human respiratory distress syndrome (Jyonouchi et al, 1998, Pagano and Barazzone-Argiroffo, 2003). A long term oxygen inhalation results in parenchymal, epithelial and endothelial injury. Oxygen-induced lung injury is characterized by extensive alveolar damage leading to disruption of the alveolo-capillary barrier, pulmonary edema, and pleural effusion (Clark a Lambertsen, 1971, Klein 1990, Chen et al, 1995, Pagano and Barazzone-Argiroffo, 2003). Hyperoxia-induced lung damage can be considered as a bimodal process resulting from direct action of increased intracellular and extracellular reactive oxygen species (ROS) and from the accumulation of inflammatory mediators within the lungs (Pagano and Barazzone-Argiroffo, 2003). ROS released from activated macrophages and leukocytes or formed in the pulmonary epithelial and endothelial cells, damage the lungs through their ability to react with and damage essential biomolecules, including enzymes, membrane lipids, and nucleic acids (Jenkinson, 1993) and initiate cascades of pro-inflammatory reaction propagating pulmonary and systemic stress (Christofidou–Solomidou and Muzykantov, 2006).

[1]Department of Pathophysiology and [2]Department of Pathological Anatomy, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

Address of correspondence: M. Brozmanova, MD, PhD, Dept of Pathophysiology, Jessenius Faculty of Medicine, Comenius University, Sklabinska 26, SK-037 53 Martin, Slovakia
Phone: +421.43.4238213, Fax: +421.43.4134807

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Because the lungs are directly exposed to high levels of oxygen there is no doubt that respiratory epithelium including sensory nerve endings is a major target for oxidative injury that is manifested in lung function changes including cough. Information about hyperoxia related cough response is still limited. On the basis of our previous experiments repeatedly demonstrating the inhibitory effect of 100% oxygen breathing for 60 h on cough in awake guinea-pigs (Brozmanova et al, 1996, Brozmanova et al, 2002, Brozmanova et al, 2004), it seems that 100 % oxygen plays a key role in the development of airway and lung damage with peripheral or central down-regulation of cough reflex (Kollarik and Undem, 2003).

Many investigators have found decreased amounts of antioxidants and elevated levels of prooxidants in animal model of ARDS, which suggests that an oxidant-antioxidant imbalance can contribute to the pathogenesis of acute lung injury (Oury et al, 2002). Because of oxidative stress is involved in the pathophysiology of inflammatory airways diseases, we can only speculate that supplemented antioxidants probably reduce oxidant status, inflammatory cytokines or gene expression and therefore antioxidant therapy would seem to be a logical rationale for treating or preventing hyperoxic lung damage (Cao et al, 1999, Blesa et al, 2003, Christofidou-Solomidou and Muzykantov, 2006).

The nonenzymatic antioxidants, most of which have low molecular weights and are able to directly and efficiently quench free radicals, constitute an important aspect of the body's antioxidant mechanism. Antioxidant action of flavonoids has been attracted attention of many investigators and good deals of studies on it were reported (Cao et al, 1999, Prior and Cao, 2000, Brusselmans et al, 2005, Cao et al 2005). Flavonoids are a group of naturally occurring phytochemicals abundantly present in fruits and other plants, and beverages such as wine and tea. In the past two decades, flavonoids have gained enormous interest because of their beneficial health effects such as antiinflammatory, antiallergic, cardioprotective, anticancer, antiproliferative, antiangiogenic and neuroprotective activities. Some of these benefits are explained by the potent antioxidant effects of flavonoids, which include metal chelation and free radical scavenging activities. Other physiological effects of flavonoids include facilitation the regeneration of vitamin E, increased vitamin C and carotene levels. Other authors have described flavonoids that protect the lungs against hyperoxia (Oury et al, 2002).

Therefore the aim of this study was to investigate whether the fruit extract with high levels of flavonoids and resveratrol affect airway function in guinea pigs after long-term oxygen therapy. The cough reflex intensity was regarded as an indicator of airway dysfunction.

Methods

Animal model and experimental groups

The study was performed on 24 adult male Trik guinea-pigs (250–350 g) which were supplied by Department of Experimental Pharmacology of the Slovak Academy of Science (Dobra Voda, Slovakia). Before starting the experiment animals were kept in the animal house with food and water ad libitum and with standard air conditioning system. The experimental protocols were approved by the institutional ethics committee and comply with Slovakia and European Community regulations for use of laboratory animals.

The animals were randomly assigned to one of the following three protocol groups: Flavin7-hyperoxia group (n=8) – pretreated with flavonoids for 14 days and subsequently exposed to 100 % O_2 for 60 h, hyperoxia group (n=8) – received vehicle, saline instead of antioxidants and was exposed to 100 % O_2 for 60 h, and control group (C, n=8) – exposed to ambient air under the same condition as the hyperoxic group.

Flavin7 (2 ml/kg b.w., including flavonoids 35 mg/kg b.w. and resveratrol 0.035 mg/kg b.w., Vita Crystal Slovakia Ltd.) was administered orally by a micropitte as a single daily dose for 14 days before and 2 days during hyperoxia.

Exposures either to oxygen or air were performed individually in a sealed glass chambers. Oxygen concentration was maintained by flow of 100 % oxygen through the chamber at 15 l/min and was periodically monitored by an oxygen analyzer (Permolyt 3, Veb Junkalor, Germany). Other biophysical parameters of the chamber environment were the following: temperature 22–24 °C, humidity 55–65 %, CO_2 concentration 0.2 vol% and O_2 concentration in ambient air 21 %.

Citric acid-induced cough and evaluation

The complete procedure of chemically-induced cough was previously described (Brozmanova et al, 2006). Briefly, unanaesthetized animals were individually placed into a bodyplethysmograph box (type 855, Hugo Sachs Electronic, Germany) and were exposed to citric acid aerosol (Lachema, Czech Republic) generated via a jet nebulizer (Pariprovocation test I, Pari Starneberg, Germany) in doubly increasing concentration (from 0.05 to 1.6 M) for 30 s. The interval between separate exposures was 1 min. Respiratory changes in the airflow were measured using pneumotachograph (Godart, Germany) with Fleish head connected to the head chamber. The appearance of cough was detected with a microphone placed in the roof of the head chamber. Pneumotachograph changes and the cough sounds were simultaneously recorded. The cough was evaluated on the basis of the sudden enhancement of expiratory airflow accompanied by typical cough sound. The cough sound was analyzed from software system according (Xiang et al, 1998) using spectral analysis of respective sounds. To quantify the intensity of cough, the cough response was expressed as the total number of coughs during all citric acid challenges.

A control cough challenge was done in all groups of animals (1st cough challenge) at the onset of the experiment. The next provocation of cough was performed after antioxidant treatment before hyperoxia (2nd cough challenge), and a final cough challenge was performed at 60 h of exposure to 100 % O_2 or ambient air (3rd cough challenge).

Mechanically-induced cough and evaluation

After hyperoxia finished and last chemically-induced cough challenge was done animals of all separate groups were anesthe-

tized (Urethane, 1.1 g/kg, i.p., Riedel-de Haen AG). Just after surgical procedure involving tracheostomy and intrapleural cannula insertion, cough was induced by mechanical stimulation of laryngopharyngeal (LPh) and tracheobronchial (TBr) mucosa using a nylon fibre (Brozmanova et al, 2002).

The number of cough efforts (NE) was counted from the trace of intrapleural pressure recorded by an electromanometer. To quantify cough, the number of cough efforts during a cough bout and the intensity of cough bout (ICB=the sum of all positive deflection of intrapleural pressure during all cough efforts in the cough bout) were used.

Histological preparation

At the end of the experiment, anesthetized animals were killed by an overdose of anesthesia and samples of tracheal, bronchial and lung tissue were removed, fixed in 10 % formalin, dehydrated in sequential alcohol concentrations, and embedded in paraffin. Cross-sectional specimens were made and stained with hematoxylin and eosin. Histopathological assessment was performed by light microscopy.

Statistical analysis

The number of coughs is presented as median and interquartile range. Data of the intensity of cough bouts are expressed as mean±SE. The inter-group differences in chemically and mechanically-induced cough response were assessed with Mann Whitney U-test and one-way analysis of variance (ANOVA). If a significant difference was detected, multiple comparisons of groups were made using the Duncan multiple range test. The intra-group differences in chemically-induced cough was assessed with Wilcoxon Sign Rank Test. Significance was accepted at the $p<0.05$ level.

Results

Effects of substitution with flavonoids on citric acid-induced cough in guinea-pigs exposed to 100 % O_2.

Our findings have demonstrated that hyperoxic exposure for 60 h caused a significant inhibition in citric acid-induced cough in hyperoxia-alone group of animals without flavonoids (2 (1–6) vs 6 (4–6); p=0.041) (Fig. 1). On the other hand, flavonoids substitution (Flavin7 – hyperoxia group) does not significantly changed the number of chemically-induced coughs after hyperoxia (8 (5.5–8.5) vs 5 (4–5.5); p=0.055) although there is a tendency to increase (Fig. 1).

When cough responses were analyzed in all protocol groups of animals obtained separately after three cough challenges according to protocol, we found no significant differences both neither in normoxic (negative control) group nor in flavonoids-treated group exposed to hyperoxia (Flavin7 – hyperoxia). The significant decrease was found solely in hyperoxic group (positive control) at the end of exposure to 100% O_2 compared to previous challenges values (2 (1–6) vs 6 (4–6) vs 7 (5–8); $p<0.05$) (Fig. 2).

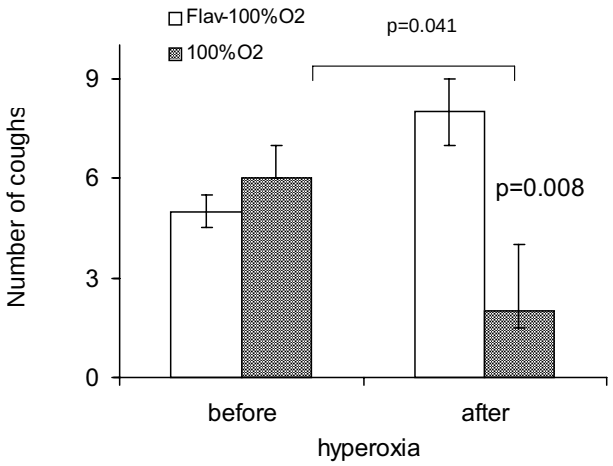

Fig. 1. Effects of hyperoxia on citric acid-induced cough in the hyperoxic group without flavonoids (100 % O_2) and in flavonoids treated group (Flav-100 % O_2). The number of coughs is expressed as median and interquartile range.

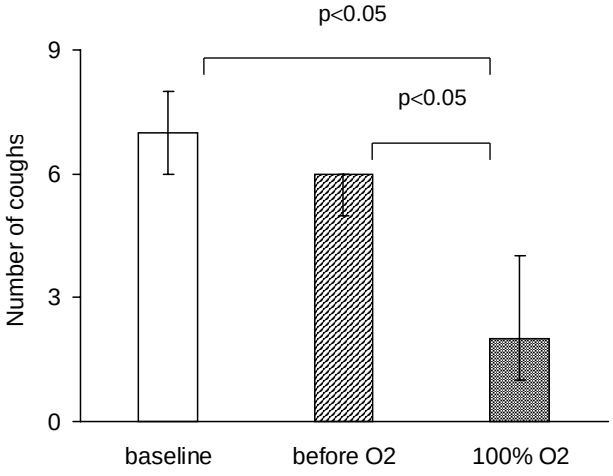

Fig. 2. Effects of hyperoxia on citric acid-induced cough in hyperoxia--alone group (positive control) at the onset of experiment, then before and after hyperoxia. The number of coughs is expressed as median and interquartile range.

Effects of substitution with flavonoids on mechanically-induced cough in guinea-pigs exposed to 100 % O_2

Our data has shown that 100 % oxygen breathing for 60 h has significantly decreased the number of coughs induced from LPh in hyperoxia-alone (100 % O_2) group compared with control (1 (0–2) vs 2.5 (1–3.5) $p<0.05$), without changes in intensity of cough bout measured from the trace of intrapleural pressure and expressed in kPa (Tab. 1). In contrast, although there were no significant differences in number of coughs among control and two tested groups of animals in mechanically-induced cough from TBr mucosa, our results revealed a tendency to increase in intensity of cough bout in flavonoids-treated group

Tab. 1. Changes in mechanically-induced laryngopharyngeal (LPh) cough in animals of Flavin7-hyperoxia group (Fla+100 % O_2), hyperoxic group (100 % O_2) and control animals (exposed to normoxia).

	Control n=8	Fla+100 % O_2 n=8	100% O_2 n=8
Number of coughs (median+IQR)	2.5(2.5)	2 (1)	1 (2)*
Intensity of cough bout (mean±SE)	94 (14)	116 (21)	77 (20)

* $p<0.05$ compared to control

Tab. 2. Changes in mechanically-induced tracheobronchial (TBr) cough in animals of Flavin7-hyperoxia group (Fla+100 % O_2), hyperoxic group (100 % O_2) and control animals (exposed to normoxia).

	Control n=8	Fla+100 % O_2 n=8	100% O_2 n=8
Number of coughs (median+IQR)	2 (2.5)	2.5 (1.5)	2 (2)
Intensity of cough bout (mean±SE)	91 (10)	113 (7)	69 (13)*

* $p<0.05$ compared to Flavin7-hyperoxia group (Fla+100 % O_2)

exposed to hyperoxia (Flavin7-hyperoxia) compared to controls and significant decrease in hyperoxia-alone (100 % O_2) group when compared with flavonoids-treated group (69 (13) vs 113 (7) $p<0.05$) (Tab. 2).

Histology

The histological examination of samples taken from the larynx, trachea, bronchi and the lungs did not recognize any appreciable differences in the intensity and quality of morphological changes between the animals of Flavin7-hyperoxia group and hyperoxia-alone group. The guinea pigs exposed to 100% oxygen for 60 h showed obvious pathological changes including hyperplasia and desquamation of the epithelium, dilatation of mucosal lymphatic and blood vessels, with the appearance of lymphocytes. More intense histopathological changes were seen in lung tissue. There was a reduction of alveolar spaces, interstitial pneumonitis and vesicular emphysema. Other changes included diffused excessive vascular dilatation, congestion, oedema, and inflammation, with aggregates of lymphocytes (not shown).

Discussion

We previously reported the inhibitory effect of hyperoxia-induced oxidative stress on cough reflex (Brozmanova et al, 1996, Brozmanova et al, 2002, Brozmanova et al, 2004) which suggests on down-regulation of cough reflex. The results of our present study confirmed our previous experiments. In spite of the fact, that up-regulation of cough in pathological condition of the airways has been detaily studied, down-regulation of cough elucidation is still limited (Carr and Lee, 2006, Widdicombe and Singh, 2006).

Hyperoxia is thought to increase the production of reactive oxygen species and disrupt the antioxidant defence mechanisms. The morphological changes of alveolar and other cells during exposure to hyperoxia have been widely described in rodents and in vitro studies (Clark and Lambertsen, 1971, Tarpy and Celli, 1995, Jyonouchi et al, 1998, Pagano and Barrazzone-Argiroffo, 2003, Buckley et al, 2005, Lofaso et al, 2007). Exposure of the rodent alveolar epithelium to high levels of oxygen results in ablation of alveolar epithelial type 1 cells and damage to the alveolar epithelial type 2 cells (Jyonouchi et al, 1998, Buckley et al, 2005). However, despite a vast literature resulting from nearly 30 years of research, the mechanisms that underlie cell injury and death, following exposure to hyperoxia, are not completely understood.

Protection of the pulmonary alveolar epithelium during oxygen therapy would be the key to the prevention of oxygen-mediated human diseases, such as bronchopulmonary dysplasia in the premature neonate. Understanding and manipulating the balance between hyperoxic damage and repair in the epithelium necessitate the use of rodent models, despite differences between human and rodent (Buckley et al, 2005).

Recently there has been a great deal of interest in polyphenolic compounds including high levels of flavonoids and resveratrol due to their beneficial cardiovascular, anticancer, antiimune and pulmonary actions of these compounds both in animal and human (Cao et al, 1999, Prior and Cao, 2000, Brusselmans et al, 2005, Buckley et al, 2005, Cao et al, 2005, Walle et al, 2007). Antioxidant action of flavonoids has been attracted attention of many investigators and their interest was mostly centered to the direct scavenging action of flavonoids against reactive oxygen species. Because flavonoids have been shown to protect the lung against hyperoxia (Oury et al, 2002), in this study Flavin7 (Vita Crystal Slovakia, Ltd.) was used as antioxidant. Flavin7 – an extract of seven fruits is a dietary supplement containing a high concentration of bioflavonoids, resveratrol, and anthocyanides. Bioflavonoid compounds, like those found in Flavin7 have a significant radical binding and antioxidant effect. Bioflavonoids also help to increase the radical binding effects of vitamins C and E and should be taken together with these vitamins. Experimental and clinical observations suggest promising product in the treatment of cardiovascular, cardiorespiratory and cancer diseases (Szentmiklósi, 2003, Ember, 2004).

In the present study we tested the hypothesis that hyperoxia-induced down-regulation of cough would be prevented by flavonoids (Flavin7) with anti-inflammatory and antioxidant properties. The doses of Flavin7 used in the study were in accordance with pilot studies of Flavin7 by Crystal Institute Ltd (Erdos and Szabo, http:/www.flavin-7.com/studies.php).

Our results showed a significant decrease in chemically-induced cough after exposure to hyperoxia that is consistent with

our previous reports (Brozmanova et al, 1996, Brozmanova et al, 2002, Brozmanova et al, 2004). Likewise, hyperoxia resulted in significant decrease in laryngopharyngeal mechanically-induced cough. These results support a suggestion that the intensity of hyperoxia-induced oxidative stress was strong enough to develop functional and morphological changes in airway mucosa including airway nerve endings responsible for mediation and modulation of cough. Histological findings are in accordance with this supposition, because there were signs of injury to airway mucosa and lung tissue in hyperoxic animals.

There is increasing evidence that oxidative stress is implicated in the development of airway inflammation and reactive oxygen species might play an important role in the modulation of airway inflammation induced by hyperoxia. Budinger and Sznajder reported that hyperoxic lung injury including damaging membrane receptors, channels, membrane of mitochondria, resulted from the activation of cellular-signaling pathways susceptible to modulation (Budinger and Sznajder, 2005). Morphological changes accompanying hyperoxia, manifesting in airway and lung damage, can contribute to decreased cough. ROS overproduction is liable to be involved in cough attenuation by hyperoxia. At the present state of knowledge, however, it is unknown what level of ROS or any other components of oxidative stress are involved in regulation of cough with its depression as a consequence. Antioxidant depletion or deficiency in antioxidants may contribute to that oxidative stress in the hyperoxia-induced cough attenuation observed in the present study.

In this study, we have demonstrated no significant changes in citric acid-induced cough in response to flavonoids pretreatment after hyperoxia. Likewise, hyperoxia was not influencing mechanically-induced laryngopharyngeal and tracheobronchial cough in animal group substituting with flavonoids.

To elicit cough, we used two different methods in this study: chemical and mechanical stimulation with different mechanisms of pathway regulating cough. While chemically-induced cough was evoked in awake animals, mechanical stimulation was performed in anesthetized ones. Another difference between the two methods was that citric acid aerosol (chemical stimulus) affected the whole surface of airways, while mechanical stimulation was restricted to confined areas.

This study was designed to evaluate the possible protective effects of flavonoids against hyperoxia-induced lung injury and our data indicate that flavonoids reversed hyperoxic attenuation of cough, probably by increasing antioxidant capacity and by regulating reactive oxygen species production.

Many other clinical and experimental studies support a beneficial effect of flavonoids against oxidative stress or improvement of lung function (Cao et al, 1999, Prior and Cao, 2000, Szentmiklosi, 2003, Ember, 2004, Christofidou-Solomidou and Muzykantov, 2006, Walle et al, 2007), but other studies have reported no clear evidence found for a protective effect of major subclasses of flavonoids on asthma and obstructive lung disease (Tabak et al, 2001, Garcia et al, 2005).

In conclusion, our study has shown that flavonoids administration inverses hyperoxia-induced cough depression. One possible explanation may be modulation of inflammatory processes by changes in lung antioxidant capacity and by blocking the production and activation of ROS. Flavonoids those found in Flavin7 may have a potential therapeutic role in the treatment of oxidative stress-related lung diseases.

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