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Low-dose theophylline enhances the anti-inflammatory effects of steroids during exacerbations of chronic obstructive pulmonary disease

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ABSTRACT

Rationale. Chronic obstructive pulmonary disease (COPD) is characterised by an abnormal inflammatory response to mainly cigarette smoke that flares up during exacerbations of the disease (ECOPD). A reduced activity of histone deacetylases (HDAC) contributes to enhanced inflammation in stable COPD. We hypothesised that HDAC activity is further reduced during ECOPD, and that theophylline, an HDAC activator, potentiates the anti-inflammatory effect of steroids in these patients.

Objectives. To investigate HDAC activity during ECOPD and the effects of theophylline on the anti-inflammatory effects of steroids in a randomised, single-blinded, controlled study.

Methods..Thirty-five patients hospitalised because of ECOPD and treated according to international guidelines (including systemic steroids) were randomised to receive (or not) low dose oral theophylline (100 mg bid). Before treatment and 3 months after discharge we measured HDAC and nuclear factor-κB (NF-κB) activity in sputum macrophages, the concentration of nitric oxide in exhaled gas (eNO), and total anti-oxidant status (TAS), TNF-α, IL-6 and IL-8 levels in sputum supernatants.

Measurements and Main results. Patients receiving standard therapy showed decreased NF-κB activity, eNO concentration and sputum levels of TNF-α, IL-6 and IL-8, as well as increased TAS during recovery of ECOPD, but HDAC activity did not change. The addition of low-dose theophylline increased HDAC activity ($p=0.02$) and further reduced IL-8 ($p=0.012$) and TNF-α concentrations ($p=0.031$).

Conclusions. During ECOPD, low dose theophylline increases HDAC activity and improves the anti-inflammatory effects of steroids.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by an enhanced inflammatory response to inhaled particles and gases, mostly cigarette smoking [1]. We have previously shown that the activity of histone deacetylases (HDAC) is decreased in patients with stable COPD, and that this can contribute to the abnormal inflammatory response that characterises the disease [2;3].

Patients with COPD often experience acute episodes of exacerbation (ECOPD) during the course of their disease. These episodes are characterised by NF-κB activation [4;5] and a burst of airway inflammation, with increased oxidative and nitrative stress [6;7], increased neutrophil counts and raised levels of several pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-8 (IL-8) and IL-6 [8-10]. Because both oxidative [11] and nitrative stress [12] can impair HDAC function, we hypothesised that HDAC activity will be further reduced during ECOPD, thus contributing to amplify baseline inflammation.

Current international guidelines recommend the use of high dose oral or intravenous steroids (in combination with inhaled bronchodilators) for the treatment of ECOPD [1]. Low concentrations of theophylline enhance HDAC activity and have anti-inflammatory effects, both in asthma and COPD [13-15], that have been shown to add clinical benefits and safety in the long term treatment of COPD [16].

We have previously shown that an impaired HDAC activity reduces the anti-inflammatory effects of steroids in patients with stable COPD [2;3], and that this can be reversed by low-dose theophylline [17]. We hypothesised here that theophylline can also enhance HDAC activity and improve steroid responsiveness during ECOPD.

Accordingly, in this prospective, randomised, single-blinded, controlled study we determined, as the primary outcome, the activity of HDAC in sputum macrophages of patients hospitalised because of ECOPD, and we investigated whether the addition of low-dose oral theophylline to standard therapy enhances its activity and the anti-inflammatory effects of steroids.

METHODS

Patients and Ethics

Patients were recruited within the first 24 hours of admission from the Accident and Emergency department of Hospital Universitario Son Dureta from June 2005 to December 2007. Subjects were eligible if they had COPD, defined as a post-bronchodilator FEV_1/FVC ratio <70% determined at stability according to GOLD criteria [1], a smoking history >15 pack-years, and a diagnosis on admission of ECOPD. The latter was made by the emergency physician, who was unaware of the study, and was based on the patient´s symptoms and complementary tests, namely arterial blood gases, electrocardiogram, chest X-ray and routine blood tests. Patients with a history of asthma, bronchiectasis,

carcinoma of the bronchus, pneumonia, or heart failure were excluded. Patients were also excluded if they were unable to provide an adequate sputum sample or if they were already treated with theophylline, anti-inflammatory therapy for chronic inflammatory diseases, such as Crohn's disease or rheumatoid arthritis, or had received antibiotics or oral steroids during the previous four weeks. Comorbidities were assessed by the Charlson co-morbidity index. All patients were informed of the nature and purpose of the study and gave their written consent. The study was approved by the local Ethics Committee.

Study design

On admission all patients received treatment according to international guidelines [1] that included nebulised bronchodilators (beta-agonist and anti-cholinergic), systemic steroids (oral or intravenously) in all patients with or without antibiotics according to Anthonisen´s criteria [18]. When the patient accepted to participate in the study, spirometry, arterial blood gases, exhaled nitric oxide (eNO) measurements, and induced sputum samples were obtained during the first 24 hours of hospitalisation. Patients were then randomised to receive standard therapy with or without theophylline (100 mg b.i.d.) that was maintained until the end of the study. At discharge, both groups received treatment with inhaled corticosteroids, long-acting beta agonist and long-acting anticholinergic. Randomisation was made by a research nurse, on the basis of a computer-generated randomisation list, and the investigators who analysed the data were unaware of the treatment arms.

An outpatient clinic visit was scheduled three months after discharge. Clinical stability was accepted if the patient had not needed to change his regular treatment. If another ECOPD episode occurred after discharge, patients were re-scheduled three months later. During this outpatient visit, measurements obtained during hospitalisation were repeated. If the patient did not attend the visit, the investigator team contacted the patient to confirm survival.

Lung function

Forced spirometry (GS, Warren E. Collins, Braintree, MA, USA) was obtained in all participants according to international guidelines [19]. Spirometric reference values were those of a Mediterranean population [20]. Previous lung function was used to confirm the diagnosis of COPD, when available (n=39).

Exhaled nitric oxide measurement

The measurement of eNO was performed with a chemiluminescence analyzer (Sievers Instruments Inc. Model 280NOA, Boulder, CO, USA) connected to a Teflon tube, following European Respiratory Task Force recommendations [21], as previously described in our group [22].

Sputum sampling and preparation

Sputum was induced and processed following standard methodology [23]. Briefly, sputum was incubated with four times its weight of 0.01M DTT in HBSS, at 4°C for 15 minutes. Sputum dilution was proportional to the collected weight,

thus standardising between time points and patients the supernatants used for assay of proteinases and other markers. The volume of HBSS was then doubled (ten-fold dilution of original sputum sample) and incubated for a further 5 minutes. The suspension was then filtered through 50 µm nylon gauze to remove mucus and debris without removing any of the cells and centrifuged at 790x*g* for 10 minutes. The cell free supernatant was removed and stored at -70°C. The cell pellet was re-suspended. Total cell count was determined with a Neubauer hemocytometer. The trypan blue exclusion method was used to determine cell viability. Cytospins were made from the cell suspension and stained with Diff-Quik to obtain a differential cell count. We considered inadequate sputum samples those with less than a million cells and therefore they were excluded from analysis. Cells were incubated in a 6 well plate (multiwell primaria surface modified polystyrene, Falcon 35.3846) at a density of $1x10^6$ cells/well in 2 mL of medium (RPMI 1640, 10%FCS, L-Glu). After 4 hours supernatant was removed and macrophages were scraped from the plate and lysed for protein extraction.

Cytokine determination

Cytokine concentration (TNF-α, IL-6, and IL-8) in sputum supernatant was determined by flow cytometry (CBA, Human inflammation Kit, BD Biosciences, San Jose, CA, USA) following manufacturer's instructions. Assay sensitivity, as expressed by the manufacturer, were as follows: 3.6 pg/ml for IL-8, 2.5 pg/ml for IL-6, and 3.7 pg/ml for TNF-α.

Total Antioxidant Status (TAS)

TAS in sputum was measured by a colorimetric test (Randox Laboratories Ltd, Crumlin, UK) as previously described by our group [24].

HDAC activity

HDAC activity of sputum macrophages nuclear extracts was measured with a non-isotopic assay using a fluorescent derivative of epsilon-acetyl lysine (HDAC fluorescent Activity Assay Kit, BIOMOL, Plymouth, PA) as previously described [17].

NF-κB activity

NF-κB activation was assessed in sputum macrophages nuclear extracts using the TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, USA) as previously described [25].

Statistical Analysis

Results are expressed as means ± SEM (or medians and ranges in non-normally distributed variables). HDAC activity, NF-κB activation values, IL-8, IL-6, TNF-α, eNO, TAS and FEV1 values were normally distributed and were compared between groups using two-way ANOVA. Analyses were performed for the intention to treat population (ITT). A p value of < 0.05 (two-tailed) was

considered statistically significant. Analysis was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

RESULTS

Clinical data

Of the 42 patients originally recruited, 7 were excluded within the first 24 hours due to inadequate sputum sample (n=5), absence of airway obstruction in the first spirometry (n=1) and voluntary study withdrawal (n=1). Except for the patient excluded due to post-bronchodilator FEV_1/FVC of 78%, the other 6 patients were not statistically different than the patients that were included in the study. The remaining 35 patients (all male) entered the study and were randomised to either standard treatment (19 patients) or standard treatment plus theophylline 100 mg bid orally (16 patients). Table 1 shows the baseline demographic, clinical and functional characteristics of all participants. In the theophylline group 10 patients were receiving treatment with a combination of inhaled corticosteroids and long-acting beta agonist and 6 were receiving tiotropium, whereas in the control group this happened in 11 and 9 patients respectively. History of previous exacerbations revealed that, in the theophylline group, 3 patients had had 2 or more exacerbations and 3 had had one exacerbation in the previous year whereas in the control group this figure was 2 and 5 patients respectively.

Table 1. Demographic and clinical characteristics of COPD patients at the time of admission. (Abbreviations: FEV_1: forced expiratory volume in 1 second, ICS: inhaled corticosteroids)

	All (n=35)	Theophylline (n=16)	Standard therapy (n=19)
Age	67.6±1.3	66.7±1.7	68.2±2.1
Gender (M/F)	35/0	16/0	19/0
Pack-year	58.6±4.3	62.9±6.9	54.1±6.4
Active smoking	15 (42%)	8 (50%)	7 (37%)
Charlson index	1.83± 0.2	1.77± 0.3	1.88± 0.2
FEV_1 (L)	1.03± 0.1	0.99±0.08	1.11±0.09
FEV_1 (%)	38.4±2.1	39.2±3.8	37.7±1.9
FEV_1/FVC (%)	46.39±1.9	45.34± 3.3	47.89±2.7
Salbutamol reversibility (%)	5.17±1.32	7.33±1.65	3.89±1.61
ICS (nº)	21 (60%)	11 (68%)	10 (53%)
PaO_2 (mmHg)	56.4±1.6	53.7± 2.4	58.6±1.7
$PaCO_2$ (mmHg)	45.6±1.7	48.4±2.5	42.7±2.2

There were no differences in basic and liver function laboratory parameters between the two groups at the time of admission. All patients were treated with systemic corticosteroids which were tapered during the admission. The dose and duration of treatment was not standardised, and it was individualised upon symptoms and physical examination. All patients had a prescription of oral prednisone at discharge. Median total dose and median total time of corticosteroids was 590 mg (range 400-920) and 18 days (range 15-30) respectively, in the theophylline group and 600 mg (range 425-1000) and 19 days (range 15-32) in the standard therapy group. These figures were not statistically different. Patients in the theophylline group were treated during a median time of 102 days (range 94-140). Compliance was assessed by measuring theophylline levels at the end of the study. Of the 35 patients studied, 27 were treated with antibiotics (12 in the theophylline group and 15 in the standard therapy group, p=0.15). Antibiotic given were amoxyclav in 19 patients, levofloxacyn in 6 patients and trimetropin/sulfametoxazol in 2 patients.
Four patients in the theophylline group and 3 patients in the control group had a positive sputum culture taken at the time of admission. The bacteria isolated were Pseudomona aeruginosa (2), Stenotrophomona sp. (1), Haemophilus influenzae (2), Streptococus pneumoniae (1) and Proteus mirabilis (1). Two patients died during hospitalisation, both in the standard treatment arm. Length of hospital stay was not different between the group receiving theophylline or standard therapy (6.31±0.27 vs.7.35±1.05 days, respectively p=0.38). After discharge, 2 patients in the theophylline group (12,5%) and 5 in the standard therapy group (26%) had a new ECOPD. Theophylline was maintained during

the new admission and both patients received systemic steroids and antibiotics (one of them amoxyclav and the other levofloxacyn) and were discharged after 7 and 10 days of admission. Median time to new exacerbation was 36 days (range 32-40) in the theophylline group and 32 days (range 12-46) in the control group. There was an additional death in the standard therapy group during follow-up. Some patients were lost during follow up due to unwillingness to participate (n=2), discontinuation of theophylline because of side-effects (n=1), or inadequate sputum sample (n=3). As a result, 16 out of the 19 patients in the standard treatment arm, and 10 out of the 16 in the theophylline arm were studied again when clinically stable (Figure 1). Compared to measurements obtained at the emergency room, FEV_1 improved by 24% (95% confidence interval (CI) 2-45%) during clinical stability in patients receiving standard therapy and by 37% (95% CI,1-63%) in those receiving theophylline on top ($p<0.01$ between groups, mean differences -0.40 vs. -0.25 L).

Anti-inflammatory effects of standard therapy

NF-κB activation was reduced during recovery of ECOPD by 34% (95%CI 23.8-44%) compared to exacerbation in the group receiving standard therapy (Figure 2, panel A). In parallel, there was a statistically significant reduction in eNO concentration and in the sputum levels of TNF-α, IL-6 and IL-8 (Figure 3). Sputum TAS increased during recovery compared to exacerbation by 70% (95%CI -4.0 -145%; 0.55±0.1 vs.0.95± 0.2 mmol/L) although HDAC activity did not change (Figure 2, panel B).

Effects of theophylline

Mean plasma levels of theophylline during follow up were 4,6±1.2 mg/L. Two patients receiving theophylline experienced mild nausea and 2 reported headache. The reduction in NF-κB activation or the increase in TAS seen in the standard therapy group were not influenced by the addition of low-dose theophylline (Figure 2, panel A). TAS in sputum supernatant increased by 105.9% (95%CI 56-155%; 0.37±0.1 vs. 0.69±0.2 mmol/L) from exacerbation to stable phase but no statistical differences with the standard therapy group was found (see above). By contrast, it resulted in a statistically significant increase in HDAC activity (Figure 2, panel B) compared to standard therapy group that was accompanied by further reductions in the sputum concentrations of TNF-α, and IL-8 (Figure 3).

DISCUSSION

This study shows that low-dose theophylline enhances the activity of HDAC and the anti-inflammatory effects of steroids in male COPD patients with sputum production hospitalised because of an exacerbation of the disease.

Previous studies

We have previously shown that HDAC activity is reduced in patients with stable COPD, that this contributes to steroid resistance [2;3] and that both HDAC activity [15] and steroid sensitivity [17] can be restored by low dose theophylline. The present study extends these previous observations for the first

time to patients hospitalised because of an acute exacerbation of the disease (ECOPD).

Oxidative stress reduces HDAC activity in patients with stable COPD [11]. This is associated with histone-4 acetylation of the IL-8 promoter, which in turn increases IL-8 transcription [3], and with enhanced NF-κB mediated gene expression [25]. Increased IL-8 synthesis contributes to neutrophil recruitment, a key pathogenic event in COPD [26]. Oxidative stress in known to increase during ECOPD [7] but its potential effects upon HDAC activity have not been investigated in this setting before.

The anti-inflammatory effects of theophylline have been previously described, and the proposed mechanism of action vary from phosphodiesterase inhibition [27], mediator inhibition [28], increased apoptosis [29] or inhibition of NF-κB [30]. These direct anti-inflammatory effects, however, occur at statistically significant higher concentrations of theophylline than those used in clinical practice (>20mg/L). In contrast, at low doses, theophylline increases HDAC activity [17], reduces neutrophil concentration in sputum [31] and large airways [32] and enhances the anti-inflammatory effects of glucocorticoids [15]. These biological effects of low-dose theophylline have been described after long-term treatment [33], but can be seen after just 4 weeks of treatment [34]. They have not been investigated before in patients hospitalised because of ECOPD.

Interpretation of novel findings

We found that from COPD exacerbation to clinical stability, NF-κB activation and the concentration of several inflammatory markers in sputum decreased, and that the addition of low-dose theophylline decreased the concentration of IL-8 and TNF-α even further. We propose that these differential effects may be due to a specific effect of theophylline on HDAC activity for these two particular genes (Figure 2 and 3).

The effect of theophylline on HDAC activity can be explained by different mechanisms. First, oxidative stress causes HDAC dysfunction [11] and, in keeping with previous studies [6;7], we found increased oxidative stress during ECOPD, as indicated by reduced TAS. However, it is unlikely that this contributes to the differences observed in patients receiving theophylline because TAS recovery was similar in both groups, with no statistical differences between them. Second, increased nitrative stress may generate peroxynitrite, which nitrates tyrosine residues on HDAC2 and impairs enzyme activity [12]. As previously shown [22], we found that eNO concentration, an indirect marker of nitrative stress, increased during ECOPD (Figure 3, panel A). Interestingly, however, eNO recovery during clinical stability showed a trend, although not statistically significant, to be enhanced in patients receiving theophylline (Figure 3, panel A), suggesting that reduced nitrative stress may contribute to enhanced HDAC activity (Figure 2, panel A). This would be in keeping with previous studies showing that theophylline reduces nitrative stress *in vitro* and *ex vivo* [32].
By comparing exacerbation to stable phase three months later, the effect of low-dose theophylline on HDAC activity would not only affect the inflammatory mechanisms during exacerbations but also it would have an effect on chronic

baseline inflammation that characterise COPD. However, the reduction in NF-κB activation seen at stability in both groups indicates that the burden of inflammation is different from exacerbation to stable phase and the fact that HDAC activity is not reduced at exacerbation compared to stable phase in the control group is suggesting that HDAC activity is not able to be modified by corticosteroids alone in COPD, which confirms previous observations [2, 3].

Irrespective of the underlying molecular mechanism explaining the enhanced HDAC activity induced by theophylline, it is well established that the latter can improve steroid sensitivity in COPD [17]. This would then explain the additional reduction in the inflammatory markers observed in those patients receiving low-dose theophylline on top of standard therapy, which includes treatment with steroids (Figure 3).

Potential limitations

This was a prospective, randomised, single-blinded study, but it was not placebo-controlled. The primary goal of the study was to investigate the biological effects of low-dose theophylline upon HDAC activity and inflammation in patients with ECOPD, and not to establish its potential clinical usefulness, something that, on the other hand, would have been limited anyway by the relatively small sample size of our study. An added limitation was that our population was entirely male and we had to exclude patients with inadequate sputum samples, for which we could not generelise these findings to all COPD exacerbations. Interestingly, however, despite these limitations, we observed that patients treated with low-dose theophylline on top of the recommended treatment for ECOPD had better clinical outcomes, such as FEV_1 recovery. Mortality was also decreased in this group but this effect was not statistically different between groups. Yet, these observations, in combination with the clear cut molecular effects described here, provide a strong rationale to investigate the potential clinical relevance of this therapeutic strategy in a large, randomized, double-blind, placebo-controlled trial.

Conclusions

This study demonstrates that low-dose theophylline increases HDAC activity and further reduces inflammation when added to the standard therapeutic regime of ECOPD. The clinical implications of these molecular observations deserve investigation in a larger double-blinded placebo-controlled randomised clinical trial.

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Competing interest

BGC has received lecture fees and has served on Scientific Advisory Boards for GlaxoSmithKline, AstraZeneca and Novartis.
KI is an employee of RespiVert Ltd
PB has received research funding, lecture fees and has served on Scientific Advisory Boards for GlaxoSmithKline, AstraZeneca, Boehringer-Ingelheim, Novartis, Altana and Pfizer, all of whom have an interest in new therapies for COPD.
AA has received research fundind from AstraZeneca, Pfizer, and Boehringer Ingelheim and lecture fees for speaking at conferences sponsored by GSK, AstraZeneca and Almirall during the past five years. He has also served on advisory board for GSK, Almirall and Altana.
The rest of authors declare no competing interest with this manuscript.

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FIGURE LEGENDS

Figure 1.

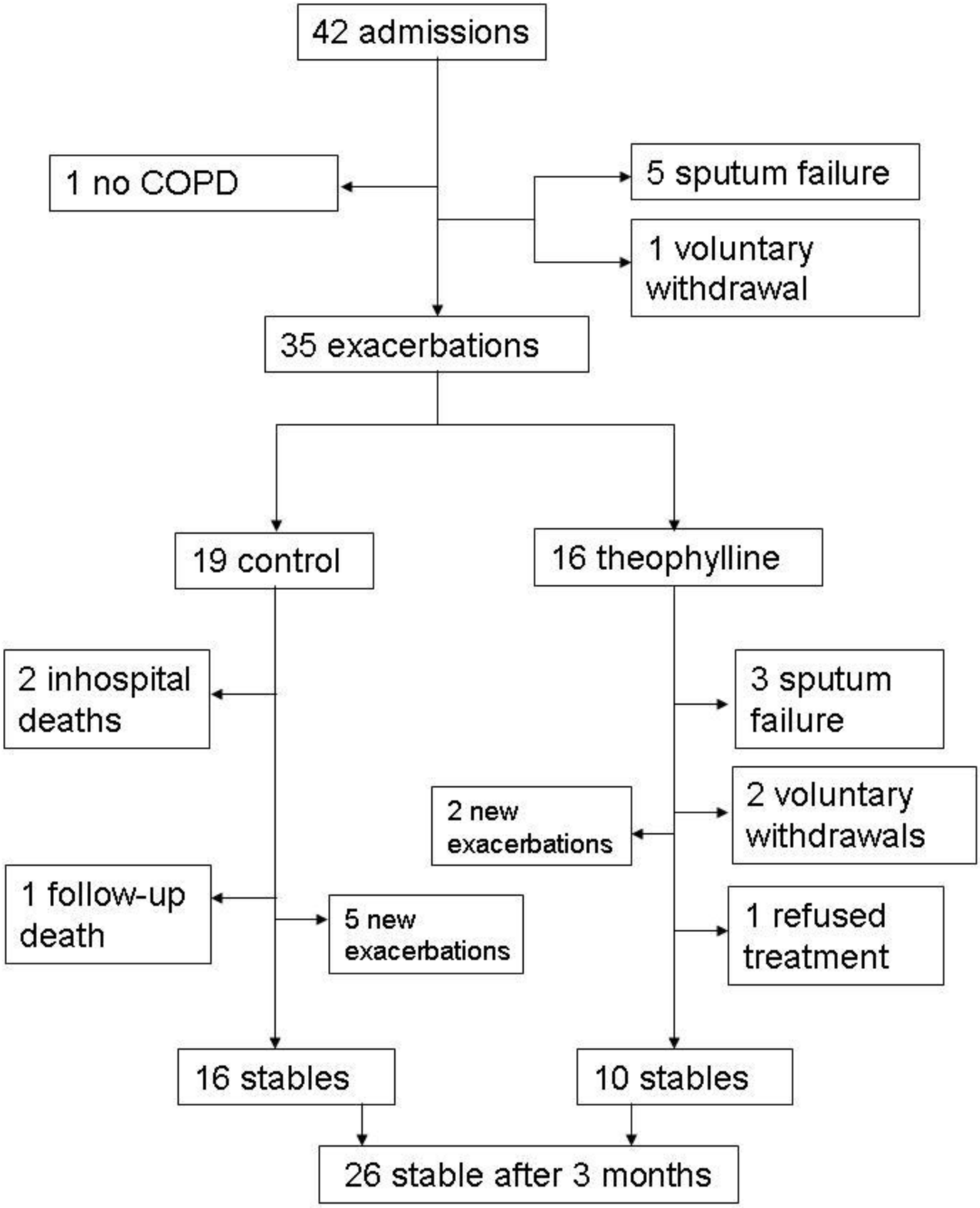
Flow diagram of study participants from recruitment, into the 2 arms, to outcomes.

Figure 2.

Mean (±SEM) values of NF-κB (panel A) and HDAC activity (panel B) in patients with ECOPD before and three months after receiving standard therapy with or without low-dose theophylline. p value refers to comparison between groups.

Figure 3.

Mean (±SEM) values of nitric oxide concentration in exhaled air (panel A), and interleukin-6 (panel B), tumor necrosis factor-α (panel C), and interleukin-8 (panel D) levels in sputum supernatants in patients with ECOPD before and after receiving standard therapy with or without low-dose theophylline. p value refers to comparison between groups.


42 admissions
1 no COPD
5 sputum failure
1 voluntary withdrawal
35 exacerbations
19 control
16 theophylline
2 inhospital deaths
3 sputum failure
2 new exacerbations
2 voluntary withdrawals
1 follow-up death
5 new exacerbations
1 refused treatment
16 stables
10 stables
26 stable after 3 months