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A combination of N-acetyl cysteine and propolis attenuates oxidative-inflammatory parameters during COPD exacerbation

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Abstract. – OBJECTIVE: The aim of this study was to determine any differences in oxidative stress and inflammation parameters in COPD patients treated with either N-acetyl cysteine (NAC) alone or with NAC in combination with propolis (NACP).

PATIENTS AND METHODS: Forty COPD patients in the exacerbation phase were enrolled into the study and were treated with either NAC (NAC group; n=20) or NACP (NACP group; n=20) twice daily for one month. Redox status was determined by measuring superoxide anion ($O_2^{\cdot-}$), advanced oxidation protein products (AOPP), total oxidative status (TOS), prooxidative-antioxidant balance (PAB), malondialdehyde (MDA), ischemia modified albumin (IMA) and several other antioxidant markers: superoxide dismutase (SOD), paraoxonase 1 (PON1), total sulfhydryl groups (SHG) and total antioxidant status (TAS). Interleukins 6, 8 and 17 were measured as markers of inflammatory status.

RESULTS: Both groups had similar socio-demographic and clinical characteristics. After treatment significantly higher SHG [0.446 (0.395-0.516) *vs.* 0.292 (0.270-0.325), $p<0.001$] and significantly lower TOS – 50.6 [49.7-53.4 *vs.* 73.2 (50.9-84.6), $p<0.05$] – and IMA [0.650 (0.629-0.682) *vs.* 0.709 (0.667-0.756), $p<0.05$] – were found in the NACP group compared to the NAC group. Factorial analysis indicated a larger oxidative stress-inflammatory load in the NAC group after treatment.

CONCLUSIONS: From an oxidative stress and inflammatory status perspective, treatment with NACP was more successful than with NAC. The inclusion of propolis into therapy for COPD patients, especially those in the exacerbation phase, could prove beneficial.

Key Words:
COPD, Exacerbation, Redox status, Inflammation, NAC, Propolis.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by decreased airflow through the airways and symptoms in the form of shortness of breath, coughing and wheezing. The main reasons for increased burden caused by COPD globally are cigarette smoking and environmental pollution. The disease is characterized by frequent exacerbations leading to gradual deterioration of lung function and quality of life[1]. According to WHO data compiled in 2019 stated that COPD was the 3rd most frequent cause of death[2]. Official data from the Institute of Public Health of Serbia, published in the Health Statistical Yearbook (2019) showed that COPD was the 5th leading cause of death[3]. The highest mortality increase associated with obstructive lung disease (6.8%) was seen during the period 2010-2019[3]. Current COPD therapy is aimed at reducing the number and frequency of exacerbations. For this reason, research into novel therapeutic strategies, including auxiliary therapy is important[4]. Inflammation and oxidative stress play an important role in the pathophysiology of COPD[1,5]. IL-17, produced by Th17 cells, is a key cytokine for both the activation of neutrophils and their chemotaxis towards the lung. IL-17 induces the expression

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of other proinflammatory cytokines: TNF-α, IL-1β, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor and IL-8 and has a role in airway remodeling by stimulating production of the profibrotic cytokines IL-6 and IL-11[6,7]. Data show that patients with COPD have a significantly higher level of IL-17, which may be helpful in the evaluation of the severity of COPD and for prediction of clinical outcome[7].

Oxidative stress is an essential part of the inflammatory response culminating in the production of free radicals, i.e., reactive oxygen species (ROS). Free radicals activate transcription factors, such as NF-κB, activator protein-1 and other signal transduction pathways, such as mitogen-activated protein (MAP) kinases and phosphoinositide-3-kinase (PI-3K), leading to enhanced release of proinflammatory cytokines: TNF-α, IL-8, IL-1 and IL-6. ROS burden reduces the synthesis of elastin and collagen affecting the structure of lung extracellular matrix components, such as hyaluronate. Oxidative stress can induce the formation of lipid peroxides and the accumulation of its degradation products, including malondialdehyde, F2-isoprostanes, acrolein and 4-hydroxy-2-nonenal, toxic substances and powerful neutrophil chemoattractant. An increased level of these species results in acute inflammation, increased mucus secretion, pulmonary tissue damage and lead to clinical symptoms such as cough, bronchoconstriction and increased risk of infection. Excessive inflammatory signals coupled with high levels of ROS lead to pathological abnormalities and functional changes within the lungs[5,8].

N-acetyl cysteine (NAC) is water-soluble, resistant to oxidation, well absorbed and well tolerated. NAC catabolism leads to the formation of cysteine and reduced glutathione. Increased cysteine after NAC absorption arises from either NAC deacetylation or from NAC-catalyzed reduction of endogenous cysteine. Increased endogenous cysteine leads to stimulation of glutathione synthesis, a rise in the activity of glutathione-dependent enzymes and general antioxidant activity[9]. Besides its mucolytic properties, NAC maintains a balance between reduced and oxidized glutathione and protects the airways from oxidative stress and inflammation[8]. The recent finding of NAC's capability to act as an anti-inflammatory agent, especially towards the suppression of IL-6 activation is interesting from a COPD perspective[10].

Propolis is a complex mixture of different compounds that synergistically contribute to the plethora of its reported effects. The main constituents are polyphenols, primarily flavonoids, cinnamic acid derivates, such as caffeic acid (3,4-hydroxycinnamic acid) and its esters, terpenoids, coumarins, sesquiterpenes, steroids and amino acids[11]. Propolis has many biological effects including immunomodulatory, antibacterial, fungicidal, anti-inflammatory, analgesic and anesthetic. Bioflavonoids, the constituents of propolis, have both antioxidant and anti-inflammatory activity[12]. Flavonoids, especially galangin and quercetin, are primarily responsible for the anti-inflammatory effect of propolis by inhibition of both cyclooxygenase and lipoxygenase activity and the reduction of PGE_2 levels. Caffeic acid inhibits the synthesis of arachidonic acid and inhibits the activity of COX1, COX2, myeloperoxidase and lipoxygenase. Caffeic acid and its phenethyl ester (CAPE) inhibits T cell activation and cytokine production. CAPE is also a potent inhibitor of NF-κB. Propolis reduces lipid peroxidation induced by free radicals while increasing superoxide dismutase activity[13].

The aim of this current study was to determine the inflammatory and redox status of COPD patients during disease exacerbation (as baseline) and after one month of supplementation of either NAC alone or NAC in combination with propolis.

Patients and Methods

Study Subjects

The study included 40 subjects (12 women and 28 men) with an average age of 63.8 ± 10.9 (36-87) years and with COPD diagnosis in the exacerbation phase. Patients were recruited at the Clinic for Pulmonology, Clinical Centre of Serbia in Belgrade from November 2018 to May 2019. The study was approved by the Ethical Committee of the Clinical Centre of Serbia (November 2018; approval number 832/25). Patients' assignment into the two groups was randomized from the outset. Two physicians recruited the patients and used a random number table with numbers 1-20. Every number corresponded to defined therapy (NAC+propolis or NAC). The patients were numbered according to their order of arrival into the clinic. Exacerbation phase therapy included antibiotics (second generation cephalosporins,

quinolones or macrolides); corticosteroids when appropriate (methylprednisolone). Randomized patients with COPD were divided into two groups for additional antioxidative/mucolytic therapy: one group (20 patients) received a combination of N-acetylcysteine and standardized dry propolis extract (600 mg + 80 mg) – the NACP group, and the other group (20 patients) received N-acetylcysteine (600 mg) – the NAC group; twice daily for one month. All patients were informed about the study goals and procedures. Those that accepted to participate in the study signed an informed consent form.

Determination of Parameters of Oxidative Stress and Inflammation

Oxidative stress parameters were determined at the Department of Medical Biochemistry and the Department of Bromatology, Faculty of Pharmacy, University of Belgrade. Inflammatory parameters were determined at the Institute of Virology, Vaccines and Serum, Torlak, Belgrade, (Department of Research and Development). Blood was sampled twice; at the beginning of the study and after one month of supplementation. Redox status parameters were determined on an ILAB 300+ analyzer (Instrumentation Laboratory, Milan, Italy). Superoxide anion radical ($O_2^{\cdot-}$) (a prooxidant) and advanced oxidation protein products (AOPP) were measured. The Auclair and Voisin method was used to measure $O_2^{\cdot-}$. AOPP were determined by the Witko-Sarsat method. Total oxidative status (TOS), i.e., the concentration of total prooxidants of hydrogen peroxide (H_2O_2) and lipid hydroperoxides in blood, was determined by spectrophotometry using o-dianisidine. Determination of PAB (prooxidative-antioxidant balance) simultaneously measured the load of prooxidants and the antioxidant capacity. The concentration of malondialdehyde (MDA), as an end degradation product of lipid hydroperoxides, was measured as thiobarbiuric acid-reactive substance by spectrophotometry (based on the absorption maximum of the complex formed between malondialdehyde and other TBARS with thiobarbituric acid at 535 nm). The enzymes superoxide dismutase (SOD) and paraoxonase 1 (PON1), the concentration of total sulfhydryl groups (SHG), as well as the total antioxidant status (TAS) were determined. Plasma SOD activity was determined using epinephrine as a substrate, while PON1 activity was measured with paraoxon as a substrate. SHG concentration in serum was determined using 0.2 mmol/L 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), TAS was determined by spectrophotometry with ABTS as a chromogen. All methods have been modified, optimized, and implemented in our laboratory and published elsewhere[14].

Cytokine Level Determination

IL-17 was measured (in duplicate) by enzyme-linked immunoassay (ELISA), Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's recommendations. IL-6 and IL-8 were measured (in duplicate) by ELISA (Immunotools, Friesoythe, Germany), according to the manufacturer's recommendations. The locking step was performed with Reagent Diluent (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS v.20; SPSS Corp., Armonk, NY, USA). Differences in the parameters before and after supplementation within a group were tested using non-parametric Wilcoxon's paired test and between different groups with Student's *t*-test, where appropriate. Analysis of covariance was used in order to eliminate the influence of the differences in baseline values of redox status parameters and also to produce adjusted parameters values. Subsequent analysis was performed using nonparametric Kruskal-Wallis test and Mann-Whitney as its *post-hoc* test. Multiple linear regression (MLR) analysis was performed for nominal difference in 4 redox status parameters (AOPP, IMA, $O_2^{\cdot-}$ and SHG) which showed significant change after supplementation as dependent variables, and all sociodemographic, clinical and biochemical parameters as independent predictors, with backward selection. MLR enabled selection of the best model with correspondingly adjusted R^2 values, which gives percentage of model influence on dependent variable variability. Principal component analysis (varimax rotation) reduced the number of examined variables to a smaller number of factors, which were grouped according to similar variability. The data included normally distributed variables and non-normally distributed variables after the logarithmic transformation. Factors were extracted only if their eigenvalues were larger than 1. Variables with factor

loadings larger than 0.5 were used for factors interpretation and the number of factors were restricted to 3. From extracted factors the scores were calculated and used as separate variables in subsequent analysis. If the p-values <0.05 the result was considered statistically significant.

Results

Table I shows the socio-demographic and clinical characteristics of the patients. There were no statistically significant differences between the groups in terms of age, gender, smoking habits, alcohol consumption or the duration of COPD. The patients were COPD stage 2 or 3 according to GOLD criteria and had cardiovascular system-related comorbidities. Baseline CRP concentration, eosinophil ratio (%) FEV1, comorbidities and therapy distribution did not differ between the two groups. Physicians made the decision about antibiotic therapy according to CRP value (all patients with CRP above 30 mg/L received antibiotics as additional therapy regardless of their regular therapy for COPD). Purulent sputum was one of the main criteria for patients' inclusion into this study as NAC possesses mucolytic characteristics.

Redox Status Parameters

The most prominent result after supplementation of COPD patients during the exacerbation phase was a significant increase in SHG concentration in the NACP group. SHG concentration in the NAC group remained unchanged. Other redox status parameters did not change in the NACP group after supplementation. Significant increases in several prooxidant parameters; AOPP, $O_2^{\cdot-}$ and IMA were found after supplementation with NAC (Table II). Due to the initial more favorable redox status of the NAC group compared to the NACP group, we performed adjustments to the values of these parameters at the end of the study and found significant difference in several parameters (significantly higher SOD activity, TOS and IMA and significantly lower SHG in the NAC compared to the NACP group after supplementation).

Table I. Demographic characteristics in two experimental groups of patients.

Variable		NACP	NAC	p
N		20	20	/
Age, years		66.2 ± 11.0	62.6 ± 9.8	0.276
Men/Women, n (%)		15 (75)/5 (25)	13 (65)/7 (35)	0.490
Smokers, n (%)		7 (35)	8 (40)	0.744
Alcohol consumption, n (%)		2 (10)	5 (25)	0.212
Duration of COPD, years[#]		5 (2.2-9.5)	8 (4.5-11.5)	0.099
FEV1 (%)		54.7 ± 22.3	44.6 ± 13.2	0.134
CRP, mg/L		19.7 (8.3-38.0)	27.5 (2.0-80.0)	0.996
Eosinophils (%)		1.8 (1.6-4.4)	1.2 (0.6-9.2)	0.700
GOLD stage, n (%)	2	12 (60)	6 (30)	
	3	8 (40)	14 (70)	0.056
Comorbidities n (%)				0.682
Arterial hypertension		11 (55)	9 (45)	
Cardiovascular disease&		7 (35)	3 (15)	
Diabetes Mellitus		2 (10)	2 (10)	
Therapy, n (%)				0.451
Bronchodilators[†]		12 (60)	14 (70)	
Corticosteroids^		5 (25)	8 (60)	
Antibiotics‡		8 (40)	7 (35)	
Antihypertensive		11 (55)	5 (25)	
Statin		2 (10)	1 (5)	
Biguanidine		2 (10)	1 (5)	
Antiagregatory/anticoagulant		2 (10)	5 (25)	
Antianginose/antiaritmics		1 (5)	3 (15)	

NACP- N-acetylcysteine + propolis group; NAC- N-acetylcysteine group. Data presented as means and SD, or percentage, except parameter with [#]Medians (25.-75%) because of non-normal distribution; p-Student t-test (age) or Chi-square test (frequency data) or Mann-Whitney U test ([#]); [†]One or more types of bronchodilators such as LAMA-long-acting muscarinic antagonists, LABA long-acting beta agonists, methylxantines; ^Corticosteroids: methylprednisolone; [‡]Antibiotics: cephalosporines 2[nd] generation, quinolones, macrolides, amoxicilline; &-atrial fibrillation, angina pectoris.

Table II. Comparison of parameters of oxidative stress and antioxidant protection in two groups at the beginning of the study and after one months of supplementation.

Parameter	Baseline		After 1 month supplementionl		
	NACP	NAC	NACP	NAC	*p*
SOD, U/L	53 (26-111)	114 (98-133)^{aa}	64 (50-104)	110 (99-123)^{cc}	< 0.001
SHG, mmol/L	0.371 (0.251-0.495)	0.263 (0.220-0.329)	0.446 (0.395-0.516)^{a}	0.292 (0.270-0.325)^{ccc}	< 0.001
TAS, µmol/L	730 (476-1032)	894 (714-1128)	785 (738-850)	808 (759-871)	0.333
PON1, U/L	283 (136-693)	386 (276-639)	345 (221-710)	465 (361-706)	0.102
AOPP, µmol/L	53.5 (40.0-78.5)	45.5 (35.5-77.5)a	61.2 (51.3-74.7)	56.6 (50.4-76.3)^{b}	0.046
PAB, U/L	104 (86-120)	82 (72-101)^{a}	97 (88-105)	87 (81-97)	0.049
MDA, µmol/L	5.30 (4.63-5.78)	4.67 (3.96-5.74)	4.99 (4.94-5.06)	4.95 (4.88-5.06)	0.647
Superoxide anion, µmol/L	30.5 (19.0-76.0)	22.0 (15.5-33.0)^{a}	34.2 (31.9-41.2)	32.5 (31.1-34.6)^{b}	0.019
TOS, µmol/L	51.2 (30.7-83.3)	79.7 (48.1-99.7)^{a}	50.6 (49.7-53.4)	73.2 (50.9-84.6)c	0.042
IMA, AU	0.693 (0.588-0.839)	0.556 (0.501-0.714)^{a}	0.650 (0.629-0.682)	0.709 (0.667-0.756)^{b,c}	0.030

Abbreviations: TAS, total antioxidative status; SHG, total sulfhydryl groups; SOD, superoxide dismutase ; PON1, paraoksonase 1; AOPP, advanced oxidation protein products; PAB, prooxidative-antioxidant balance; O_2.–;superoxide anion radical; MDA, malondialdehyde; AU – absorbance unit; *p*-Kruskal-Wallis test; $^{a, aa}p$ <0.05, 0.01 *vs*. NACP baseline, respectively; ^{b}p <0.05 *vs*. NAC baseline; $^{c, cc, ccc}p$ < 0.05, 0.01, 0.001 *v*s. NACP after 1 month supplementation.

Inflammatory Markers

Of the four inflammatory cytokines measured in this study, only IL-8 in the NACP group showed a significant increase after supplementation. Baseline IL-8 did not differ between both two groups (Table III). In the NAC group IL-17 was higher both at baseline and after supplementation compared with the NACP group.

Spearman's non-parametric correlation analysis (data not shown here) between inflammatory markers and redox status parameters after supplementation revealed significant positive correlation between IL-6 and IL-8 in both groups; NACP (ρ=+0.682, p<0.01) and NAC groups (ρ= +0.840, p<0.01). In the NACP group IL-6 inversely correlated with MDA (ρ= –0.515, p<0.05), while in the NAC group TAS directly correlated with IL-6 (ρ= +0.566, p<0.05) and IL-8 (ρ= +0.570, p<0.05).

MRL selected the best models by combining nominal difference in 4 redox status parameters revealing 46.1%, 69.0%, 82.2% and 36.0% influence of dIMA, $dO_2^{\cdot-}$, dAOPP and dSHG, respectively. Supplementation significantly influenced dIMA, $dO_2^{\cdot-}$ and dSHG while it didn't show relation with dAOPP level (Table IV).

Principal component analysis focused on redox status and inflammatory parameters. The results are presented in Table V. Kaiser-Meyer-Olkin measure of sampling adequacy (0.502) and Bartlett's test of sphericity (χ^2 = 212, p<0.001) indicated that factors selection was adequate. Three extracted factors explained 59% of the total vari-

Table III. Inflammatory parameters in two groups at the beginning of the study and after one months of supplementation.

Parameter	Baseline		After 1 month supplementation		
	NACP	NAC	NACP	NAC	*p*
IL6	26.0 (12.8-69.1)	25.0 (17.3-95.0)	20.0 (13.4-62.2)	20.7 (15.2-86.7)	0.629
IL8	87.9 (59.4-126)	73.2 (44.8-184)	119.0^{a} (57.1-240)	71.6^{c} (43.4-192)	0.041
IL17	11.2 (10.3-18.6)	20.4^{aaa} (17.1-25.2)	12.0 (9.1-15.7)	20.5 ccc (15.7-25.2)	< 0.001

p-Kruskal-Wallis test; $^{a, aaa}p$<0.05, 0.001 *vs*. NACP baseline, respectively. $^{c, cc, ccc}p$ < 0.05, 0.01, 0.001 *vs*. NACP after 1 month supplementation.

Table IV. Multiple linear regression (MLR) analysis models (backward selection) for difference (d) of redox status parameters caused by NAC-related supplementation.

Parameter/model	Unstandardized coefficients		Standardized coefficients	p
	B	Std. Error	Beta	
dIMA (adj. $R^2 = 0.461$)				
Age, years	0.008	0.003	0.483	0.011
Gender	0.237	0.079	0.526	0.007
IL8, pg/mL	-0.001	0.000	-0.341	0.048
SHG, mmol/L	-0.524	0.195	-0.459	0.013
PON1 IU/L	0.000	0.000	0.369	0.040
PAB IU/L	-0.004	0.002	-0.467	0.028
TOS µmol/L	0.002	0.001	0.681	0.011
NAC/NACP supplement type	**-0.186**	**0.088**	**-0.471**	**0.049**
dO_2^- (adj. $R^2 = 0.690$)				
SHG, mmol/L	61.2	30.0	0.233	0.051
PAB IU/L	-0.506	0.216	-0.266	0.027
Superoxide anion, µmol/L	-0.808	0.115	-0.802	< 0.001
NAC/NACP supplement type	**-26.7**	**10.2**	**-0.298**	**0.015**
dAOPP (adj. $R^2 = 0.822$)				
Gender	20.7	8.1	0.196	0.035
IL8, pg/mL	0.000	0.000	0.861	< 0.001
AOPP, µmol/L	-0.477	0.158	-0.239	0.005
PAB, U/L	0.391	0.163	0.198	0.023
MDA, µmol/L	8.627	3.390	0.201	0.017
dSHG (adj. $R^2 = 0.360$)				
SH grupe mmol/L	-0.473	0.161	-0.468	0.006
PAB U/L	-0.003	0.001	-0.357	0.031
NAC/NACP supplement type	**-0.164**	**0.055**	**-0.473**	**0.006**

All parameters included in models are baseline values; dIMA, dO_2^-, dAOPP, dSHG: difference in redox status parameters caused by supplementation, respectively; adj. R^2: adjusted correlation coefficient produced by the best model selected by MLR analysis.

ance of all parameters. The first factor, which we termed inflammatory-protein factor, included positive loadings of IL-6, IL-8 and AOPP and explained 26% of the total variance. The second factor was associated with positive loadings of TAS and negative loadings of SHG and O_2^- and we termed it prooxidant-antioxidant factor. This factor explained 17% of the total variance. The third factor was termed inflammatory-oxidative stress factor and it explained 16% of the total variance. It demonstrated positive loadings of PAB and TOS and negative loadings of IL-17. We also saved scores produced from three factors in PCA analysis and compared them between the NACP and NAC groups after supplementation. We found a significantly higher ranking of the third factor (inflammatory-oxidative stress factor) in the NAC group compared with the NACP group (p=0.043, Mann-Whitney U test).

Table V. Extracted factors by PCA.

Factors	Included variables	Factor variability
Inflammatory-protein factor	IL-6 (0.933) IL-8 (0.942) AOPP (0.932)	26%
Prooxidant-antioxidant factor	SHG (-0.734) TAS (0.560) O_2^- (-0.701)	17%
Inflammatory-oxidative stress factor	IL-17 (-0.709) PAB (0.704) TOS (0.521)	16%

Abbreviations: PCA, principal component analysis; AOPP, advanced oxidation protein products; PAB, prooxidant antioxidant balance; SH, sulphydryl groups. TAS, total antioxidant status; TOS, total oxidant status; IL-6,8,17 – interleukines 6,8,17.

Discussion

In this study we examined the oxidative-inflammatory status of COPD patients in the exacerbation phase of the disease and the effects of NAC (alone or in combination with propolis) after one month of supplementation in the subsequent

stable phase of the disease. During COPD exacerbation a decrease in antioxidant capacity occurs, either because of the increased demand for antioxidants or because of their diminished production due to the disease chronicity and whole-body exhaustion. Antioxidant depletion may be associated with increased release of ROS from neutrophils during their "oxidative burst", which is part of the immune system's combat mode against invading pathogens[15]. NAC is able to react with the electrophilic groups of free radicals because of the free cysteine thiol group that acts as an antioxidant. In order to efficiently neutralize reactive groups, the cellular balance between reduced and oxidized glutathione must be maintained high; the homeostatic GSH to GSSG ratio is about 100)[16]. The amino acid cysteine is a major determinant of GSH synthesis. Therefore, as NAC increases the amount of cysteine, either by deacetylation of NAC or by the reduction of endogenous cysteine[17-19], it is clear why supplementation with NAC could be important and desirable in a situation when GSH demand is very high. An increase in endogenous cysteine leads to a stimulation of glutathione synthesis and a subsequent increase in antioxidant activity[20]. In addition, NAC has a mucolytic effect, by reducing disulphide bonds (S-S) to free sulfhydryl groups (SH), which can no longer participate in cross-linking. NAC affects the elasticity and viscosity of mucus in addition to its production and secretion. This mucolytic effect is associated with a significant reduction in exacerbations[15,21]. Calzetta et al[10] stated that NAC has anti-inflammatory potential. One of the beneficial effects of propolis extract is its anti-inflammatory effect, derived from different flavonoids, above all galangins. Galangins inhibit two proinflammatory enzymes; inducible nitric-oxide synthase (iNOS) and the inducible isoform of cyclooxygenase enzyme (COX2), which reduces synthesis of large amounts of NO and proinflammatory prostaglandins during acute inflammation, respectively[22]. Caffeic acid phenethyl ester (CAPE), which is one of the components within propolis, inhibits the release of arachidonic acid from the cell membrane and reduces the activity of COX1 and COX2. In addition, CAPE enhances the anti-inflammatory effect of galangin[8,23]. During acute COPD exacerbation, GSH levels are reduced due to insufficient response of reduced γ-glutamylcysteine synthetase (γ-GCS) activity to oxidative stress or due to direct depletion by ROS released by activated neutrophils[24]. Zeng et al[25] showed that during acute exacerbation, in addition to the level of GSH, SOD activity also reduced, resulting in an imbalance between oxidative stressors and antioxidants. After oral administration, NAC is deacetylated to cysteine, which is a precursor for glutathione synthesis[26]. NAC's effects depend both on the dose and the route of administration. Even at a low dose of 600 mg once per day, a temporary increase in GSH was observed. Using a higher dose of 600 mg three times a day, a permanent increase in GSH was found[21,27]. Herein, subjects were treated with 600 mg of NAC or a combination of 600 mg NAC and 80 mg propolis extract twice daily, which is considered a low dose. Calzetta et al[10] noted that higher dosage enabled greater anti-inflammatory effects of NAC, especially towards IL-6 inhibition. Herein, we found a trend of increased SHG, although the statistically significant increase was seen only in the NACP group. We propose that this was connected with the activity of propolis. Our results agree with those by Zhao et al[28], since propolis increased SHG. Propolis components artepillin and drupanin, are capable of directly neutralizing ROS and to prevent GSH depletion. In addition, pinocembrin and quercetin, the most abundant flavonoids of propolis, are known to induce gene transcription of gamma-glutamylcysteine synthetase (γ-GCS) which is responsible for GSH synthesis[29].

Our previous study[30] regarding COPD patients and their redox status in different phases of the disease (exacerbation and stable, i.e., hospital discharge) revealed significant depletion of measured antioxidant parameters (SOD, TAS, PON1) and increased prooxidants ($O_2^{\cdot-}$, TOS, PAB, AOPP) in the exacerbation phase compared with healthy controls. COPD patients from our previous study needed hospitalization because of exacerbation severity. In our current study the patients were not hospitalized. This could, at least in part, explain the difference in results. Moreover, COPD patients from our earlier study did not receive any additional therapy except for conventional antibiotics and corticosteroids.

Some antibiotics and corticosteroids can affect oxidative stress by altering enzyme activity. Some may inhibit glucose-6-phosphate dehydrogenase (G6PD) which plays an important role in maintaining a high NADPH/$NADP^+$ ratio in cells. NADPH is important for the regeneration of GSH from GSSG. Since there was no significant difference in initial therapy between the two groups its potential impact on both groups was likely to be the same[31-33]. SOD is a very important

enzyme acting against free radicals. It analyses the transformation of $O_2^{\cdot-}$ to H_2O_2 and molecular oxygen. Data on SOD activity in COPD patients in previous studies[25,34,35] are diverse. Zeng et al[25] noticed decreased SOD activity in patients with COPD. Montaño et al[34] found the opposite. Lei et al[35] showed that the effects of NAC were tissue-specific, and that the application of NAC had no significant effect on plasma SOD activity. Although in our study there was no significant change in SOD activity in both groups over time, a comparison of the two groups showed that slightly higher SOD activity was found in the NACP group while in the NAC group there was a slight decrease. It is important to note that baseline values in the NAC group were significantly higher than in the NACP group. The ratio remained the same even after adjustment. Our results are in agreement with those from Curti et al[36], who performed an animal study with brown propolis supplementation. A significant increase in SOD-1 activity was noted, while catalase and glutathione peroxidase activities remained unchanged. The proposed mechanism of SOD-1 activation is explained either through an increase in free radicals, as a consequence of the stimulation of this specific defense mechanism directly or through activation of NF-κB by propolis.

TAS measures the total reactivity of all reducing substances in the plasma, representing the first line of defense against free radicals in the systemic circulation. COPD patients generally have reduced TAS, especially during exacerbations[37-39] as in our previous study[30]. The TAS decrease was evident and pronounced at the point of hospital discharge when in a clinically stable state. Some *in vitro* studies have shown that NAC increases TAS[40]. However, Zukowski et al[41] found NAC led to TAS reduction in blood whilst TAS was elevated in target tissue in rats. Propolis prevented a decrease in TAS in an animal study[42], in a model of kainic acid-mediated excitotoxicity. We found that TAS did not change significantly in either group, although we noticed a slight increase in the NACP group and a minor fall in the NAC group (Table II). This finding was unexpected, but it could be explained by an initial TAS decrease caused by an acute disease process and later on NAC and/or propolis initiated antioxidant level normalization. Several prooxidants (AOPP, $O_2^{\cdot-}$, PAB, IMA) were significantly increased in the NAC group compared to baseline while the decrease in prooxidants (PAB, MDA, TOS, IMA) did not reach statistical significance. No difference in the above-mentioned parameters was noted in the NACP group compared to baseline values (Table II).

Treatment with propolis resulted in significantly lower TOS and IMA values at the end of the study. Pascual et al[43] showed that propolis has strong antioxidative (free radical scavenging) activity against alkoxy radicals and to a lesser extent against $O_2^{\cdot-}$. Salehi et al[44] explained the relationship between the structure of propolis' flavonoids and their activity as both $O_2^{\cdot-}$ and hydroxyl radical scavengers and chain radical reactions disruptors. The mechanism being the donation of hydrogen atoms to the peroxy radicals, transforming them to flavonoid radicals that react with free radicals. Polyphenolic compounds reduce the activity of enzymes involved in the formation of ROS such as xanthine oxidase, protein kinase and NADPH oxidase while increasing the levels of SOD and GSH. This could explain, at least in part, a more powerful oxidative stress diminishing effect of NAC-propolis combination compared to NAC alone.

Among the inflammatory markers measured in this study (IL-6, IL-8 and IL-17), only IL-8 increased significantly in the NACP group after supplementation compared to its baseline value. Zhang et al[45] found that high IL-8, but not IL-6 or TNF-α, persisted even after COPD stabilization following disease exacerbation. This finding is not completely understood, but it is likely that the mechanisms which modulate IL-8 expression are different from the other known cytokines measured. IL-17 correlates with COPD severity and its value is inversely correlated with airflow restriction measured by spirometry[46]. Using this background information, we can conclude that the NAC group of patients, randomly assigned to only NAC treatment, had COPD exacerbation slightly more severe compared to patients in the NACP group, according to our measurements (Table III).

MLR analysis, tailored to explain the influence of other parameters on redox status differences caused by supplementation, showed (among the models tested) that both NACP and NAC significantly influenced variability in IMA, $O_2^{\cdot-}$ and SHG change, whilst the AOPP change remained treatment type-independent (Table IV). Factorial analysis (Table V) enabled us to group a large number of variables into three factors which explained almost 60% of variability in all the examined parameters. The third factor (inflammatory-oxidative stress fac-

tor) after transferred in PCA generated score, was significantly higher in the NAC group compared to the NACP group.

This result implies larger disease sequelae loadings in the NAC group compared to NACP group. Ran et al[47] found disturbances in amino acid and lipid metabolism and consequential changes in energy producing metabolic processes together with redox imbalance in COPD patients leading to local and systemic inflammation through the NF-kB pathway activation, and *vice versa*. This partially explains our findings regarding the higher burden of inflammation-oxidative stress in the NAC patients. NAC in combination with propolis proved to be a more appropriate choice of therapy for COPD exacerbation phases.

The limitations of this study include: the small number of patients, which could be partially excused by the by the complexity and number of parameters measured and the overall concept of the study. The concept needs to be re-examined in a larger study in order to gain further confidence in the results.

Conclusions

The current COPD therapy is not capable to completely treat neither the cause nor disease symptoms. Therapy supplementation with NAC and propolis could be a rational choice for disease management.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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