DNA damage protection by bulk and nano forms of quercetin in lymphocytes of patients with chronic obstructive pulmonary disease exposed to the food mutagen 2-amino-3-methylimidazo [4,5-f]quinolone (IQ).

Khaled Habas[1], Mhamoued Abdulmwli[1], Eşref Demir[2], Badie K. Jacob [3], Mojgan Najafzadeh[1], Diana Anderson[1]

[1]Division of Medical Sciences, Faculty of Life Sciences, University of Bradford, Richmond Road, Bradford, West Yorkshire, BD7 1DP, UK.

[2] Giresun University, Faculty of Engineering, Department of Genetics and Bioengineering, 28200-Güre, Giresun, TURKEY.

[3] Bradford Royal Infirmary, Bradford Teaching Hospitals NHS Foundation Trust, Duckworth Lane, Bradford, West Yorkshire, BD9 6RJ, UK.

* To whom correspondence should be addressed:

Tel: + 44 (01274) 233569, E-mail: d.anderson1@bradford.ac.uk

Keywords: 2-amino-3-methylimidazo [4,5-f]quinolone, DNA damage, endogenous antioxidant catalase, exogenous antioxidant flavonoid quercetin, Micronuclei.

Abstract

Chronic obstructive pulmonary disease (COPD) in humans, describes a group of lung conditions characterised by airflow limitation that is poorly reversible. The airflow limitation usually progresses slowly and is related to an abnormal inflammatory response of the lung to toxic particles. COPD is characterised by oxidative stress and an increased risk of lung carcinoma. The 2-amino-3-methylimidazo [4,5-f]quinoline (IQ) is one of a number of mutagenic/carcinogenic heterocyclic amines found mainly in well-cooked meats which are thus part of the regular diet. Antioxidants are very important in order to protect the cells against oxidative damage. The aim of the present study was to assess the effects of IQ on the level of DNA damage and susceptibility to a potent mutagen in peripheral blood cells of COPD patients. DNA damage and the frequency of micronuclei were evaluated using the Comet and micronucleus assays, respectively. Differential expressions of both mRNA and protein of the endogenous antioxidant enzyme catalase were evaluated with quantitative polymerase chain reaction (qPCR) and Western blot analysis, respectively. Furthermore, the effect of bulk and nano forms of quercetin and their combination with IQ were examined. Results of the present study clearly demonstrated that micronucleus (MNi) frequency in the peripheral blood lymphocytes exhibited a positive correlation with the DNA damage as evident from the different Comet assay parameters. Increase of the endogenous antioxidant catalase also showed there was a stimulation of this enzyme system by IQ. Whereas, the endogenous antioxidant quercetin significantly reduced oxidative stress in COPD patients and healthy individuals.

Introduction

Oxidative stress has important implications for numerous events of chronic obstructive pulmonary disease (COPD) pathophysiology and has been shown to be one of the major features in the pathogenesis of COPD (Rabe et al., 2007). The sources of increased oxidative stress in the respiratory compartment of COPD patients has been shown to be associated with an increased burden of inhaled oxidants from environmental exposures and increased generation of reactive oxygen species (ROS) by several inflammatory and structural cells of the airways (Rahman, 2005), Such an increase in the number of reactive nitrogen species (RNS) which are released during leukocyte and macrophage production (Rahman and Adcock, 2006). ROS are highly reactive molecules which are produced from oxygen metabolism and can damage cell structures such as, nucleic acids and proteins resulting in alterations in their functions (Birben et al., 2012). The results of oxidative stress can occur by direct or indirect ROS-mediated damage of nucleic acids and proteins and are associated with carcinogenesis (Trachootham et al., 2009).

Studies have reported that cigarette smoking is the main risk factor in COPD patients, but up to one-third of COPD patients have never smoked, implying that there are other factors involved (Varraso and Camargo, 2014; Yoshida and Tuder, 2007). Comparatively little attention has been paid to these other factors, such as intake and their alteration could decrease the global burden of COPD (Varraso and Camargo, 2014). A high intake of meat, mainly red and processed meat has been related to an increased risk of a number of chronic diseases such as cancer (Zheng and Lee, 2009). Heterocyclic aromatic amines (HCAs) are a group of mutagenic and/or carcinogenic compounds found in cooked meats which play a critical role in the etiology of human cancer (Felton et al., 2007a; Knize and Felton, 2005). These compounds are formed during the cooking process of meat from the reaction of creatinine and amino acids and can also generate ROS (Kizil et al., 2011). It has also been

shown that they can cause oxidation of proteins and DNA, leading to oxidative stress, DNA damage and can alter biological functions (Felton et al., 2007b; Knize and Felton, 2005). A study has shown that one potential mechanism between processed meat intake and the risk of COPD is due to nitrites to added meat products as preservatives and colour fixatives (Jakszyn et al., 2004). These nitrites could generate reactive nitrogen species (RNS) which increase inflammatory procedures in the airways and lung parenchyma, resulting in DNA damage and nitrosative stress (Varraso and Camargo, 2014). Another study showed that nitrosative stress can affect pulmonary function and is associated with the pathogenesis of COPD (Ricciardolo et al., 2006). A further study has suggested another possible mechanism of advanced glycation end-product (AGEs). This protein can occur naturally and is formed during heat processing and has been found to be associated with pro-inflammatory actions (Vlassara and Uribarri, 2014). The 2-Amino-3-methylimidazo [4,5-f] quinolone (IQ) is one of several HCAs carcinogens and is classified by the International Agency of Research on Cancer (IARC) as 2A (Jakszyn et al., 2004).

The balance between these probably carcinogenic compounds and the protective actions of the antioxidant defences system, particularly those derived from intake, might play a critical role in pulmonary function and the ultimate development of COPD (Varraso and Camargo, 2014). The cellular redox homeostasis is maintained by a complex endogenous antioxidant defence system, and includes endogenous antioxidant enzymes such as catalase. It is well established that the antioxidant defence system in humans is intricate and should aid to control the levels of ROS while permitting the valuable roles of ROS to perform cell signalling and redox regulation (Halliwell, 2011). The exogenous antioxidants derived from fruit and vegetables have shown that they support the endogenous antioxidant defence. The flavonoid antioxidants, like quercetin are presently considered to be one of the main exogenous antioxidants. Clinical studies imply that eating a diet rich in fruit, vegetables,

whole grains, legumes, and omega-3 fatty acids can help humans in disease prevention (Domej et al., 2014a).

In the present study, the DNA damage was evaluated using the Comet and micronucleus assays in peripheral blood lymphocytes of patients with COPD disease and healthy individuals, using the nanoparticle (NP) and bulk versions of the flavonoid , quercetin. The mRNA and protein expression levels of catalase were also examined using qPCR and Western blot. We also examined the changes in oxidant-antioxidant imbalance of the catalase gene related to counteracting the aggregation of oxidative stress in human lymphocytes in patients with COPD. Thus, trying to map pathways affected by IQ exposure and suggesting a possible mechanism for its cytotoxic behaviour.

Materials and methods

Chemicals

IQ (CAS No. 5346-56-5) were purchased from Toronto Research Chemicals, North York, Ont., Canada (Lage, Germany) and quercetin (≥95% purity; CAS No. 117-39-5) were purchased from the Sigma-Aldrich Company Ltd. (Sigma Chemical Ltd., Gillingham, UK). All other chemicals used in the different tests were from Sigma-Aldrich Company Ltd. (Sigma Chemical Ltd., Gillingham, UK). Prior to their use IQ and quercetin were dissolved in DMSO and excipient, respectively. The structural formulae of the tested chemicals are shown in Figure 1.

Collection of samples

After informed consent, approximately 10 ml heparinised blood was taken by venepuncture from the COPD patients at the Department of Ambulatory Care Unit (ACU), Bradford Royal Infirmary (BRI) Hospital, Bradford, UK. Healthy control individuals' samples were taken

within the University of Bradford, UK (West Yorkshire, UK). Ethical permission was obtained from Leeds East Ethics Committee (Reference no: 12/YH/0464) and the University of Bradford's Sub-Committee for Ethics in Research involving Human Subjects (Reference no.: 0405/8).

Isolated Lymphocytes

Three millilitres whole blood was diluted 3:3 with 0.9% saline and total of this dilution was carefully layered on top of three ml of Lymphoprep (Axis-Shield, Norway) in 15 ml falcon tubes. The tubes were centrifuged for 20 minutes at 800 × g. Lymphocytes were harvested, washed with 10 ml saline and centrifuged again for 15 min at 500 × g at room temperature. Cells were re-suspended in Roswell Park Memorial Institute (RPMI) 1640 Medium and used for the *in vitro* experiments.

Cell viability

The viability of lymphocytes was checked before and after treatment at the concentrations chosen for each experiment. Viability was measured by the Trypan blue exclusion test indicating intact cell membranes (Phillips, 1973). Ten microliters of 0.05% Trypan blue were added to 10 µl of cell suspension and the percentage of cells excluding the dye was estimated using an improved Neubauer haemocytometer (Poolzobel et al., 1992).

Treatment of lymphocytes

Isolated lymphocytes (2×10^5) from COPD patients (n=10) and healthy controls (n=10) were treated without metabolic activation for 30 minutes (Comet assay). There was also a negative control (untreated cells) and positive control (60 µM H_2O_2). For (qPCR and Western blot) in RPMI at 37 ºC either with different form (bulk and nano) of quercetin (40µM) in the presence of IQ (140 µM) for 2 h. Lymphocytes from healthy individuals served as the negative control

group. After treatment, the cells were used in the Comet assay to detect DNA damage primarily single-strand breaks and alkali labile sites, and in the MNi assay to detect cytogenetic DNA damage. The antioxidant enzyme catalase was evaluated by qPCR and Western blot assays to quantify mRNA and protein levels respectively.

Comet assay

The Comet assay was processed with slight modifications according to Tice (Tice et al., (2000) and OECD, (2016)). In brief, the lymphocytes were mixed with prewarmed 0.5% low melting agarose (LMP) (Invitrogen, Paisley, UK: 15517-022). This cell suspension was transferred to slides pre-coated with 1% normal melting point (NMP) agarose, and covered with a coverslip. The slides were solidified on an ice block for 5 min, the coverslip was removed, and slides were incubated in cold lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 and 10% DMSO added just before use) and kept overnight at 4°C. The slides were placed on a horizontal gel electrophoresis platform to allow the DNA to unwind in a cold fresh electrophoresis buffer (300 mM NaOH and 1 mM Na2EDTA, pH ~13.5) at 4°C, and electrophoresis was performed at 4°C for 30 min. The slides were then neutralized with a 400 mM Tris (pH 7.5) buffer for 5 min. The slides were stained with ethidium bromide (Sigma) and covered with a coverslip. Slides were examined by a computerized image analysis system (Comet 6.0; Andor Technology, Belfast, UK). One hundred cells were scored per sample (50 cells from each slide); Olive tail moment (OTM) and % tail DNA were measured as DNA damage parameters.

The cytokinesis block micronucleus (CBMN) assay

Five hundred microliters of whole blood was added to a T25 cm^2 Corning culture flask containing 4.5 ml RPMI-1640 medium supplemented with 1% of Penicillin-streptomycin, 15% Foetal bovine serum and 25 mM HEPES and L-Glutamine with end concentrations of

15 and 1%, respectively, followed by 100 µl of phythaemagglutinin (PHA) (2.5%). In the next 24 h, 50 µl of excipient (original solution) was added to the negative control. 50 µl of mitomycin C (0.4 µM) was added to the positive control. 140 µM of IQ and 25 µM quercetin from both bulk and nano forms were added to the rest of the flasks. Cultures were incubated at 37°C in the presence of 5% CO_2 for 44 h. After 44 h, cytochalasin-B (6 µg/ml, Sigma) was added and the cultures were incubated for another 28 h. The CBMN test preparations were performed according to Fenech et al. (Fenech et al., (2003 and 2007) and OECD (2016)). Various cytological parameters were evaluated: including cell mitotic status, mononucleated cells (MonoNC), binucleated cells (NC), multinucleated cells (MultiNC), other nuclear anomalies such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were also evaluated as biomarkers of genotoxic events. MNi, NBPs, and NBUDs were scored in NC up to 500 cells. 500 other cells were scored to calculate the percentages of each type of cells: Mono, NC, and MultiNC. The nuclear division index (NDI) was used as an indicator of the cytotoxicity and the rate of mitotic division and the calculation was used as described by Fenech, (2007).

Total RNA isolation and cDNA synthesis

Total RNA was extracted from lymphocytes (2×10^5) using the GenElute Mammalian Total RNA Purification kit (Sigma-Aldrich, UK). The RNA was treated with DNase I (Sigma-Aldrich, UK) to remove any DNA contaminants. The purity of total RNA was determined by measuring the absorbance at 260 and 280 nm ($A_{260/280}$) ratios, using a NanoDrop™ Spectrophotometer. Random hexamer primed reverse transcription reactions were performed for 400 ng of total RNA in a 20 µl setup using ImProm-II™ Reverse Transcription System reaction following the manufacturer's instructions (Promega). The samples were eluted in 30 µL elution solution and stored at −20 °C until analysis by qPCR.

Quantitative PCR

The reactions were performed using the StepOnePlus™ real-time PCR instrument (Applied Biosystems). The qPCR was used to measure the mRNA expression level of catalase in lymphocytes. Each reaction was prepared in triplicate and consisted of 5 µl of 10 × SYBR® Green PCR Master Mix (Applied Biosystems), 12.5 pmol each of forward and reverse primers, and 2 µl of cDNA template, making up to a 10 µl final volume per well. The qPCR was initially conducted at 50°C and 95°C for 2 and 20s, respectively, followed by amplification of the template for 40 cycles (each cycle involved 15 s at 95°C and 30s at 60°C). The data were analysed by StepOne™ Software v 2.2.2. The cycle threshold (Ct) mean value for the target gene was used to calculate the relative expression with the relative quantification (RQ) value and formula: $RQ = 2^{-\Delta CT} \times 100$, where $\Delta C_T = C_T$ of target gene - C_T of an endogenous housekeeping gene. Evaluation of $2^{-\Delta CT}$ indicates the fold change in gene expression, normalized to the internal control (β-actin) which enables the comparison between differently treated cells.

Western blot analysis

The cells were lysed within the wells using lysis buffer (60 mM Tris, 2 % SDS, 100 mM DTT) on ice for 10 min and protein concentrations were then determined with a commercial kit (Bradford Protein Assay, Biorad, UK). Samples were subjected in 10 % SDS polyacrylamide gel and separated proteins were transferred to a PVDF membrane. The blots were blocked with blocking buffer (Tris-buffered saline, 0.5 % skim milk powder, and 0.1 % Tween-20) for 1 h at room temperature, and subsequently incubated overnight at 4°C with primary antibody against catalase with anti- catalase (1:2000; Abcam, Cambridge, UK), and mouse monoclonal anti-GAPDH (1:1,000; Abcam, Cambridge, UK). The blots were washed three times in TBST (TBS, 0.1 % Tween-20). The blots were then incubated with the secondary horseradish peroxidase-conjugated antibodies (1:1,000; Cell Signaling

Technology, UK) for 1h at room temperature. The bands were visualized using enhanced chemiluminescence assay kit (GE Healthcare, UK) in the Biorad ChemiDoc MP imaging system and density measurements were made using commercial software (Bio-Rad Quantity One).

Statistical Analysis

All the experiments were performed in duplicate and repeated at least three times. Results are expressed as means ± SEM of three experiments and data were analysed using one-way analysis of variance with Dunnett's post hoc test to determine significance relative to control; for all experiments, a $p \leq 0.05$ was considered significant.

Results

The responses of lymphocytes from healthy individuals and COPD patients to IQ for the Comet assay parameters OTM and % tail DNA are shown in Table I, Figures 2 and 3. A significant increase was shown in OTM and % tail DNA in the lymphocytes from healthy individuals from 19.24 (OTM) and 47.99 % (% tail DNA) compared to the untreated control groups to 4.26 (OTM) 13.02 % (% tail DNA), respectively, when cells were treated with 140 µM IQ. Thus, cells were treated with 140 µM IQ and 25 µM Q-NPs for 30 mins and showed significant decreases in OTM and % tail DNA from 19.24 to 8.75 (OTM) and 47.99 to 17.63 (% tail DNA) respectively (***$p \leq 0.001$).

Lymphocytes from COPD patients also showed that a significant increase in OTM and % tail DNA from 27.11 (OTM) and 49.18 % (% tail DNA) compared to the untreated control groups to 5.25 (OTM) and 19.62 % (% tail DNA), respectively, when cells were treated with 140 µM IQ. Furthermore, cells treated with 140 µM IQ and 25 µM Q-NPs for 30 mins showed significant decreases in OTM and % tail DNA from 27.11 to 10.14 (OTM) and 49.18 to 26.63 (% tail DNA) respectively *(***$p \leq 0.001$*). Results also showed that following

exposure to IQ in the presence of quercetin bulk form and NPs, the levels of OTM and % tail DNA significantly decreased ($^{\#\#}p \leq 0.01$ *and* $^{\#\#\#}p \leq 0.001$ respectively) in lymphocytes from healthy individuals and CODP patients. The results also indicated that there was a statistically significant difference between the two forms *(*$^{**}p \leq 0.01$) Table I Figure 2 and 3.

Our results in the CBMN assay also indicated that exposure to IQ showed clear evidence of cytogenetic damage in the groups of healthy individuals and COPD patients. In the CBMN assay, various parameters were evaluated: binucleated cells (BN), multinucleated cells (MN), binucleated with micronuclei (BN with MNi), nucleoplasmic bridges (NPBs) and nuclear buds (N-Buds). Table II showed that there was no significant difference in the Comet length and MNi frequency in both groups after treatment with IQ. The results also showed that following exposure to IQ in the presence of bulk and NPs forms, significantly reduced the MNi frequencies by both forms. Also quercetin showed significant differences between the bulk and nano forms ($^{**}p \leq 0.01$). Thus, the extent of DNA damage by the Comet assay in lymphocyte cells was perfectly mirrored by the CBMN assay and MNi frequency can be used with the same effectiveness in the early detection of DNA damage in COPD patients.

To determine whether catalase overexpression by ROS was reversible, cells were treated with IQ and the levels of catalase mRNA was determined in lymphocytes from COPD patients. Figure 4 shows that IQ significantly increased mRNA expression of catalase compared with untreated cells over 2 h. Thus, lymphocytes treated with 140 µM IQ in the presence of 25 µM Q-BPs for 2 h showed significant decreases in the mRNA expression level of catalase compared with the same cells treated with 140 µM IQ and 25 µM Q-NPs for 2 h (Figure 4). Catalase expression was also measured at the protein level by the quantitative Western blot method in COPD patients at 2 h. A statistically significant increase was shown in the levels of catalase protein at 2 h ($^{***}p<0.001$). Thus, lymphocytes treated with 140 µM IQ in the presence of 25 µM Q-BPs for 2 h showed significant decreases in the protein level of catalase

compared with the same cells treated with 140 µM IQ and 25 µM Q-NPs for 2 h ($^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001$ respectively) (Figures 5 A and B). Also quercetin showed significant differences between the bulk and nano forms *(*$^{**}p \leq 0.01$).

Discussion

Exposure to exogenous environmental carcinogens has become one of the most important causes of human cancers. Monitoring the DNA damage response in pathologies has been increasingly performed and might add another aspect to their clinical appearance. In addition to representing a potential target for therapeutic intervention in the treatment of COPD disorders. It is known that oxidative stress is a major pathogenetic component of the airway inflammation that is characteristic for COPD (Domej et al., 2014b). The increased oxidative burden present in COPD has been found to be associated with a range of pathogenic processes. These started with inactivation of antiproteases and enhancing bronchial inflammation by activating redox-sensitive transcription factors such as mucus hypersecretion and damage to pulmonary cells, activation of macrophages, neutrophils and fibroblasts (Hansel and Barnes, 2009; Rabe et al., 2007).

The present study has examined DNA damage in the Comet and micronucleus assays in peripheral blood lymphocytes of healthy individuals and patients with COPD using the nanoparticle (NP) and bulk suspensions of the quercetin. In the Comet assay and in the MN assay, the results clearly showed that there was significantly an increase in the Comet parameters (OTM and % Tail DNA) and an increase in number of MNi in cells treated with IQ when compared to their untreated lymphocytes in healthy individual and COPD patients. Furthermore, DNA damage was a decrease in lymphocytes from healthy individuals and COPD patient after treatment with Q-BPs and Q-NPs and showed a significant reduction in DNA damage in both the Comet and MN assays. Based on this result, we also studied

whether there is regulation of catalase enzyme activity by IQ at the protein or gene expression levels.

HCAs like IQ have been shown to be potent mutagens and exert strong carcinogenic effects in mammalian tissues (Sugimura, 2002; Sugimura et al., 2000). HCAs have also shown different types of DNA damage by interacting directly with DNA through the formation of covalent adducts. Most of the HCAs undergo metabolic activation by N-hydroxylation of the exocyclic amine group to producer the arylnitrenium ion, which is the significant metabolite implicated in toxicity and DNA damage (Hatch et al., 2001). HCAs have also induced genetic damage that can lead to mutations. These mutations are a vital event for the beginning of carcinogenesis; reducing these could be an important key of prevention for cancers (De Flora and Ferguson, 2005).

A study has shown that IQ induced significant DNA damage in lymphocytes from inflammatory bowel disease (IBD) patients as a result of oxidative stress (Najafzadeh et al., 2009). Flavonoids are important antioxidants and play a significant role in carcinogen deactivation *in vivo* (Dolara et al., 2005). Flavonoids like quercetin are known to modulate effects of HCAs in human lymphocytes and sperm *in vitro* (Anderson et al., 1998). The results in this study indicated that quercetin may have a beneficial role in lowering IQ toxicity. Another important finding of this study was that quercetin (NPs) had higher reduction effects at 25 µM concentration than quercetin (BPs). This implied that NPs have distinctive physicochemical properties display toxic effects that are different from their bulk counterparts (Harman et al., 2002; McDonald et al., 2005).

The impact of IQ on the endogenous antioxidant enzyme catalase was additionally tested in lymphocytes from COPD patients with respect to the generation of oxidative stress such as ROS. The results showed that catalase significantly increases in mRNA expression levels and

protein activity may play an essential role in induced DNA damage in COPD patients. Consistent with the decreased level of DNA damage was reduced catalase expression at both the protein and mRNA levels in the present of quercetin at BPs and NPs forms. The results provide evidence that the effects of IQ could contribute to ROS generation and plays an important role in regulating catalase expression and in the pathogenesis of COPD. To our knowledge, this is the first *in vitro* study showing that IQ causes DNA damage by enhancement of ROS, endogenous and exogenous antioxidants involved. In cells, ROS are the primary cause of oxidative damage, which has long been identified as an initiating factor and altering antioxidant molecule function in the pathogenesis of COPD (McGuinness and Sapey, 2017). Catalase is one of the major endogenous antioxidant enzymes involved in defending cells from the damaging effects of ROS and disruption of normal cellular homeostasis via redox signalling could result in cancer (Bhattacharyya et al., 2014). The functional polymorphisms of the catalase gene may significantly be involved in the pathogenesis of COPD. Stimulation or reduced levels of catalase or non-functionality of the catalase gene may lead to oxidative stress and initiate stress-related pathways. Also, the endogenous antioxidant quercetin significantly reduced oxidative stress in healthy individuals and COPD patients.

Together, these results show that IQ-mediated ROS generation was adequate to recruit additional scavenger enzymes to defend diseased cells against oxidative stress-mediated DNA damage. Thus, IQ significantly stimulated the catalase pathway in human healthy in COPD patients.

Conclusion

To our knowledge, no study has assessed the association of high intake of well-done meat intake with COPD risk. These results are consistent with the mutagenic/carcinogenic effect of

IQ in animal models and suggest that HCA exposure may contribute to the development of the pathogenesis in COPD disease. The ability of flavonoids to reduce oxidative stress and induce human protective enzyme systems appear promising, *in vitro* in lymphocytes of COPD patients as well as healthy individuals, Thus, flavonoids and flavonoid-containing foods could be very beneficial in reducing oxidative DNA damage of COPD patients and so reduce the risk of potential cancer and other human illness.

Declaration of interest

The corresponding author confirmed that the authors have no conflicts of interest with this manuscript.

Acknowledgement

The authors would like to thank the clinical staff of Bradford Royal Infirmary Hospital and St. Luke's Hospital for their assistance in this study.

The Sponsorship by the Libyan Government of a PhD studentship to Khaled Habas is gratefully acknowledged. The Sponsor played no part in the conduct of the work or the writing of the manuscript.

References

Anderson, D., et al., 1998. Flavonoids modulate Comet assay responses to food mutagens in human lymphocytes and sperm. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis. 402**,** 269-277.

Bhattacharyya, A., et al., 2014. Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases. Physiological Reviews. 94**,** 329-354.

Birben, E., et al., 2012. Oxidative stress and antioxidant defense. World Allergy Organization Journal. 5**,** 9.

De Flora, S., Ferguson, L. R., 2005. Overview of mechanisms of cancer chemopreventive agents. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 591**,** 8-15.

Dolara, P., et al., 2005. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 591**,** 237-246.

Domej, W., et al., 2014a. Oxidative stress and free radicals in COPD--implications and relevance for treatment. Int J Chron Obstruct Pulmon Dis. 9**,** 1207-24.

Domej, W., et al., 2014b. Oxidative stress and free radicals in COPD implications and relevance for treatment. International Journal of Chronic Obstructive Pulmonary Disease. 9**,** 1207-1224.

OECD, 2016.Test No. 489: In Vivo Mammalian Alkaline Comet Assay Test. OECD Publishing.

OECD, 2016. Test No. 487: In Vitro Mammalian Cell Micronucleus Test. OECD Publishing.

Felton, J. S., et al., 2007a. Mutagenic potency of food-derived heterocyclic amines. Mutat Res. 616**,** 90-4.

Felton, J. S., et al., 2007b. Mutagenic potency of food-derived heterocyclic amines. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis. 616**,** 90-94.

Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. Nature Protocols. 2**,** 1084-1104.

Fenech, M., et al., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Research-Genetic Toxicology and Environmental Mutagenesis. 534**,** 65-75.

Halliwell, B., 2011. Free radicals and antioxidants - quo vadis? Trends Pharmacol Sci. 32**,** 125-30.

Hansel, T. T., Barnes, P. J., 2009. New drugs for exacerbations of chronic obstructive pulmonary disease. Lancet. 374**,** 744-755.

Harman, T., et al., 2002. Quantum dot superlattice thermoelectric materials and devices. science. 297**,** 2229-2232.

Hatch, F. T., et al., 2001. Extended quantitative structure-activity relationships for 80 aromatic and heterocyclic amines: Structural, electronic, and hydropathic factors affecting mutagenic potency. Environmental and Molecular Mutagenesis. 38**,** 268-291.

Jakszyn, P., et al., 2004. Development of a food database of nitrosamines, heterocyclic amines, and polycyclic aromatic hydrocarbons. Journal of Nutrition. 134**,** 2011-2014.

Kizil, M., et al., 2011. A review on the formation of carcinogenic/mutagenic heterocyclic aromatic amines. J Food Process Technol. 2**,** 2.

Knize, M. G., Felton, J. S., 2005. Formation and human risk of carcinogenic heterocyclic amines formed from natural precursors in meat. Nutr Rev. 63**,** 158-65.

McDonald, S. A., et al., 2005. Solution-processed PbS quantum dot infrared photodetectors and photovoltaics. Nature Materials. 4**,** 138-142.

McGuinness, A. J. A., Sapey, E., 2017. Oxidative Stress in COPD: Sources, Markers, and Potential Mechanisms. Journal of Clinical Medicine. 6.

Najafzadeh, M., et al., 2009. Flavonoids inhibit the genotoxicity of hydrogen peroxide (H2O2) and of the food mutagen 2-amino-3-methylimadazo[4,5-f]-quinoline (IQ) in lymphocytes from patients with inflammatory bowel disease (IBD). Mutagenesis. 24**,** 405-411.

Phillips, H., 1973. Dye exulusion tests for cell viability. Tissue culture: methods and applications.

Poolzobel, B. L., et al., 1992. Systemic Genotoxic Effects of Tobacco-Related Nitrosamines Following Oral and Inhalational Administration to Sprague-Dawley Rats. Clinical Investigator. 70**,** 299-306.

Rabe, K. F., et al., 2007. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med. 176**,** 532-55.

Rahman, I., 2005. The role of oxidative stress in the pathogenesis of COPD: implications for therapy. Treat Respir Med. 4**,** 175-200.

Rahman, I., Adcock, I. M., 2006. Oxidative stress and redox regulation of lung inflammation in COPD. Eur Respir J. 28**,** 219-42.

Ricciardolo, F. L. M., et al., 2006. Reactive nitrogen species in the respiratory tract. European Journal of Pharmacology. 533**,** 240-252.

Sugimura, T., 2002. Food and cancer. Toxicology. 181**,** 17-21.

Sugimura, T., et al., 2000. Complex factors pertinent to human hazard risk. Food borne carcinogens: heterocyclic amines.**,** 349-359.

Tice, R. R., et al., 2000. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis. 35**,** 206-221.

Trachootham, D., et al., 2009. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 8**,** 579-91.

Varraso, R., Camargo, C. A., 2014. Processed meat consumption and lung health: more evidence for harm. European Respiratory Journal. 43**,** 943-946.

Vlassara, H., Uribarri, J., 2014. Advanced Glycation End Products (AGE) and Diabetes: Cause, Effect, or Both? Current Diabetes Reports. 14.

Yoshida, T., Tuder, R. M., 2007. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. Physiol Rev. 87**,** 1047-82.

Zheng, W., Lee, S. A., 2009. Well-Done Meat Intake, Heterocyclic Amine Exposure, and Cancer Risk. Nutrition and Cancer-an International Journal. 61**,** 437-446.

Table I DNA damage induced *in vitro* in lymphocytes from healthy individuals and COPD patients by the food mutagen IQ (140 µM) its reduction by flavonoid supplementation with 25 µM of quercetin (Q). Data represent the means ± SE obtained from ten healthy individuals and ten patients with COPD.

	Treatment	Olive tail moment (OTM) Mean ± SE	% Tail DNA
Healthy Individuals	Untreated	4.26 ± 0.51	13.02 ± 0.43
Positive Control (PC)	60 µM H_2O_2	19.56 ± 0.71***	40.21± 1.51***
	IQ 140 µM	19.24± 0.72 ***	45.61 ± 1.49 ***
	25 µM and Q-BPs	15.80 ± 1.55 ##	32.10 ± 0.90 ##
	25 µM and Q-NPs	8.75 ± 0.59 ###	17.99 ± 1.25 ###
COPD Patients	Untreated	5.25 ± 0.68	21.46 ± 0.85
Positive Control (PC)	60 µM H_2O_2	22.88± 0.57***	41.50± 1.31***
	IQ 140 µM	27.11 ± 0.56 ***	47.18 ± 1.09 ***
	25 µM and Q-BPs	16.40 ± 1.02 ##	37.07 ± 0.61 ##
	25 µM and Q-NPs	10.14± 0.67 ###	22.99 ± 0.46 ###

Q-BPs= Bulk quercetin, Q-NPs= Nano quercetin, PC= Positive control, ***$p < 0.001$ versus untreated cells and a positive control (PC) of 60 µM H_2O_2. ##$p < 0.01$and ###$p < 0.001$ indicates cells treated with 140 µM IQ and 25µM Q-BPs and 25µM Q-NPs. ***P < 0.01 indicates comparison of bulk and nano.*

Table II The CBMN assay detects the outcome of lesions such as micronuclei, nucleoplasmic bridges or nuclear buds in lymphocytes by the food mutagen IQ (140 µM) and its reduction by flavonoid supplementation with 25 µM of quercetin (Q). Data represent the means ± SE obtained from three healthy individuals and three patients with COPD.

Treatment	NID	% NC	MNi	NPBS	Buds	% MonoNC
			Mean ± SE			
Healthy individuals						
Untreated	1.3 ± 0.40	80 ± 1.71	4 ±.0.03	2 ± 0.06	1 ± 0.04	58.4 ± 1.22
Mitomycin C (PC)	1.4 ± 0.12	67.2± 1.46	13 ± 0.53 ***	4 ± 0.13	3 ± 0.07	70.4 ± 1.02
IQ 140 µM	1.4 ± 0.04	69.6 ± 1.23	19 ± 0.72 ***	2 ± 0.04	3 ± 0.02	62.8 ± 1.54
25 µM and Q-BPs	1.3 ± 0.14	73.6 ± 1.45	14 ± 0.78 ##	4 ± 0.12	5 ± 0.17	60.6 ± 2.43
25 µM and Q-NPs	1.3 ± 0.34	75.6 ± 1.98	9 ±0.56 ###	7 ± 0.17	7 ± 0.23	61 ± 2.5
COPD Patients						
Untreated	1.3 ± 0.02	71.5 ± 1.46	3 ± 0.57	2 ± 0.07	1 ± 0.02	67.4 ± 1.09
Mitomycin C (PC)	1.4 ± 0.04	57.2 ± 1.09	12 ± 0.43 ***	5 ± 0.16	5 ± 0.67	75.3 ± 1.12
IQ 140 µM	1.4 ± 0.05	72.4 ± 1.05	18 ± 0.75 ***	2 ± 0.06	5 ± 0.12	72.4 ± 1.05
25 µM and Q-BPs	1.4 ± 0.23	78.6 ± 1.34	15 ± 0.89 ##	4 ± 0.18	4 ± 0.16	62.5 ± 0.78
25 µM and Q-NPs	1.4 ± 0.12	72.6 ± 2.34	9 ±0.34 ###	7 ± 0.13	3 ± 0.12	67 ± 1.96

Q-BPs= Bulk quercetin, Q-NPs= Nano quercetin, PC= Positive control, NDI= Nuclear division index, NC=Binucleated cells, % NC, is% expressed out of all types of 500 cells scored. MNi=Micronuclei score/500 cells each of NC, NPBs=Nucleoplasmic bridges and NBUDs=Nuclear buds.***$p < 0.001$ versus untreated cells and a positive control (PC) of 0.4 M of Mitomycin C (MMC). ##$p < 0.01$and ###$p < 0.001$ and indicates cells treated with 140 µM IQ and 25µM Q-BPs and 25 µM Q-NPs.

Figure legends

Figure 1. Chemical structure of IQ and quercetin

Figure 2. DNA damage measured as mean OTM before and after treatment with bulk and nano forms of quercetin in human lymphocytes from healthy individuals and COPD patients in the Comet assay. n= (10 in each group). *** P < 0.001 when compared with untreated lymphocytes and a positive control (PC) of 60 µM H2O2. ##p < 0.01 and ###p < 0.001 indicates cells treated with 140 µM IQ and 25 µM Q-BPs and Q-NPs. **P < 0.01 indicates comparison of bulk and nano.

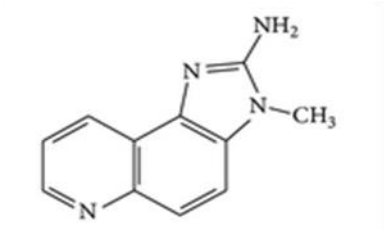
Figure 3. DNA damage measured as mean % tail DNA before and after treatment with bulk and nano forms of quercetin in human lymphocytes from healthy individuals and COPD patients in the Comet assay. n= (10 in each group). *** P < 0.001 when compared with untreated lymphocytes and a positive control (PC) of 60 µM H2O2. ##p < 0.01 and ###p < 0.001 indicates cells treated with 140 µM IQ and 25 µM Q-BPs and Q-NPs. **P < 0.01 indicates comparison of bulk and nano.

Figure 4. Effect of IQ on the catalase mRNA expression in lymphocytes from COPD by qPCR. The COPD lymphocytes were incubated with or without IQ (140 µM), and with bulk and nano form of quercetin (25 µM and Q-BPs and 25 µM and Q-NPs) in the presence of IQ (140 µM) for 2 h for total RNA isolation and the expression of the catalase mRNA were determined by qPCR. Data from three different COPD patients (n=3). Figure shows change of catalase mRNA level at each time point. *** P < 0.001 when compared with untreated lymphocytes. ##p < 0.01 and ###p < 0.001 indicates cells treated with 140 µM IQ and 25 µM Q-BPs and Q-NPs. **P < 0.01 indicates comparison of bulk and nano.

Figure 5 A and B. Effect of IQ on catalase expression in lymphocytes from COPD patients by Western blot. COPD lymphocytes were incubated with or without IQ (140 µM), and with bulk and nano form of quercetin (25 µM and Q-BPs and 25 µM and Q-NPs) in the presence of IQ (140 µM) for 2 h. (A): shows a representative blot indicating a protein band demonstrating catalase. (B): data (mean ± SE) are shown as a bar graph of densitometry data from three different COPD patients (n=3). *** P < 0.001 when compared with untreated lymphocytes. ##p < 0.01 and ###p < 0.001 indicates cells treated with 140 µM IQ and 25 µM Q-BPs and Q-NPs. **P < 0.01 indicates comparison of bulk and nano.

Figure 1

2-amino-3-methylimidazo [4,5-f]quinoline (IQ)



Quercetin

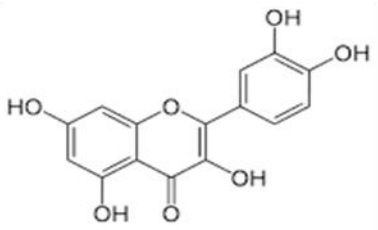


Figure 2

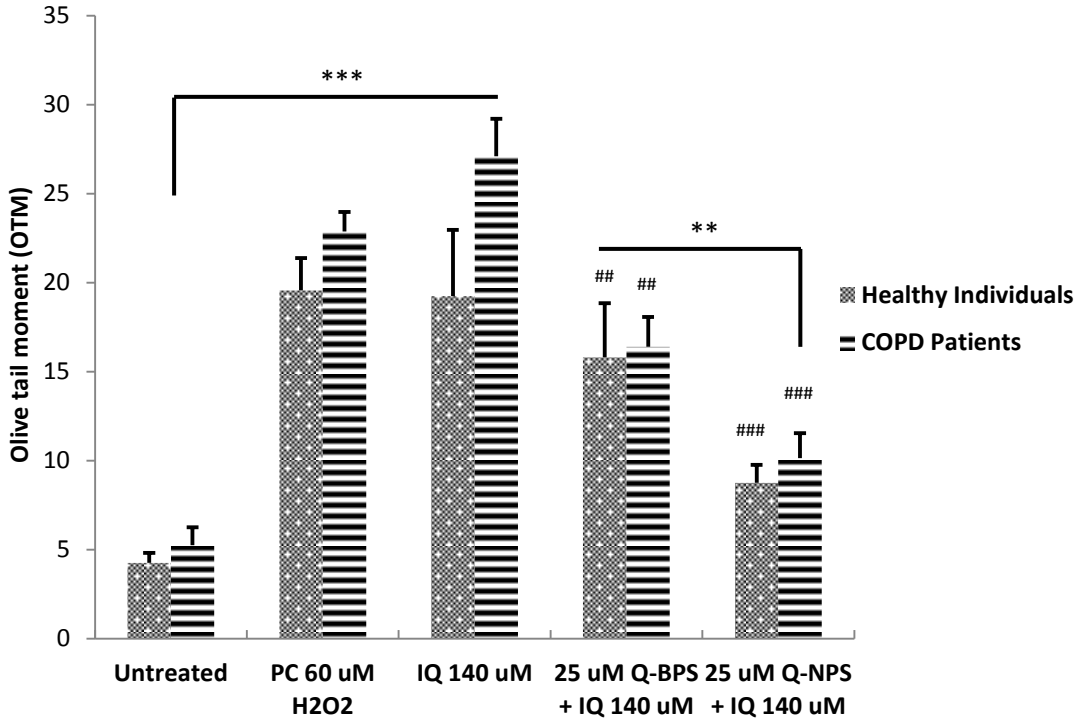

35
30
25
20
15
10
5
0
Olive tail moment (OTM)
***
**
##
##
###
###
Healthy Individuals
COPD Patients
Untreated
PC 60 uM H2O2
IQ 140 uM
25 uM Q-BPS + IQ 140 uM
25 uM Q-NPS + IQ 140 uM

Figure 3

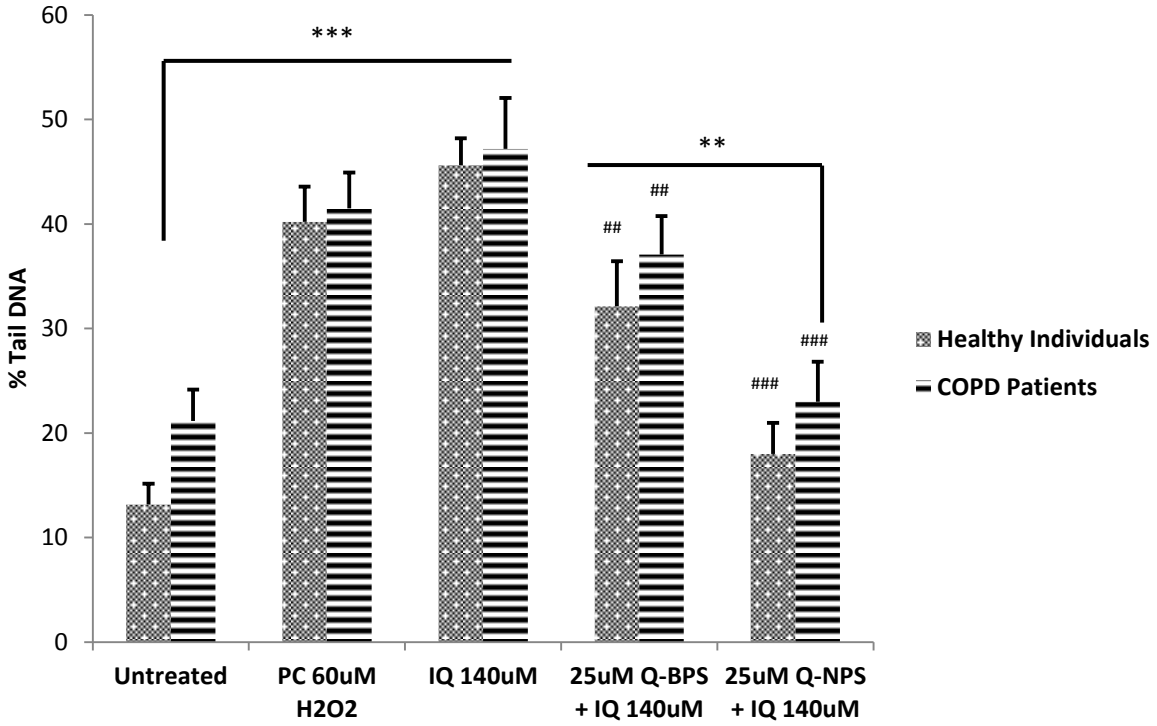

60
50
40
30
20
10
0
% Tail DNA
***
**
##
##
###
###
Untreated
PC 60uM H2O2
IQ 140uM
25uM Q-BPS + IQ 140uM
25uM Q-NPS + IQ 140uM
Healthy Individuals
COPD Patients

Figure 4

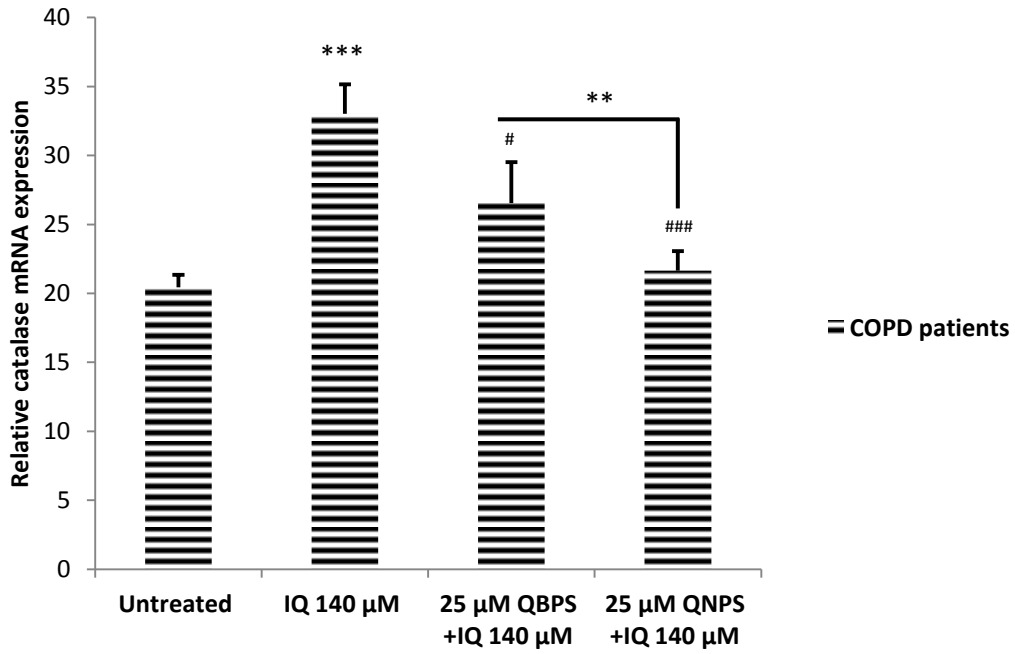
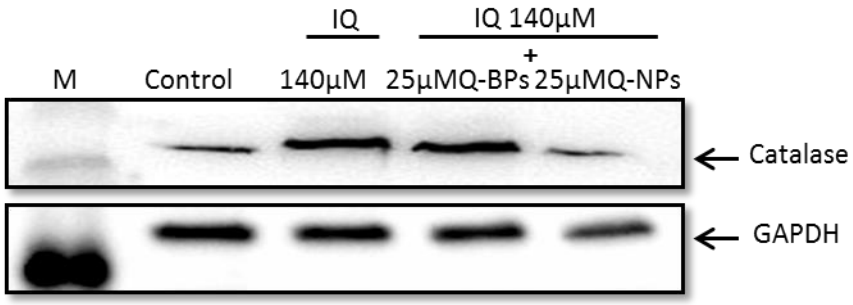
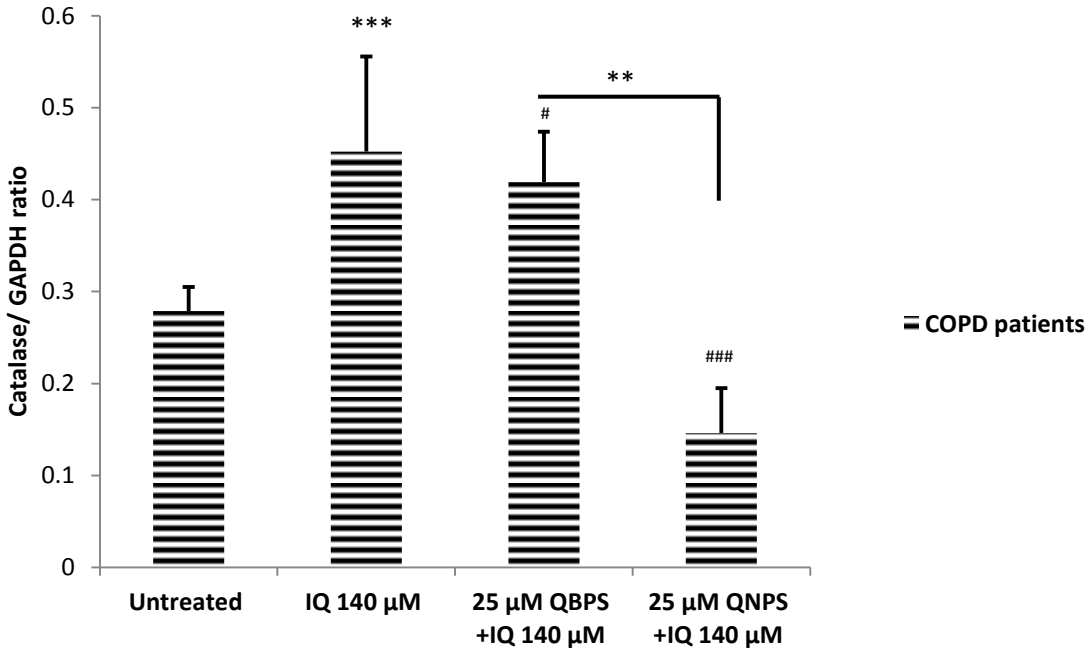

Relative catalase mRNA expression
40
35
30
25
20
15
10
5
0
***
**
#
###
Untreated
IQ 140 µM
25 µM QBPS +IQ 140 µM
25 µM QNPS +IQ 140 µM
COPD patients

Figure 5

A


IQ
IQ 140µM
+
M
Control
140µM
25µMQ-BPs
25µMQ-NPs
Catalase
GAPDH

B


0.6
0.5
0.4
0.3
0.2
0.1
0
Catalase/ GAPDH ratio
***
**
#
###
Untreated
IQ 140 µM
25 µM QBPS +IQ 140 µM
25 µM QNPS +IQ 140 µM
COPD patients