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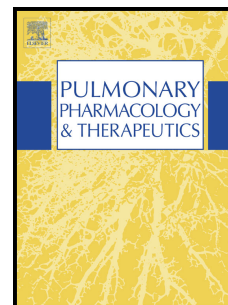
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## APOCYNIN DECREASES HYDROGEN PEROXIDE AND NITRATE CONCENTRATIONS IN EXHALED BREATH IN HEALTHY SUBJECTS

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### Abstract

The imbalance between reactive oxygen species (ROS) synthesis and antioxidants might be involved in the pathogenesis of many inflammatory diseases. NADPH oxidase, an enzyme responsible for ROS production, may represent an attractive therapeutic target to inhibit for the treatment of these diseases.

Apocynin is an inhibitor of activation of NADPH oxidase complex present in the inflammatory cells.

In double blind, placebo controlled, cross-over study, we investigated the effect of nebulized apocynin on ROS synthesis in 10 nonsmoking healthy volunteers. Apocynin (6 ml of 0.5 mg/ml) was administered by nebulization and its effects on H<sub>2</sub>O<sub>2</sub>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> generation were assessed after 30, 60 and 120 minutes by collecting exhaled breath condensate (EBC) samples using an EcoScreen analyzer. Additionally, respiratory parameters have been evaluated, utilizing spirometry and DLCO. We also analyzed peripheral blood differential counts and NO<sub>2</sub><sup>-</sup> serum level, cough scale control and blood pressure as safety parameters.

Apocynin caused reduction of H<sub>2</sub>O<sub>2</sub> concentration in EBC as compared to placebo, after 60 min. of inhalation (0.18 µM vs. 0.31 µM, p<0.05) as well as after 120 min. (0.2 µM vs. 0.31 µM, p<0.05). Similarly, apocynin significantly decreased concentration of NO<sub>3</sub><sup>-</sup> as compared to placebo, after 60 and 120 min. (6.8 µM vs. 14.4 µM and 6.5 µM vs. 14.9 µM respectively,

$p < 0.05$ ). Apocynin was well tolerated and no adverse events have been observed throughout the study.

Thus, as apocynin significantly influence ROS concentration, it might have also antiinflammatory properties. As it is safe, it may have a potential to become a drug in airway inflammatory diseases treatment.

Keywords: apocynin, NADPH oxidase, ROS, NOS, EBC

## Introduction

Asthma is a chronic airway disease, characterized by airway inflammation and hyperresponsiveness [1]. Human airways from healthy subjects contain cells that are able to release reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ) and peroxynitrite [2, 3], i.e. polymorphonuclear leukocytes (PMNs), eosinophils or macrophages.

Reactive oxygen species have a strong impact on homeostasis and are thought to play an important role in inflammation in asthma and COPD [4]. ROS play a key role in initiation as well as amplification of inflammation in asthmatic airways. When imbalance between reactive oxygen species concentration and the endogenous antioxidant system emerges, oxidative stress occurs. Oxidant-antioxidant imbalance leads to pathophysiological effects associated with asthma such as vascular permeability, mucus hypersecretion, smooth muscle contraction, and epithelial shedding [4, 5]. The concentrations of nitric oxide and hydrogen peroxide in exhaled air are increased in asthmatics and it is considered to reflect the state of airway inflammation [6]. ROS in the airways come from various enzymes expressed in airway cells.

The EBC collection used in this study is a safe method of gaining information regarding respiratory fluids. Since the pattern of breathing is normal, it is safer than FVC measurements, which may provoke bronchospasm in some asthmatic patients. It is noted that some people tend to hyperventilate especially at the beginning of EBC collection, but this has not led to any adverse event [7]. Though the methodology of EBC has not yet been standardized, and thus may constitute a some methodological bias that may hamper a solid interpretation of the data, it is still very useful and accurate method to gather research material.

In the respiratory system  $H_2O$  may be released both from inflammatory cells and epithelial cells. Hydrogen peroxide is a putative marker of airway inflammation in exhaled air. Stimulated alveolar macrophages from asthmatics generate more reactive oxygen species than alveolar macrophages from healthy subjects [8].

The main ROS, is superoxide, generated through one-electron reduction of oxygen molecule. It reacts with enzymes, producing a series of other ROS, including  $H_2O_2$ . Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a principal enzyme responsible for the superoxide production [9].

NADPH oxidase (EC 1.6.3.1) is a complex, containing p47phox and p67phox subunits, membrane-bound cytochrome b558 (composed of gp91phox or its homologues), p22phox, and a small G protein rac [10]. It generates superoxide anion after activation, i.e. aggregation of all subunits. The agent that is able to inhibit the release of superoxide anion by NADPH oxidase by inhibiting activation NADPH oxidase itself is apocynin (4-hydroxy-3-methoxyacetophenone, acetovanillone) [11]. It blocks migration of p47phox to the mitochondrial membrane, pivotally involved in initiating assembly of the functional NADPH oxidase complex. Apocynin is a naturally occurring agent, isolated from roots of plants *Picrorhiza kurroa* and *Apocynum cannabinum*. It is an acetophenone with a molecular weight of 166.17 and forms needles upon crystallization from water. It possesses a faint vanilla odor and has a melting point of 115°C and has very low toxicity [12]. Apocynin reveals multiple biological aptitudes, such as neuroprotective features [13, 14], beneficial effects on arteriosclerosis and hypertension [15-17], inhibition of cancer cells migration [18] and scavenger properties [19]. Particularly, it displays powerful antiinflammatory and antioxidant effects in a variety of cell and animal models as well as in human studies [20, 21]. There is only one paper characterizing nebulized apocynin influence on humans [21].

Through inhibiting superoxide production, apocynin may affect decrease of  $H_2O_2$ ,  $NO_2^-$  and  $NO_3^-$  generation, as  $O_2^{\cdot-}$  is a pivotal element of ROS and NOS formation [22-24]. Nitric oxide (NO), is generated by nitric oxide synthase (NOS, EC 1.14.13.39), from L-arginine. In physiological concentrations, half-life time of endothelium-derived NO particle is about 1-30 s. *In vivo*, NO is easily oxidized to  $NO_2^-$ , which half-life in blood is 110s, and afterwards, transforms into stable nitrate  $NO_3^-$  (half-life time = 8 h) [25, 26]. In some study, significant correlations between EBC nitrite and selected lung function parameters, symptoms and airway hyper-reactivity is presented [27] while in other, that EBC nitrite was a good indicator for asthma severity [28]. Exhaled nitric oxide has the potential to be a valuable marker of inflammation at different lung depths, making it attractive as a noninvasive monitoring tool.

The most probable mechanism of the inhibitory effect of apocynin is the reactivity of apocynin radical with thiol compounds. Additionally, apocynin needs to be activated (oxidized) in the presence of  $H_2O_2$  and myeloperoxidase (MPO), abundant in neutrophils, to

inhibit the NADPH oxidase [29]. The oxidation effects in conversion to a dimer, which has been shown to be more efficient than apocynin itself monomer [18].

Through inhibiting source of ROS, apocynin has, among others, strong antiinflammatory properties. By preventing generation of the most serious radical -  $O_2^{\cdot-}$ , it obviates formation of other ROS as well as RNS (reactive nitrogen species). Hence, it may play important role in treatment of airway inflammatory disease, like bronchial asthma [21].

Although therapeutic strategies focusing on the elimination of superoxide have been developed in animal models and in clinical cases, the effects of such reagents are still unclear [11, 30-32]. Therefore, in this study, we investigated whether inhalation of apocynin may influence hydrogen peroxide, nitrite and nitrate concentrations in airways in healthy subjects. Simultaneously, we analyzed safety parameters and adverse events of nebulized apocynin in the applied dose.

## Material and methods

### Study Design

The study had a double-blind, placebo-controlled, *cross-over* design, consisted of 2 visits, separated 30 to 60 days. If during the first visit the drug was used, in the second visit a subject nebulized placebo or *vice versa*. The summary of the study design with the detailed characteristics of the performed analyses are shown in Fig. 1a. and 1b. Before and after procedure safety measures (arterial blood pressure, heart rate value, and cough scale) were performed.

The appropriate methods have been chosen for the determination of  $H_2O_2$ ,  $NO_2^-$  and  $NO_3^-$  in EBC to detect a significant difference in a healthy non-smoking population. The methods are imperfect, but accurate enough to display the differences between the state of airways before and after apocynin nebulization.

The study protocol was approved by the local Ethics Committee (no. RNN/12/08/KE) and written consent was obtained from every subject prior to the study.

### Subjects

Ten healthy, nonsmoking volunteers participated in the study (mean age 32 years, 6 men, 4 women, no atopy). This number of subjects is typical for phase I RCTs (randomized clinical trials) [21, 33, 34]. The volunteers had not suffered from any infectious diseases including upper respiratory tract infections for at least 3 months prior to the study (exclusion criterion). They were free of any medication and routine physical examination was normal (inclusion

criterion). The study has begun at 8 a.m. The volunteers were asked not to do any exercise for one day. They were free of any medication and routine physical examination was normal.

#### Exhaled Breath Condensate (EBC) collection

EBC was collected using a modification of the method described previously by Doniec et al. [35], and regarding ERS Task Force on EBC [7]. The subjects breathed spontaneously through a mouthpiece for 20 min. Each subject wore a nose clip during this procedure. EBC was collected by using a condenser (EcoScreen; Erich Jaeger Viasys; Hoechberg, Germany) that yielded nongaseous components of expiratory air. Subjects breathed through a mouthpiece connected to the condenser, and were asked to breathe at a normal frequency and tidal volume, wearing a nose clip, for a period of 20 min. Approximately 2 ml of condensate was collected and immediately stored at  $-80^{\circ}\text{C}$  in the laboratory for maximum 7 days, until the procedure of  $\text{H}_2\text{O}_2$ ,  $\text{NO}_2^-/\text{NO}_3^-$  measurement [36].

#### Apocynin inhalation

6 ml of apocynin of total dose 3 mg (0.5 mg/ml dissolved in sterile 0.9% NaCl as the study drug) [21] has been nebulized for 15-20 min. through the mouthpiece with using of a nose clip. A nebulizer Pulmo Aide AP-50 (DeVilbiss; Richmond, VA) was used (mass median aerosol diameter 3.1  $\mu\text{m}$ , output 0.3 ml/min.).

#### Determination of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in EBC

The  $\text{H}_2\text{O}_2$  concentration in EBC was measured according to the method applied previously by Nowak et al. [2] Briefly, 600  $\mu\text{l}$  of EBC was mixed with 600  $\mu\text{l}$  of HRP solution (1 U/ml) containing 100 mM homovanillic acid and was incubated for 60 min. at  $37^{\circ}\text{C}$ . Then, the sample was mixed with 150  $\mu\text{l}$  0.1 M glycine-NaOH buffer (pH 12.0) with addition of 25 mM EDTA. The homovanillic acid oxidation product as a measure of the amount of  $\text{H}_2\text{O}_2$  was determined spectrofluorimetrically using a Perkin Elmer Luminescence Spectrometer LS-50B (Norwalk, CT, USA). Excitation was at 312 nm and emission was measured at 420 nm. The lower limit of  $\text{H}_2\text{O}_2$  detection was 0.08  $\mu\text{M}$ . The intra-assay variability was 1-2.1% for standard solutions of 0.05-0.75  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . Data were expressed in  $\mu\text{M}$  [2].

#### Determination of nitrite ( $\text{NO}_2^-$ ) in EBC

Determination of  $\text{NO}_2^-$  with Griess solution was performed by micromethod, carried out in 98-well plates, according to Griess' method [37].  $\text{NO}_2^-$  standards (concentration range: 0.5–

12.5  $\mu\text{mol/l}$ ) were prepared freshly in deionized water and kept on ice prior to use. 60  $\mu\text{l}$  of  $\text{NaNO}_2$  standard or EBC were dispensed in duplicate. Control wells contained 60  $\mu\text{l}$  of deionized water. The volume was made up to 100  $\mu\text{l}$  with phosphate-buffered saline (PBS) pH 7.2. For colour development, the samples were incubated for 10 min. at room temperature with 40  $\mu\text{l}$  of Griess solution A (58.07 mmol/l sulphanilamide, Sigma, St. Louis, MO, USA in 2 M sulphuric acid) and then with 40  $\mu\text{l}$  of Griess solution B (38.58 mmol/l naphthylethylenediamide dihydrochloride in deionized water; Sigma, St. Louis, MO, USA). The absorbance was measured at 562 nm utilizing a microplate reader Sunrise (Tecan, Männedorf Germany). The concentration of  $\text{NO}_2^-$  in the samples was calculated utilizing the  $\text{NO}_2^-$  standard curves [37].

As  $\text{NO}_2^-$  and  $\text{NO}_3^-$  are present on every laboratory surface, including glassware and pipette tips, precautions were taken to avoid contamination of the sample. Any material that might come in contact with EBC, including devices used for collection, processing and assaying EBC was thoroughly rinse with highly pure (distilled/de-ionised) water [7].

#### Determination of nitrate ( $\text{NO}_3^-$ ) in EBC

We modified the method described by Dziedzic et al. [37], for measurement of  $\text{NO}_3^-$  using the NADPH- nitrate reductase (EC 1.6.6.2 from *Aspergillus* species, Sigma, St. Louis, MO, USA). Sixty microliters of EBC was added in duplicate to a flat bottom 96 well plate. Control wells contained 60  $\mu\text{l}$  of deionized water. The samples were mixed with 30  $\mu\text{l}$  of NADPH-nitrate reductase (250 mU/ml dissolved in deionized water) and 10  $\mu\text{l}$  of NADPH solution (0.625 mg/ml in deionized water, Sigma, St. Louis, MO, USA). The plates were incubated for 30 min. at room temperature. Then, the samples were mixed with Griess solutions and the absorbance was measured by microplate reader Sunrise (Tecan, Männedorf Germany).  $\text{NO}_3^-$  concentration was calculated utilizing the method described previously by Dziedzic et al. [37].

#### Determination of nitrite ( $\text{NO}_2^-$ ) in serum

The concentration of  $\text{NO}_2^-$  in serum was measured according to the method of Griess, described by Doganay et al. [38]. 100  $\mu\text{l}$  of serum was added in duplicate to an Eppendorf tube. Control wells contained 100  $\mu\text{l}$  of deionized water. The samples were mixed with 50  $\mu\text{l}$  of PBS (PH 7.2), 50  $\mu\text{l}$  of NADPH-nitrate reductase (250 mU/ml in deionized water) and 17  $\mu\text{l}$  of NADPH solution (0.625 mg/ml in deionized water). The plates were incubated for 30 min. in room temperature. Then, the samples were mixed with 125  $\mu\text{l}$  of Griess A solution



(incubated for 10 min.) and with 125 µl of Griess B solution. Afterwards, 250 µl of 10 % TCA were added, shaken vigorously, and centrifuged for 15 min., 20,000 x g. The absorbance was measured by microplate reader Sunrise (Tecan, Männedorf Germany).

#### Safety parameters

Peripheral blood differential counts were measured using an ABX Micros OT 45 system (Horiba ABX, Montpellier, France). Master-Laboratory Screen (Jaeger Master Screen Body System, Wuerzburg, Germany) was used for lung functional tests including forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), single breath carbon monoxide diffusing capacity corrected for hemoglobin and alveolar volume (TLCOc), according to ERS/ATS standards (2006).

The frequency of coughing was assessed using a 10 cm coughing visual analogue scale with the extremes marked 1 - 'I never cough' and 10 - 'I am coughing all the time'. The visual analogue scales were administered before and after apocynin/placebo nebulization [39].

#### Statistical analysis

The data from the study were analyzed utilizing Statistica software package (v.8.0; StatSoft Inc., Tulsa, OK). The distribution of all examined variables was checked for normal distribution by Kolmogorov-Smirnov test. For NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, and H<sub>2</sub>O<sub>2</sub> concentration data were normally distributed. A two tailed p-value<0.05 was considered to indicate statistical significance using student t-test. Data are expressed as mean ± SEM and standard deviation.

### **Results**

#### Safety measures

There were no significant changes in blood pressure, heart rate value and cough scale before and after apocynin or placebo application (p>0.05). No serious and non-serious adverse events were observed throughout the study.

Furthermore, we have analyzed an effect of apocynin inhalation on some parameters of blood differential counts (e.g.: white blood cells, red blood cells, hemoglobin, hematocrit, platelets, lymphocytes, monocytes and granulocytes) (Table 1). Neither apocynin nor placebo nebulization influenced blood differential counts.

Inhalation of apocynin or placebo aerosol had any effect neither on respiratory parameters (FEV<sub>1</sub>, FVC, PEF), nor on DLCO parameters (TLCOc/VA), (p>0.05), (Table 1).

### Hydrogen peroxide concentration in EBC

Analysis of breath condensates has shown an influence of apocynin inhalation on  $\text{H}_2\text{O}_2$  production in comparison to placebo inhalation.  $\text{H}_2\text{O}_2$  concentration after 60 minutes of apocynin inhalation comparing to placebo inhalation was significantly lower (mean:  $0.18 \mu\text{M}$  vs.  $0.31 \mu\text{M}$ ,  $p < 0.05$ ). Moreover, this trend remains after 120 min. after apocynin application ( $0.20 \mu\text{M}$  vs.  $0.31 \mu\text{M}$ ) (Fig. 2a).

### Nitrite and nitrate concentration in EBC

The mean concentrations of  $\text{NO}_2^-$  in EBC collected from volunteers were statistically significantly lower comparing to placebo, after 60 and 120 min. after nebulization ( $2.88 \mu\text{M}$  vs.  $3.44 \mu\text{M}$  and  $2.32 \mu\text{M}$  vs.  $3.3 \mu\text{M}$  respectively,  $p < 0.05$ ). Nevertheless, both apocynin and placebo caused parallel effect (Fig. 2b) on nitrite concentration in exhaled breath condensate. Apocynin caused significant changes in  $\text{NO}_3^-$  concentration in EBC as well. Moreover, we observed a significant decrease of  $\text{NO}_3^-$  concentration 60 and 120 min. after apocynin inhalation (Fig. 2c.), whereas placebo caused augmentation of  $\text{NO}_3^-$  concentration in these timepoints ( $6.8 \mu\text{M}$  after apocynin vs.  $14.4 \mu\text{M}$  after placebo and  $6.5 \mu\text{M}$  vs.  $14.9 \mu\text{M}$  respectively,  $p < 0.05$ ). Before placebo inhalation, the concentration of nitrate was higher than before apocynin nebulization.

### Serum - $\text{NO}_2^-$ concentration

The analysis of the whole group of volunteers showed that there is no nitrite concentration change in serum of healthy subjects after apocynin nebulization ( $p > 0.05$ ).

## **Discussion**

In this study, we investigated the effect of nebulized apocynin administration at the dose of 3 mg on simultaneously hydrogen peroxide, nitrite and nitrate generation as well as safety parameters in healthy subjects. Using EBC technique we have confirmed that apocynin decreases  $\text{H}_2\text{O}_2$  exhalation. Apocynin caused a significant decline of  $\text{H}_2\text{O}_2$  concentration after 60 minutes of inhalant administration, not causing any side-effects. Furthermore, the inhibitory potency of this drug last at least 2h after apocynin nebulization.

The use of apocynin as an inhibitor of the activation of the NADPH oxidase complex is based on the inhibition of the assembly process, as the migration of the p47phox component to the membrane is impeded in its presence [40]. It is also known that the oxidation of apocynin plays an important role in its inhibitory effect.

Thus our results stay in agreement with literature, as apocynin has been shown to act as a strong antiinflammatory agent. Muijsers et al. showed that apocynin inhibited peroxynitrite formation in murine macrophages model. Though this experiment was performed *in vitro*, apocynin revealed a potential value to limit peroxynitrite formation in inflammatory conditions *in vivo* [11]. Among others, in airways, there are some pivotal sources of superoxide radical – NADPH oxidase in inflammatory cells [41], inhibited by apocynin, mitochondrial sources [42, 43] and arachidonic acid (AA) metabolism [44].

Peters et al investigated the effect of nebulized apocynin on ozone-induced bronchial hyperresponsiveness *in vivo* in humans. They demonstrated that apocynin reduced ozone-induced airway hyperresponsiveness in mild asthmatics [21]. Furthermore, Hougee et al. observed that oral administration of apocynin had inhibitory effects on cyclooxygenases similar to the non-steroidal anti-inflammatory drug (NSAID) ibuprofen [45]. Nowak et al showed that only one third of never smoked subjects continuously exhale detectable amounts of  $H_2O_2$  [2], and additionally, that exhalation of  $H_2O_2$  by healthy never smoked subjects correlates with the ability of blood phagocytes to produce ROS in general [36]. Nevertheless, our results stay in agreement with Guatura's report [46], demonstrating that healthy nonsmoking subjects exhale detectable concentration of hydrogen peroxide. We showed here that it can be subsequently reduced by apocynin, not evoking any harmful effects. Such results may suggest that apocynin might be used to alleviate inflammatory process in some diseases.

Both  $H_2O_2$  and NO are associated with oxidative stress, which is defined as an imbalance between oxidants and antioxidants, and is thought to be involved in airway inflammation and respiratory diseases [47]  $H_2O_2$  is generated from superoxide anion by the action of superoxide dismutase in neutrophils, eosinophils, macrophages and epithelial cells, and in particular, by eosinophils in asthma [48-50]

Exhaled nitric oxide (eNO) is a marker of airway inflammation [1]. Although NO is produced in the airways by a variety of cells, large amounts may be generated from those involved in the acute and chronic inflammatory responses [51]. NO plays a role in pulmonary host defence mechanisms, and is thought to have both bactericidal and bacteriostatic effects [52]. Nitric oxide is difficult to measure because it is a free radical which reacts rapidly with oxygen, superoxide, water, thiols, amines, and lipids to form products with biochemical activities ranging from bronchodilation to cytotoxicity [53, 54]. Nitrates and nitrites are products of nitric oxide metabolism, which can be detected in EBC.

Since simultaneous  $\text{NO}_2^-$  and  $\text{NO}_3^-$  measurements provide better indication of NO production in respiratory system, in our study both of those parameters were investigated [37].

Unexpectedly, there were considerable differences in  $\text{NO}_2^-$  and  $\text{NO}_3^-$  concentrations after apocynin as well as after placebo nebulization. Though  $\text{NO}_2^-$  concentration slightly increased after 30 minutes of inhalation and after 60 min it decreased, the same effect has been observed when apocynin and placebo were nebulized. These results confirm previous data [29], which displayed that, apocynin at first stimulates ROS formation, however, after a certain period, the inhibition of ROS yield occurs. The same trend might appear in case of placebo because of the activation of alveolar phagocytes caused by mild exertion connected with EBC collection, or because of nebulization effect.  $\text{NO}_2^-$  may also originate from alveolar epithelium [55]. Still, there is not much data showing EBC time point study. Nevertheless, Bodini et al., [56] also observed that the levels of several analyzed parameters, i.e. nitrotyrosine, in EBC changed after placebo treatment at the two time points of the study. These results might suggest that there is a need to evaluate an influence of EBC collection on inflammatory parameter levels as an effort which might accompany EBC collection may also have an influence on EBC contents.

Furthermore, our results suggest, that apocynin causes significant decrease of  $\text{NO}_3^-$  concentration. Apocynin entailed gentle growth of  $\text{NO}_3^-$  level, as reported before [29], but then it firmly reduced it. These data confirm antiinflammatory capabilities of apocynin, which have been claimed in literature. Additionally, no adverse effects occurred during and after apocynin nebulization.

According to Szkudlarek et al. [36], ability of blood polymorphonuclear leukocytes (PMNs) to produce ROS, may mirror  $\text{H}_2\text{O}_2$  release from cells localized in the close neighborhood of lower airways epithelial lining fluid. Nevertheless, this rule is not reflected in case of nitrite. Our research displayed, that there was no significant alteration of  $\text{NO}_2^-$  concentration in blood serum after apocynin inhalant application. Additionally, no changes in blood differential counts have been noticed. This may suggest that nebulized apocynin does not diffuse into the blood, and acts locally [32, 57].

Apocynin did not affect respiratory parameters as well. No lung functional tests parameters have been changed after apocynin administration. Peters et al, [21] also showed that the change in  $\text{FEV}_1$  immediately followed the ozone exposure in asthmatics, was not significantly different between apocynin and placebo group.

The fact that apocynin has no influence on DLCO might show that it not causes changes in airways gas diffusion. These findings may have important clinical implications for the safety

of patients with inflammatory diseases like asthma or COPD. Nevertheless, further investigations, involving patients with airway inflammatory diseases, are needed, especially as apocynin does not cause any severe and non-severe adverse effects.

We are aware of several limitations that apply to our study. The study contained relatively small group of subjects (n=10), however, there are many significant studies on such few objects. Additionally, we shortly intend to continue extend our research.

We are also aware, that except for nitrite concentration, nitrate and hydrogen peroxide should have been investigated in serum. Nevertheless as apocynin showed not to infiltrate into blood and influence on  $\text{NO}_2^-$  concentration, we concluded that it is case might be omitted.

One of the current limitations of EBC measurements is the low concentration of many biomarkers so that their measurement is limited by the sensitivity of assays.

One relative disadvantage of EBC measurements is that they require a subsequent analysis and it is likely that there will be important advances in on-line detection of particular biomarkers using sensitive biosensors. On the other hand, an EBC collection allows detecting several biomarkers at one time, without harmful actions to the patients. It also could become a useful tool for monitoring and screening of healthy individuals for possible early pulmonary tissue damage. EBC collection is a completely noninvasive way of sampling the respiratory tract that can be repeated frequently within short intervals without adverse events with good reproducibility in EBC volume and mediator concentration for several tested markers

Moreover, the fact that there is not EBC collection standard might have triggered differences in research, dependently on technique and equipment applied [7]. Therefore, we adapted a modification of the method described previously by Nowak et al. [2], as in this study results have been repetitive.

The significant limitations in our study are the differences in baselines before nebulization.

Ideally, baseline values should not differ. The possible explanation of this fact might be variability among individuals. Nevertheless, the concentrations difference is not statistically significant.

In conclusion, we displayed that 3 mg of nebulized apocynin decreased  $\text{H}_2\text{O}_2$  and  $\text{NO}_3^-$  concentrations in EBC of healthy subjects. Nebulized apocynin was well tolerated and no adverse events were observed throughout the study. Thus, it might be a valuable antiinflammatory agent, acting locally in airways with very low toxicity and neutral to other physiological parameters. Nevertheless, there is a need to evaluate the antiinflammatory and safety potential of apocynin, not only in healthy subjects but also in the state of disease.

**Conflict of Interest:**

The authors declare that there are no conflicts of interest.

**References:**

- [1] Kharitonov SA, Gonio F, Kelly C, Meah S, Barnes PJ. Reproducibility of exhaled nitric oxide measurements in healthy and asthmatic adults and children. *Eur Respir J* 2003;21:433-8.
- [2] Nowak D, Kalucka S, Bialasiewicz P, Krol M. Exhalation of H<sub>2</sub>O<sub>2</sub> and thiobarbituric acid reactive substances (TBARs) by healthy subjects. *Free Radic Biol Med* 2001;30:178-86.
- [3] Ricciardolo FL, Di Stefano A, Sabatini F, Folkerts G. Reactive nitrogen species in the respiratory tract. *Eur J Pharmacol* 2006;533:240-52.
- [4] Masood A, Nadeem A, Mustafa SJ, O'Donnell JM. Reversal of oxidative stress-induced anxiety by inhibition of phosphodiesterase-2 in mice. *J Pharmacol Exp Ther* 2008;326:369-79.
- [5] Adcock IM, Caramori G, Chung KF. New targets for drug development in asthma. *Lancet* 2008;372:1073-87.
- [6] Ko FW, Leung TF, Hui DS. Are exhaled breath condensates useful in monitoring asthma? *Curr Allergy Asthma Rep* 2007;7:65-71.
- [7] Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, Becher G, van Beurden WJ, Corradi M, Dekhuijzen R, Dweik RA, Dwyer T, Effros R, Erzurum S, Gaston B, Gessner C, Greening A, Ho LP, Hohlfeld J, Jobsis Q, Laskowski D, Loukides S, Marlin D, Montuschi P, Olin AC, Redington AE, Reinhold P, van Rensen EL, Rubinstein I, Silkoff P, Toren K, Vass G, Vogelberg C, Wirtz H. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* 2005;26:523-48.
- [8] Rysz J, Stolarek RA, Luczynski R, Sarniak A, Wlodarczyk A, Kasielski M, Nowak D. Increased hydrogen peroxide concentration in the exhaled breath condensate of stable COPD patients after nebulized N-acetylcysteine. *Pulm Pharmacol Ther* 2007;20:281-9.
- [9] Zalba G, San Jose G, Moreno MU, Fortuno MA, Fortuno A, Beaumont FJ, Diez J. Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. *Hypertension* 2001;38:1395-9.
- [10] Cotter MA, Cameron NE. Effect of the NAD(P)H oxidase inhibitor, apocynin, on peripheral nerve perfusion and function in diabetic rats. *Life Sci* 2003;73:1813-24.

- [11] Muijsers RB, van Den Worm E, Folkerts G, Beukelman CJ, Koster AS, Postma DS, Nijkamp FP. Apocynin inhibits peroxynitrite formation by murine macrophages. *Br J Pharmacol* 2000;130:932-6.
- [12] Luchtefeld R, Luo R, Stine K, Alt ML, Chernovitz PA, Smith RE. Dose formulation and analysis of diapocynin. *J Agric Food Chem* 2008;56:301-6.
- [13] Tang LL, Ye K, Yang XF, Zheng JS. Apocynin attenuates cerebral infarction after transient focal ischaemia in rats. *J Int Med Res* 2007;35:517-22.
- [14] Wang Q, Tompkins KD, Simonyi A, Korthuis RJ, Sun AY, Sun GY. Apocynin protects against global cerebral ischemia-reperfusion-induced oxidative stress and injury in the gerbil hippocampus. *Brain Res* 2006;1090:182-9.
- [15] Holland JAaJ, D.K. Prevention of atherosclerosis using NADPH oxidase inhibitors. United States Patent 1999;5:902,831.
- [16] Meyer JW, Schmitt ME. A central role for the endothelial NADPH oxidase in atherosclerosis. *FEBS Lett* 2000;472:1-4.
- [17] Hamilton CA, Brosnan MJ, Al-Benna S, Berg G, Dominiczak AF. NAD(P)H oxidase inhibition improves endothelial function in rat and human blood vessels. *Hypertension* 2002;40:755-62.
- [18] Klees RF, De Marco PC, Salaszyk RM, Ahuja D, Hogg M, Antoniotti S, Kamath L, Dordick JS, Plopper GE. Apocynin derivatives interrupt intracellular signaling resulting in decreased migration in breast cancer cells. *J Biomed Biotechnol* 2006;2006:87246.
- [19] Heumuller S, Wind S, Barbosa-Sicard E, Schmidt HH, Busse R, Schroder K, Brandes RP. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. *Hypertension* 2008;51:211-7.
- [20] Hu L, Zhang Y, Lim PS, Miao Y, Tan C, McKenzie KU, Schyvens CG, Whitworth JA. Apocynin but not L-arginine prevents and reverses dexamethasone-induced hypertension in the rat. *Am J Hypertens* 2006;19:413-8.
- [21] Peters EA, Hiltermann JT, Stolk J. Effect of apocynin on ozone-induced airway hyperresponsiveness to methacholine in asthmatics. *Free Radic Biol Med* 2001;31:1442-7.
- [22] Kawasaki S, Sakai Y, Takahashi T, Suzuki I, Niimura Y. O<sub>2</sub> and reactive oxygen species (ROS) detoxification complex composed of O<sub>2</sub>-responsive NADH:rubredoxin oxidoreductase - flavoprotein A2 - desulfoferrodoxin operon enzymes, rubperoxin, and rubredoxin in *Clostridium acetobutylicum*. *Appl Environ Microbiol* 2009.



- [23] Timoshenko AV, Dubovskaya LV, Karvatskaya OD, Zharkov VV, Andre S, Gabius HJ. NO-dependent regulation of lectin- and menadione-induced H<sub>2</sub>O<sub>2</sub> production by cells from pleural effusions of lung cancer patients and by immune cells. *Int J Oncol* 1999;14:793-8.
- [24] Neill S, Bright J, Desikan R, Hancock J, Harrison J, Wilson I. Nitric oxide evolution and perception. *J Exp Bot* 2008;59:25-35.
- [25] Goligorsky MS. Endothelial nitric oxide synthase: from structure to function in one aspartic substitution. *Kidney Int* 2009;75:255-7.
- [26] Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic Biol Med* 2007;43:645-57.
- [27] Straub DA ER, Hall GI Correlation of nitrites in breath condensate and lung function in asthmatic children. *Pediatr Allergy Immunol* 2004;15:20-5.
- [28] Robroeks CM vdKK, Jöbsis Q. Exhaled nitric oxide and biomarkers in exhaled breath condensate indicate the presence, severity and control of childhood asthma. *Clin Exp Allergy* 2007;37:1303–11.
- [29] Vejrazka M, Micek R, Stipek S. Apocynin inhibits NADPH oxidase in phagocytes but stimulates ROS production in non-phagocytic cells. *Biochim Biophys Acta* 2005;1722:143-7.
- [30] Masamune A, Watanabe T, Kikuta K, Satoh K, Shimosegawa T. NADPH oxidase plays a crucial role in the activation of pancreatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 2008;294:G99-G108.
- [31] Muijsers RB, van Ark I, Folkerts G, Koster AS, van Oosterhout AJ, Postma DS, Nijkamp FP. Apocynin and 1400 W prevents airway hyperresponsiveness during allergic reactions in mice. *Br J Pharmacol* 2001;134:434-40.
- [32] Lafeber FP, Beukelman CJ, van den Worm E, van Roy JL, Vianen ME, van Roon JA, van Dijk H, Bijlsma JW. Apocynin, a plant-derived, cartilage-saving drug, might be useful in the treatment of rheumatoid arthritis. *Rheumatology (Oxford)* 1999;38:1088-93.
- [33] Zhao JJ SY, Dobashi K, Kawata T, Ono A, Yanagitani N, Kaira K, Utsugi M, Hisada T, Ishizuka T, Mori M. The relationship between oxidative stress and acid stress in adult patients with mild asthma. *J Investig Allergol Clin Immunol*. 2008;18(1):41-5.
- [34] Jorres R, Nowak D, Grimminger F, Seeger W, Oldigs M, Magnussen H. The effect of 1 ppm nitrogen dioxide on bronchoalveolar lavage cells and inflammatory mediators in normal and asthmatic subjects. *Eur Respir J* 1995;8:416-24.
- [35] Doniec Z, Nowak D, Tomalak W, Pisiewicz K, Kurzawa R. Passive smoking does not increase hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels in exhaled breath condensate in 9-year-old healthy children. *Pediatr Pulmonol* 2005;39:41-5.



- [36] Szkudlarek U, Maria L, Kasielski M, Kaucka S, Nowak D. Exhaled hydrogen peroxide correlates with the release of reactive oxygen species by blood phagocytes in healthy subjects. *Respir Med* 2003;97:718-25.
- [37] Dziedzic B, Mazanowska-Gajdowicz J, Walczewska A, Sarniak A, Nowak D. Comparison of cadmium and enzyme-catalyzed nitrate reduction for determination of NO<sub>2</sub>-/NO<sub>3</sub>- in breath condensate. *Clin Chim Acta* 2003;335:65-74.
- [38] Doganay S, Evereklioglu C, Er H, Turkoz Y, Sevinc A, Mehmet N, Savli H. Comparison of serum NO, TNF-alpha, IL-1beta, sIL-2R, IL-6 and IL-8 levels with grades of retinopathy in patients with diabetes mellitus. *Eye* 2002;16:163-70.
- [39] Leidy NK, Rennard SI, Schmier J, Jones MK, Goldman M. The breathlessness, cough, and sputum scale: the development of empirically based guidelines for interpretation. *Chest* 2003;124:2182-91.
- [40] Barbieri SS, Cavalca V, Eligini S, Brambilla M, Caiani A, Tremoli E, Colli S. Apocynin prevents cyclooxygenase 2 expression in human monocytes through NADPH oxidase and glutathione redox-dependent mechanisms. *Free Radic Biol Med* 2004;37:156-65.
- [41] Matthiesen S, Lindemann D, Warnken M, Juergens UR, Racke K. Inhibition of NADPH oxidase by apocynin inhibits lipopolysaccharide (LPS) induced up-regulation of arginase in rat alveolar macrophages. *Eur J Pharmacol* 2008;579:403-10.
- [42] Zmijewski JW, Lorne E, Banerjee S, Abraham E. Participation of mitochondrial respiratory complex III in neutrophil activation and lung injury. *Am J Physiol Lung Cell Mol Physiol* 2009.
- [43] Poderoso JJ. The formation of peroxynitrite in the applied physiology of mitochondrial nitric oxide. *Arch Biochem Biophys* 2009.
- [44] Hii CS, Ferrante A. Regulation of the NADPH oxidase activity and anti-microbial function of neutrophils by arachidonic acid. *Arch Immunol Ther Exp (Warsz)* 2007;55:99-110.
- [45] Hougee S, Hartog A, Sanders A, Graus YM, Hoijer MA, Garssen J, van den Berg WB, van Beuningen HM, Smit HF. Oral administration of the NADPH-oxidase inhibitor apocynin partially restores diminished cartilage proteoglycan synthesis and reduces inflammation in mice. *Eur J Pharmacol* 2006;531:264-9.
- [46] Guatura SB, Martinez JA, Santos Bueno PC, Santos ML. Increased exhalation of hydrogen peroxide in healthy subjects following cigarette consumption. *Sao Paulo Med J* 2000;118:93-8.

- [47] Psathakis K, Mermigkis D, Papatheodorou G, Loukides S, Panagou P, Polychronopoulos V, Siafakas NM, Bouros D. Exhaled markers of oxidative stress in idiopathic pulmonary fibrosis. *Eur J Clin Invest* 2006;36:362-7.
- [48] Ueno T, Kataoka M, Hirano A, Iio K, Tanimoto Y, Kanehiro A, Okada C, Soda R, Takahashi K, Tanimoto M. Inflammatory markers in exhaled breath condensate from patients with asthma. *Respirology* 2008;13:654-63.
- [49] Horvath I, Donnelly LE, Kiss A, Kharitonov SA, Lim S, Chung KF, Barnes PJ. Combined use of exhaled hydrogen peroxide and nitric oxide in monitoring asthma. *Am J Respir Crit Care Med* 1998;158:1042-6.
- [50] Conner GE, Salathe M, Forteza R. Lactoperoxidase and hydrogen peroxide metabolism in the airway. *Am J Respir Crit Care Med* 2002;166:S57-61.
- [51] Puthucherry ZA, Liu J, Bennett M, Trytko B, Chow S, Thomas PS. Exhaled nitric oxide is decreased by exposure to the hyperbaric oxygen therapy environment. *Mediators Inflamm* 2006;2006:72620.
- [52] Hucke C, MacKenzie CR, Adjogble KD, Takikawa O, Daubener W. Nitric oxide-mediated regulation of gamma interferon-induced bacteriostasis: inhibition and degradation of human indoleamine 2,3-dioxygenase. *Infect Immun* 2004;72:2723-30.
- [53] Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 1994;149:538-51.
- [54] Liu J, Thomas PS. Exhaled breath condensate as a method of sampling airway nitric oxide and other markers of inflammation. *Med Sci Monit* 2005;11:MT53-62.
- [55] Lundberg JON WE, Lundberg JM, Alving K. . Nitric oxide in exhaled air. *Eur Respir J* 1996;9:2671-80.
- [56] Bodini A, Peroni DG, Zardini F, Corradi M, Alinovi R, Boner AL, Piacentini GL. Flunisolide decreases exhaled nitric oxide and nitrotyrosine levels in asthmatic children. *Mediators Inflamm* 2006;2006:31919.
- [57] Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ. Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* 1994;11:95-102.

**Figures legend:**

Fig. 1a. Study design

Fig. 1b. Experimental diagram

Figure 2a.  $\text{H}_2\text{O}_2$  concentration in EBC of healthy nonsmokers (MEAN $\pm$  standard deviations).

Results are shown as comparison apocynin (3 mg) and placebo effects at given timepoints.

\*p<0.05

Figure 2b.  $\text{NO}_3^-$  concentration in EBC of healthy nonsmokers (MEAN $\pm$  standard deviations).

Results are shown as comparison apocynin (3 mg) and placebo effects at given timepoints.

\*p<0.05

Figure 2c.  $\text{NO}_2^-$  concentration in EBC of healthy nonsmokers (MEAN $\pm$  standard deviations).

Results are shown as comparison apocynin (3 mg) and placebo effects at given timepoints.

\*p<0.05

Safety parameters		Healthy controls			
		Placebo		Apocynin	
		Before	After	Before	After
<b>Pulmonary Function Tests</b>	<b>FEV<sub>1</sub> [L/s]</b>	4.2±3.6	4.3±3.5	4.3±3.5	4.4±3.5
	<b>FVC [L]</b>	4.8±4.1	4.9±4.0	4.5±4.1	5.0±4.0
	<b>PEF [L/min]</b>	9.5±8.6	9.3±9.0	9.4±8.2	9.6±8.3
	<b>TLCOc/VA [%]</b>	90.95±88.1	91.30±82.6	90.35±79	91.10±85.7
<b>Peripheral blood differential counts</b>	<b>WBC [10<sup>3</sup>/mm<sup>3</sup>]</b>	5.77±5.5	6.58±6.1	5.74±5.1	6.27±5.2
	<b>RBC [10<sup>6</sup>/mm<sup>3</sup>]</b>	5.00±4.6	4.62±4.9	4.88±4.5	5.01±4.6
	<b>HGB [g/dl]</b>	14.42±13.3	14.29±13.2	14.28±12.9	14.20±12.6
	<b>HCT [%]</b>	39.37±39.9	35.25±39.6	38.34±38.3	39.35±37.8
	<b>PLT [10<sup>3</sup>/mm<sup>3</sup>]</b>	224.80±192	230.80±200	223.60±190	227.50±202
	<b>LYM [10<sup>3</sup>/mm<sup>3</sup>]</b>	1.82±1.6	1.82±1.7	1.78±1.6	1.79±1.5
	<b>MON [10<sup>3</sup>/mm<sup>3</sup>]</b>	0.25±0.2	0.31±0.2	0.26±0.2	0.28±0.2
	<b>GRA [10<sup>3</sup>/mm<sup>3</sup>]</b>	3.76±3.3	4.51±4.0	3.70±3.1	4.18±3.4
<b>Blood pressure [mm Hg]</b>		117/79	119/79	114/73	119/76
<b>Heart rate value [per min.]</b>		71	69	69	71
<b>Cough [1-10]</b>		1	1	1	1

Table 1. Apocynin effects on safety parameters in group of 10 healthy subjects. PFTs parameters data shown are the median±1<sup>st</sup> QR; comparison of FEV<sub>1</sub>, PEF and FVC values are presented. No significant effect to PFTs parameters has been observed.

Peripheral blood differential counts are shown as the median±1<sup>st</sup> QR. No significant differences were found (p>0.05). No effects to blood pressure, heart rate value and cough have been observed.

