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A new antioxidant formulation reduces the apoptotic and damaging effect of cigarette smoke extract on human bronchial epithelial cells

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Abstract. – OBJECTIVE: In this study we evaluated the possible protective effect of an antioxidant formulation containing microfiltered milk derived polypeptides, Curcumin, Vitamin B2, Carnitine and N-Acetyl-cysteine (NAC) in an *in vitro* model of chronic obstructive pulmonary disease (COPD).

MATERIALS AND METHODS: Human bronchial epithelial cells (16HBE) were used in this study. Cells were treated for 24 h in the presence or absence of 10% of cigarette smoke extract (CSE) and in the presence or absence of antioxidant formulation. We evaluated cell viability by MTT assay, reactive oxygen species by flow cytometer and quantitative analysis of gene expression by Real-time PCR.

RESULTS: The data obtained showed a significant increase of cell viability in CSE-exposed cells and a significant reduction of reactive oxygen species (ROS) production compared to cells treated with only CSE. The antioxidant effects of formulation were confirmed by a decrease of inflammatory cytokines genes IL-1β, IL-6, TNFα, nitric oxide synthase gene (NOS2) and through an induction of antioxidant genes such as heme oxygenase 1 (HO-1), nuclear transcription factor erythroid 2 (NRF2) and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α).

CONCLUSIONS: The results suggest that antioxidants combination plays a protective role on oxidative stress and inflammation, in an *in vitro* model of COPD, activating key genes in response to oxidative stress and decreasing the cytokines responsible for the inflammatory pathways.

Key Words:
Antioxidants, Heme oxygenase, Inflammation, COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease characterized by airflow limitation, with a high morbidity and mortality. Epidemiological studies have shown that smoking is the most important pathogenic factor of human COPD ranking fourth in the cause of mortality worldwide[1,2].

The airflow limitation is frequently gradual and correlated with an irregular inflammatory response of the lung to noxious particles or gases[3]. Smoking cigarettes is the principal cause of COPD, but other causes might increase the risk of disease in non-smokers such as maternal smoking, childhood asthma and respiratory infections, previous tuberculosis, outdoor air pollution, exposure to second-hand smoke and genetic cause[4,5].

Cigarette smoke contains oxidant molecules, such as hydrogen peroxide, peroxynitrate and peroxynitrite[6], leading to airway epithelial cells injury, neutrophils, macrophages and lymphocytes recruitment[7]. Reactive oxygen species (ROS) represent one of the key pathophysiological triggers in the above-described mechanisms, and are responsible for parenchymal injury, if not sufficiently counterbalanced by antioxidant factors[8].

Previous studies showed that various antioxidant agents, dietary natural product-derived polyphenols and endogenous antioxidant scavenging systems, can scavenge free radicals and oxidants, regulate the expression of redox and glutathione

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biosynthesis genes, chromatin remodeling, and inflammatory gene expression[9,10-14].

In this work we evaluated the effect of a commercially available formulation (React-On, Thompson Ltd., Rome, Italy), containing microfiltered milk derived polypeptides, Curcumin, Vitamin B2, Carnitine and N-Acetyl-cysteine (NAC)[15-21], in epithelial bronchial cell line exposed to smoke cigarette extract.

Materials and Methods

Cell Cultures

Human bronchial epithelial cells (16HBE) were purchased from ATCG. The cells were maintained in culture Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA), supplemented with 10% of fetal bovine serum (FBS) (Gibco), 0.5% gentamycin (Gibco, Rockville, MD, USA), 1% glutamine (Gibco, Rockville, MD, USA) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. 16HBE cells were treated for 24 h in the presence or absence of cigarette smoke extract (CSE), respectively with "React-On" (100 μg/ml), Carnitine (1 mM), Curcumin (5 μM), Vitamin B2 (200 μM), N-Acetyl Cysteine (NAC 1 mM).

Preparation of Cigarette Smoke Extract (CSE)

For the *in vitro* studies we used cigarettes containing 10 mg of tar, 0.7 mg nicotine and 10 mg of carbon monoxide per cigarette. The derived-cigarette smoke was bubbled through a vacuum pump in 10 ml of phosphate buffered saline (PBS). The obtained solution was filtered through a 0.22 μm filter and then diluted to be used in each experiment. The concentration of CSE used in the different experiments was equal to 20%, 10%, 5% and 2.5% of the final volume used for each assay.

Measurement of Cell Viability

Cells were cultured in 96-well plates and treated for 24 h with different concentrations of CSE, respectively 20%, 10%, 5% and 2.5% and subsequently with React-On (100 μg/ml), Carnitine (1 mM), Curcumin (5 μM), Vitamin B2 (200 μM), N-Acetyl Cysteine (NAC 1 mM) in the presence or absence of 10% CSE. Successively, the medium was replaced by a solution containing bromide 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and incubated for 3 h at 37°C. Finally, it was added dimethylsulfoxide (DMSO) to all wells and the plate was read in a spectrophotometer at λ 570 nm.

Measurement of Reactive Oxygen Species (ROS)

Reactive oxygen species formation (ROS) was determined cytofluorimetrically using the MUSE Oxidative Stress kit (Millipore, Billerica, MA, USA). Briefly, 10 μl of cell suspension in 1X Muse assay buffer was added to 190 μL of working solution reagent MUSE® oxidative stress. The samples were vortexed for 3-5 s and then incubated for 30 min at 37°C, subsequently read by Muse™ Cell Analyzer (Millipore, Billerica, MA, USA).

RNA Extraction and Real-Time PCR

Trizol reagent (Life Technology, Milan, Italy) was used to extract total RNA. The extracted mRNA was subsequently converted into cDNA through a kit containing the reverse transcription (Life Technology, Milan, Italy). The quantitative analysis was performed using the One-Step Real-time PCR instrument using the SYBR Green PCR master mix (Life Technology, Milan, Italy). The primer sequences are shown in Table I. The level of expression of its mRNA was calculated using the comparative method ΔΔCt-2 as previously described[22-24].

Table I. PCR primers used.

Gene	Primer Forward	Primer Reverse
HO-1	GTGCCACCAAGTTCAAGCAG	CACGCATGGCTCAAAAACCA
GAPDH	AGACACCATGGGGAAGGTGA	TGGAATTTGCCATGGGTGGA
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-6	GAAAGCAGCAAAGAGGCACT	TTTCACCAGGCAAGTCTCCT
NRF2	TCAGCGACGGAAAGAGTATGA	CCACTGGTTTCTGACTGGATGT
PGC1α	GGTGCAGTTTTGCCAAGGAG	TTCCTTGGGGTCCAGACAGA
NOS2	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
TNFα	AAGCACACTGGTTTCCACACT	TGGGTCCCTGCATATCCGTT

Statistical Analysis

Data were presented as mean ± standard deviation (SD) of n=4 experiments performed in triplicate. The statistical significance ($p<0.05$) of differences between experimental groups was determined by analysis of *t*-test. Newman-Keuls Post hoc test was used to validate ANOVA.

Results

Effect of CSE on Cell Viability

In order to estimate preliminarly the concentration of CSE to be used, a viability test was performed respectively with 20%, 10%, 5% and 2.5% of CSE for 24 h. These set of experiments showed a significant decrease in cell viability following 20% and 10% CSE treatment (Figure 1) ($p < 0.05$). Therefore, we used a concentration of 10% of CSE for all the following experiments.

React-On Effect on Cell Viability

To evaluate the effects of React-on following exposure to CSE, cells were treated in the presence and absence of 100 μg/ml of React-On for 24 h. In addition, the effects on cell viability of different components of the React-On formulation as Curcumin, Carnitine, NAC and vitamin B2, were evaluated. Surprisingly, React-On was the only treatment conferring protection to cells from the cytotoxic effects of CSE (Figure 2). In fact, our results showed a significant increase in cell viability compared to cells treated with only CSE ($p < 0.05$).

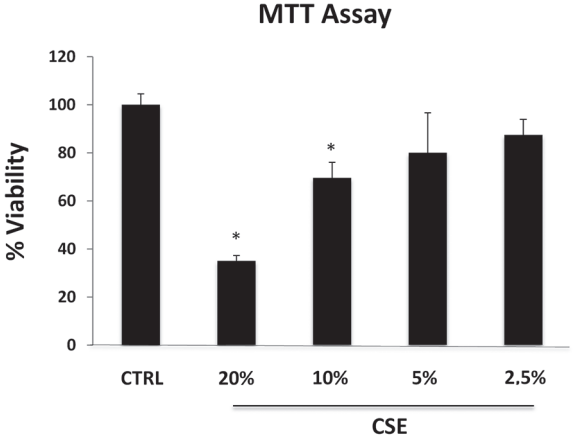

Figure 1. Viability assay measured by MTT assay after 24 h of treatment. CSE = cigarette smoke extract. *$p < 0.05$ to CTRL.

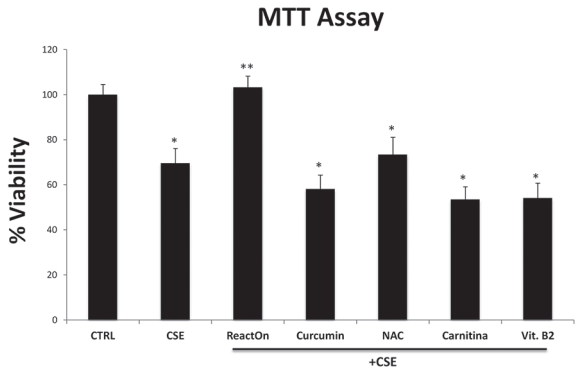

Figure 2. Viability assay measured by MTT assay after 24 h of treatment. *$p < 0.05$ to CTRL; **$p < 0.05$ to CSE.

Measuring the Formation of Reactive Oxygen Species

Figure 3 shows the results of ROS measurement. In particular, our results showed that ROS formation is induced by CSE ($p < 0.05$) and that treatment with React-On was able to decrease ROS levels ($p < 0.05$), demonstrating a protective effect from oxidative stress induced by CSE.

Expression of Cytokine Genes IL-1β, IL-6 and TNFα

In order to assess the grade of inflammation caused by CSE, we evaluated the expression of pro-inflammatory cytokine genes, interleukin 1β (IL-1 β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα). Figure 4 shows that CSE treatment was able to increase cytokines gene levels ($p < 0.05$ for all cytokines) whereas treatment with CSE and React-on led to a decrease of IL-1β, IL-6 and TNFα gene expression ($p < 0.05$).

React-On Induces HO-1, NRF2, PGC-1α and Reduces iNOS Gene Expression

Figure 5 shows that CSE and React On cotreatment were able to increase heme oxygenase-1 (HO-1) and transcription factor nuclear factor erythroid-2 (NRF2) genes levels, compared to CSE treatment alone. Moreover, cotreatment with React-On and CSE resulted in a decrease of inducible nitric oxide synthase gene (NOS2 or iNOS) and a significant increase of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α).

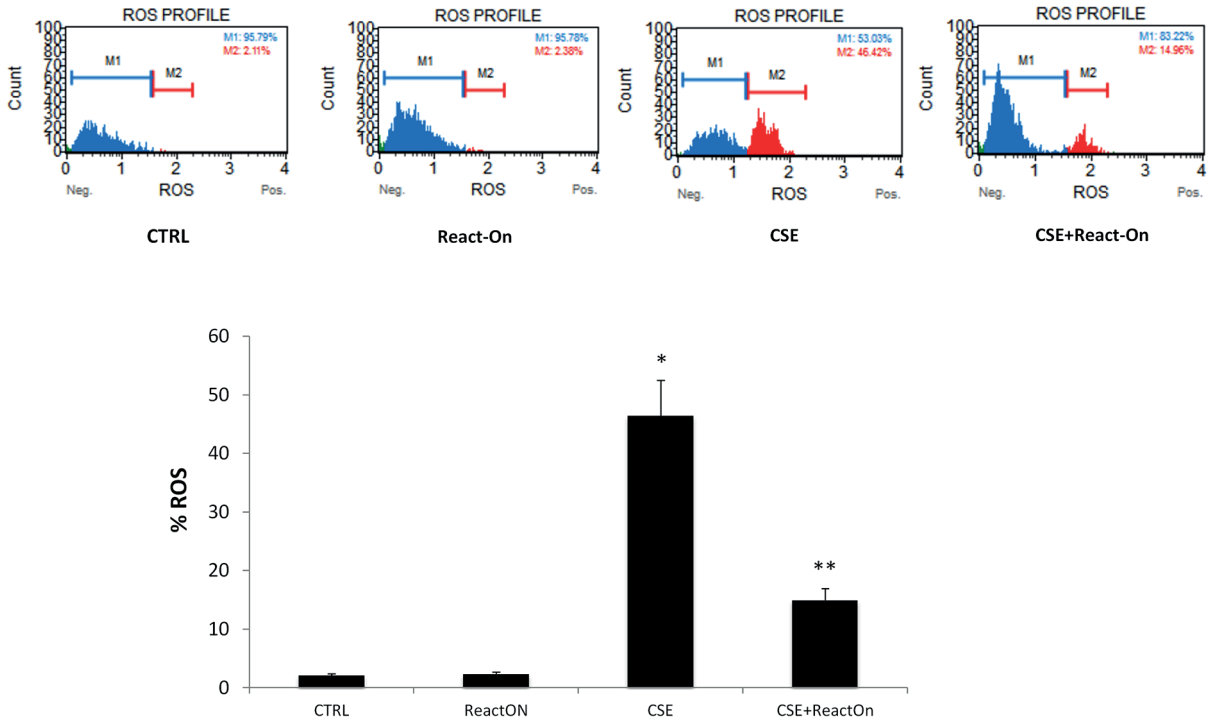

Figure 3. Effect of CSE and React-On on the ROS production analyzed by flow cytometric assay. *$p < 0.05$ to CTRL; **$p < 0.05$ to CSE.

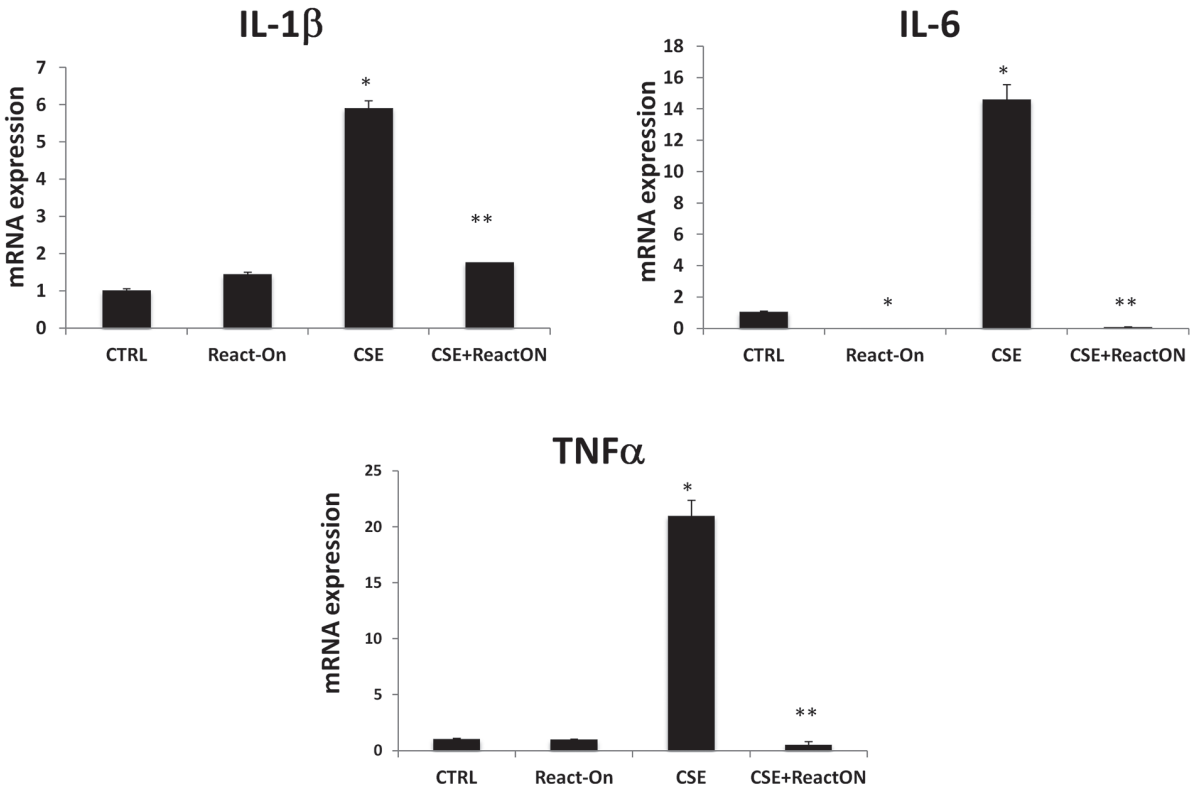

Figure 4. Effect of CSE and React-On on cytokines genes IL-1β, IL-6 and TNFα analyzed by Real-time PCR. *$p < 0.05$ to CTRL; **$p < 0.05$ to CSE

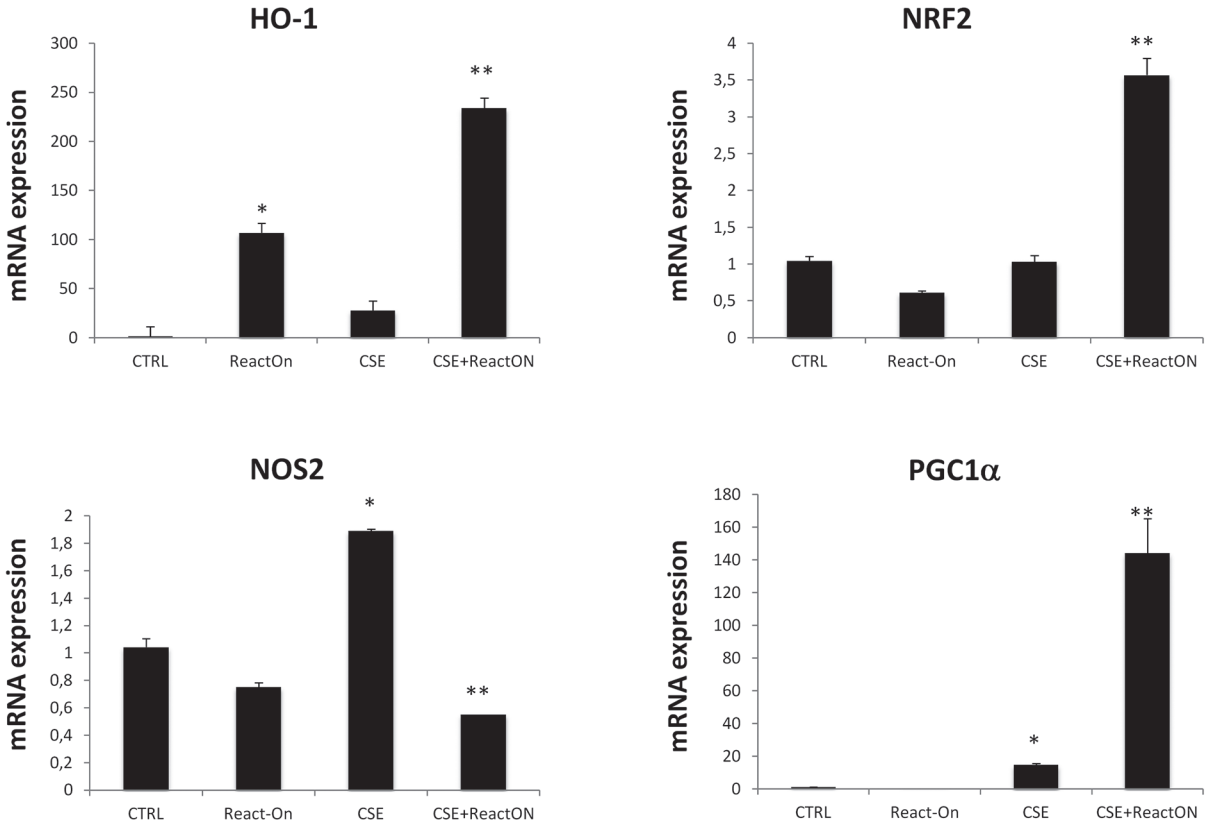

Figure 5. Effect of CSE and React-On on HO-1, NRF2, PGC-1α and iNOS gene expression analyzed by Real-time PCR. **p* < 0.05 to CTRL; ***p* < 0.05 to CSE.

Discussion

In this study we evaluated the possible protective capacity of React-On in an *in vitro* model of chronic obstructive pulmonary disease (COPD), emphysema and chronic bronchitis, reproducing the bronchial epithelium exposed to cigarette smoke[25,26]. From the data collected and consistently with previous studies, it appears that the administration, *in vitro*, of a medium containing a cigarette smoke extract was able to reduce significantly cell viability, increasing both ROS formation and inflammatory cytokines levels, such as IL-1β, IL-6 and TNFα[27,28].

The React-On formulation contains, in addition to polypeptides of microfiltered milk, Curcumin, Vitamin B2, Carnitine and NAC. These molecules are frequently present as metabolic cofactors having an important function in response to oxidative stress[10-13].

In our investigations, the above substances were also analyzed individually, in order to obtain a comparative analysis with the complete formulation.

The results, obtained after the treatment with React-On, Curcumin, Vitamin B2, Carnitine and NAC, surprisingly reveal that React-On was the only treatment able to significantly restore cell viability in CSE exposed cells. Consequently, we investigated the effect of React-On on oxidative stress induced by CSE. In this regard, the formation of ROS was measured following treatment with React-On in the presence or absence of the CSE. The data obtained showed that React-On was able to significantly reduce the production of ROS compared to cells treated with only CSE, confirming the reduction of oxidative stress induced by the product.

In order to assess the biochemical mechanism by which React-On reduces oxidative stress, it was examined the expression of HO-1 and NRF2 genes. It has been shown previously, that the expression of these genes is essential in characterizing the endogenous pathway response of cells to oxidative stress[29-34]. React-On was able to increase both genes, suggesting that the decrease of oxidative stress, observed in our results, is mediated by the induction of these genes and HO-

1 enzymatic activity[33,34]. Also, treatment with React-On following CSE exposure was able to decrease the expression of these genes confirming an anti-inflammatory role of this product. Noteworthy, our data showed that React-On induced peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) gene, which is a marker of mitochondrial biogenesis and its overexpression is associated to an increased energy production, fundamental for the cellular antioxidant response[35].

Conclusions

React-On plays a protective role on oxidative stress and inflammation in an *in vitro* model of COPD, emphysema and chronic bronchitis by reducing the formation of ROS, activating key genes in response to oxidative stress and to decrease the cytokine responsible for the inflammatory pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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