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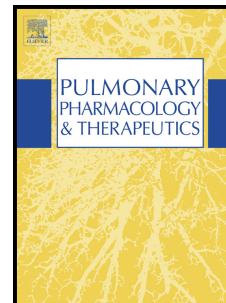
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APOCYNIN DECREASES HYDROGEN PEROXIDE AND NITRATE CONCENTRATIONS IN EXHALED BREATH IN HEALTHY SUBJECTS

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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 H₂O₂, NO₂⁻ and NO₃⁻ 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 NO₂⁻ serum level, cough scale control and blood pressure as safety parameters.

Apocynin caused reduction of H₂O₂ 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 NO₃⁻ 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 $115^\circ C$ 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°C in the laboratory for maximum 7 days, until the procedure of H_2O_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 μm , output 0.3 ml/min.).

Determination of hydrogen peroxide (H_2O_2) in EBC

The H_2O_2 concentration in EBC was measured according to the method applied previously by Nowak et al. [2] Briefly, 600 μl of EBC was mixed with 600 μl of HRP solution (1 U/ml) containing 100 mM homovanillic acid and was incubated for 60 min. at 37°C . Then, the sample was mixed with 150 μl 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 H_2O_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 H_2O_2 detection was 0.08 μM . The intra-assay variability was 1-2.1% for standard solutions of 0.05-0.75 μM H_2O_2 . Data were expressed in μM [2].

Determination of nitrite (NO_2^-) in EBC

Determination of NO_2^- with Griess solution was performed by micromethod, carried out in 98-well plates, according to Griess' method [37]. 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 μl of NaNO_2 standard or EBC were dispensed in duplicate. Control wells contained 60 μl of deionized water. The volume was made up to 100 μl with phosphate-buffered saline (PBS) pH 7.2. For colour development, the samples were incubated for 10 min. at room temperature with 40 μl of Griess solution A (58.07 mmol/l sulphanilamide, Sigma, St. Louis, MO, USA in 2 M sulphuric acid) and then with 40 μl 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 NO_2^- in the samples was calculated utilizing the NO_2^- standard curves [37].

As NO_2^- and 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 (NO_3^-) in EBC

We modified the method described by Dziedzic et al. [37], for measurement of 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 μl of deionized water. The samples were mixed with 30 μl of NADPH-nitrate reductase (250 mU/ml dissolved in deionized water) and 10 μl 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). NO_3^- concentration was calculated utilizing the method described previously by Dziedzic et al. [37].

Determination of nitrite (NO_2^-) in serum

The concentration of NO_2^- in serum was measured according to the method of Griess, described by Doganay et al. [38]. 100 μl of serum was added in duplicate to an Eppendorf tube. Control wells contained 100 μl of deionized water. The samples were mixed with 50 μl of PBS (PH 7.2), 50 μl of NADPH-nitrate reductase (250 mU/ml in deionized water) and 17 μl 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 μl 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 NO₂⁻, NO₃⁻, and H₂O₂ 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 (FEV₁, 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 H₂O₂ production in comparison to placebo inhalation. H₂O₂ concentration after 60 minutes of apocynin inhalation comparing to placebo inhalation was significantly lower (mean: 0.18 μM vs. 0.31 μM, p<0.05). Moreover, this trend remains after 120 min. after apocynin application (0.20 μM vs. 0.31 μM) (Fig. 2a).

Nitrite and nitrate concentration in EBC

The mean concentrations of NO₂⁻ in EBC collected from volunteers were statistically significantly lower comparing to placebo, after 60 and 120 min. after nebulization (2.88 μM vs. 3.44 μM and 2.32 μM vs. 3.3 μM 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 NO₃⁻ concentration in EBC as well. Moreover, we observed a significant decrease of NO₃⁻ concentration 60 and 120 min. after apocynin inhalation (Fig. 2c.), whereas placebo caused augmentation of NO₃⁻ concentration in these timepoints (6.8 μM after apocynin vs. 14.4 μM after placebo and 6.5 μM vs. 14.9 μM respectively, p<0.05). Before placebo inhalation, the concentration of nitrate was higher than before apocynin nebulization.

Serum - NO₂⁻ 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 H₂O₂ exhalation. Apocynin caused a significant decline of H₂O₂ 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 NO_2^- and 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 NO_2^- and NO_3^- concentrations after apocynin as well as after placebo nebulization. Though 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. 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 NO_3^- concentration. Apocynin entailed gentle growth of 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 H_2O_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 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 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 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 H_2O_2 and 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.

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Figures legend:

Fig. 1a. Study design

Fig. 1b. Experimental diagram

Figure 2a. H_2O_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. 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

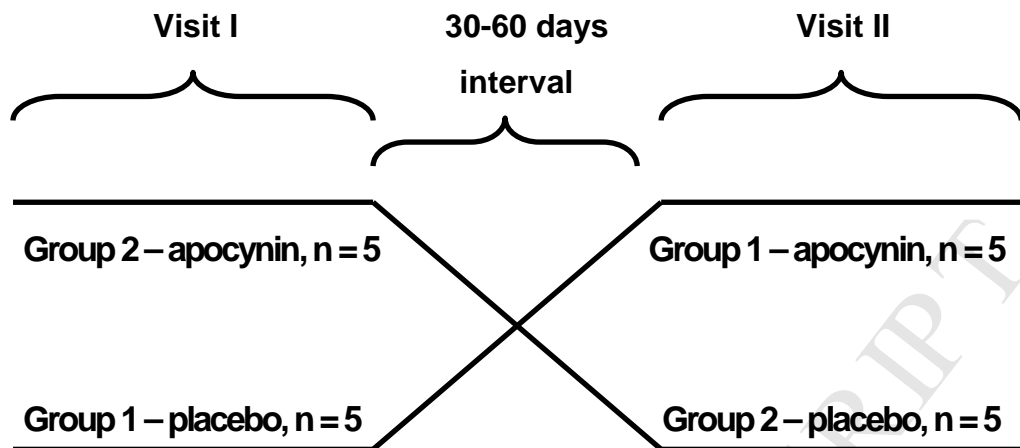
Figure 2c. NO_2^- concentration in EBC of healthy nonsmokers (MEAN \pm standard deviations). Results are shown as comparison apocynin (3 mg) and placebo effects at given timepoints. *p<0.05

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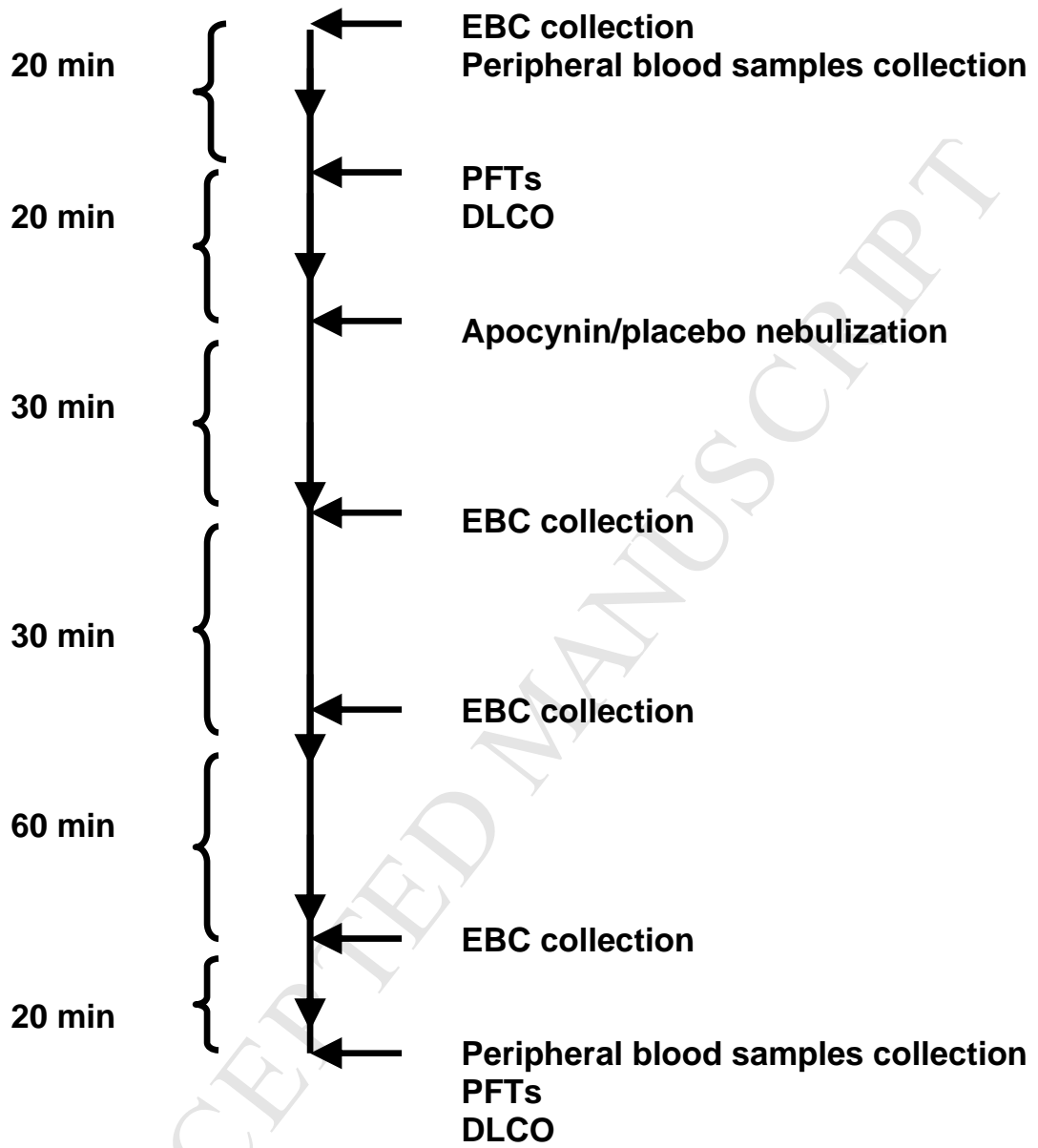
Safety parameters		Healthy controls			
		Placebo		Apocynin	
		Before	After	Before	After
Pulmonary Function Tests	FEV₁ [L/s]	4.2±3.6	4.3±3.5	4.3±3.5	4.4±3.5
	FVC [L]	4.8±4.1	4.9±4.0	4.5±4.1	5.0±4.0
	PEF [L/min]	9.5±8.6	9.3±9.0	9.4±8.2	9.6±8.3
	TLCOc/VA [%]	90.95±88.1	91.30±82.6	90.35±79	91.10±85.7
Peripheral blood differential counts	WBC [10³/mm³]	5.77±5.5	6.58±6.1	5.74±5.1	6.27±5.2
	RBC [10⁶/mm³]	5.00±4.6	4.62±4.9	4.88±4.5	5.01±4.6
	HGB [g/dl]	14.42±13.3	14.29±13.2	14.28±12.9	14.20±12.6
	HCT [%]	39.37±39.9	35.25±39.6	38.34±38.3	39.35±37.8
	PLT [10³/mm³]	224.80±192	230.80±200	223.60±190	227.50±202
	LYM [10³/mm³]	1.82±1.6	1.82±1.7	1.78±1.6	1.79±1.5
	MON [10³/mm³]	0.25±0.2	0.31±0.2	0.26±0.2	0.28±0.2
GRA [10³/mm³]	3.76±3.3	4.51±4.0	3.70±3.1	4.18±3.4	
Blood pressure [mm Hg]		117/79	119/79	114/73	119/76
Heart rate value [per min.]		71	69	69	71
Cough [1-10]		1	1	1	1

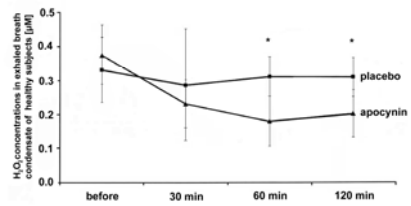
Table 1. Apocynin effects on safety parameters in group of 10 healthy subjects. PFTs parameters data shown are the median±1st QR; comparison of FEV₁, PEF and FVC values are presented. No significant effect to PFTs parameters has been observed.

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

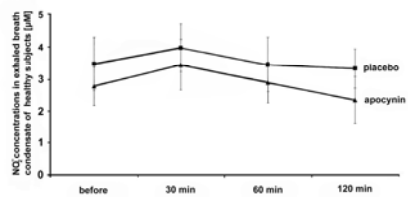


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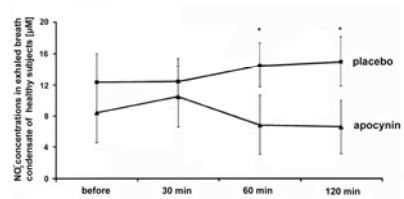




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