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J Clin Pharmacol 2005 45: 498
DOI: 10.1177/0091270004273527

The online version of this article can be found at:
http://jcp.sagepub.com/content/45/5/498

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This study aimed to test the utility of the ozone challenge model for profiling novel compounds designed to reduce airway inflammation. The authors used a randomized, double-dummy, double-blind, placebo-controlled 3-period crossover design alternating single orally inhaled doses of fluticasone propionate (inhaled corticosteroids, 2 mg), oral prednisolone (oral corticosteroids, 50 mg), or matched placebo. At a 2-week interval, 18 healthy ozone responders (>10% increase in sputum neutrophils) underwent a 3-hour ozone (250 ppb)/intermittent exercise challenge starting 1 hour after drug treatment. Airway inflammation was assessed at 2 hours (breath condensate) and 3 hours (induced sputum) after ozone challenge. Compared to placebo, pretreatment with inhaled corticosteroids or oral corticosteroids resulted in a significant reduction (mean [95% confidence interval]) of sputum neutrophils by 62% (35%, 77%) and 64% (39%, 79%) and of sputum supernatant myeloperoxidase by 55% (41%, 66%) and 42% (25%, 56%), respectively. The authors conclude that an optimized ozone challenge model (including ozone responders and ensuring adequate drug levels during exposure) may be useful for testing novel anti-inflammatory compounds in early development.

Keywords: *Ozone challenge; drug profiling; induced sputum; exhaled breath condensate*

Journal of Clinical Pharmacology, 2005;45:498-503

Experimental ozone (O_3) exposure of human subjects is known to elicit a reversible impairment in lung function, as well as acute airway inflammation, characterized primarily by submucosal infiltration of neutrophils. The inflammatory response also includes sputum neutrophilia and elevated concentrations of inflammatory mediators in bronchoalveolar lavage and induced sputum supernatants. This response can be detected as early as 1 hour following exposure and may persist for 18 to 24 hours following exposure.[1-4]

Owing to the fact that the response to ozone is well characterized, reproducible, and reversible, it has been used for testing the efficacy of anti-inflammatory compounds in preclinical animal studies.[5,6] Several investigators have also used this model for testing anti-inflammatory interventions in human subjects. Pretreatment with the macrolide azithromycin had no effect on ozone-induced neutrophilia,[7] whereas treatment with the cyclooxygenase inhibitor indomethacin for 6 days inhibited the ozone-induced impairment in pulmonary function in healthy volunteers.[8] A 4-week inhalation of the corticosteroid budesonide did not attenuate ozone-induced airway inflammation in normal subjects, but in subjects with mild asthma, budesonide significantly reduced sputum neutrophilia and CXCL-8 levels.[9,10] It is not clear whether these conflicting observations were due to the differences in study populations, the duration of treatment, the dose of steroids, or the concentration of ozone (270 vs 400 ppb).

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DOI: 10.1177/0091270004273527

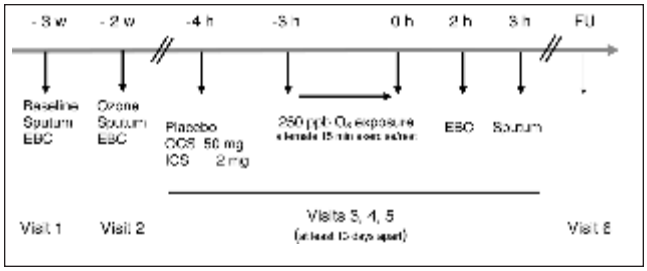

Figure 1. Study design. ICS, inhaled corticosteroids (fluticasone propionate); OCS, oral corticosteroid (prednisolone); EBC, exhaled breath condensate.

The objective of this study was to establish an optimized ozone challenge model in healthy subjects and to assess its utility for profiling anti-inflammatory compounds in early stages of clinical development. To optimize the model, we only included documented ozone responders (>10% increase in sputum neutrophils) and treated them with a single high dose of inhaled or oral corticosteroids administered immediately before the ozone/exercise challenge to ensure adequate drug exposure. In addition, subjects were challenged with a dose of ozone that is known to produce a moderate and reproducible neutrophil influx into the airways (250 ppb with intermittent exercise over 3 hours).[2]

MATERIAL AND METHODS

Study Design

We performed a randomized, double-dummy, double-blind, placebo-controlled, 3-period crossover study investigating inhaled doses of 2 mg of fluticasone propionate (inhaled corticosteroids [ICS]) and matched oral placebo, oral prednisolone (oral corticosteroids [OCS], 50 mg) and matched inhaled placebo, or both placebos (Figure 1). At visit 1, a prestudy medical examination was carried out, baseline exhaled breath condensate (EBC) was collected, and the subjects' ability to produce induced sputum was assessed. At visit 2, subjects underwent a screening ozone challenge, including EBC and sputum collection. Visit 6 consisted of a safety medical examination. The study was approved by the Ethics Committee of the Chamber of Physicians of the State of Schleswig-Holstein, and all subjects gave their written informed consent.

Study Population

Eighteen healthy, nonsmoking subjects (4 women/14 men, mean ± SD; age, 31.4 ± 8.4 years; mean forced expiratory volume in 1 second [FEV_1], 103.5 ± 13.3% pred) able to produce sputum of sufficient quality, known to be responsive to ozone (>10% absolute increase in percent sputum neutrophils over baseline), and free of respiratory tract infections for at least 3 weeks prior to tests were included (total screened: 35; unable to produce sufficient amounts of induced sputum: 6; insufficient response to ozone: 6). Subjects were not hyperresponsive to methacholine, and those with a positive skin prick test to a common allergen were tested outside their allergen season.

Ozone Exposure

Each exposure consisted of 3 hours of intermittent exercise (15-minute rest, 15-minute exercise) using equipment described previously.[2] Ozone generated from 100% oxygen was added to purified air and its concentration monitored continuously. The analyzer was calibrated by the Environmental Protection Agency of the State of Hamburg, Germany.

Sputum Induction

We followed a previously described procedure,[11] but the sputum plugs from consecutive saline inhalations were pooled and homogenized by mixing 1:4 with Sputolysin (DTT, Calbiochem, Bad Soden, Germany), followed by addition of 4 parts of phosphate-buffered saline (PBS). Mean differential cell counts were obtained from 2 observers counting 400 cells on coded cytospins. Coded supernatants (stored at –80°C) were analyzed by a contract laboratory using validated commercial enzyme-linked immunosorbent assays (ELISA). The limits of detection after dilution (to minimize potential effects of DTT and to achieve sufficient volume for measurements) were 400 pg/mL for IL-8 (CXCL-8, R&D Systems, Abingdon, UK), 40 µg/mL for total protein (Dojindo Molecular Technologies, Inc, Gaitherburg, Md), 62.5 pg/mL for IL-6 (R&D Systems), 25.6 pg/mL for TNF-α (Amersham Biosciences, Buckinghamshire, UK), and 36 ng/mL for MPO (Immundiagnostik, Bensheim, Germany).

Exhaled Breath Condensate

Exhaled breath condensate was collected during tidal breathing with a nose clip (Ecoscreen, Viasys, Hoechberg, Germany). After collection, the condensate was quickly thawed, and aliquots were stored at –80°C. The coded EBC samples were analyzed by a contract laboratory without further processing.

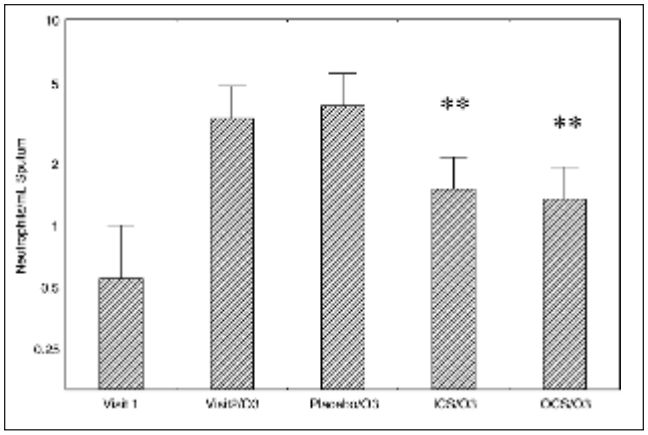

*Figure 2. Neutrophils/mL sputum at baseline (visit 1), 3 hours after ozone screening (visit 2), and 3 hours after ozone challenges following a single-dose pretreatment with placebo, 2 mg fluticasone propionate (inhaled corticosteroid [ICS]), or 50 mg prednisolone (oral corticosteroid [OCS]). **P < .001 compared to placebo treatment.*

Data Analysis

All statistical analyses were performed using SAS (Version 8.2). Each endpoint was analyzed using separate analysis of variance (ANOVA) models, adjusting for terms due to treatment period and treatment (fixed effects), as well as subject (random effect). Data were log transformed prior to analysis, where applicable, and the adjusted means for each treatment calculated together with the appropriate mean and 95% confidence intervals (CIs) for the comparisons of interest. Comparisons were made between fluticasone propionate (FP), or predisolone, with placebo. Statistical significance was declared if the 95% confidence interval for the difference did not contain 0 for untransformed data or 1 for log-transformed data.

RESULTS

Previous studies demonstrated that ozone challenge can be performed safely in humans,[2,3] provided that lung function is monitored in regular intervals to avoid marked deteriorations in lung function (decrease of forced vital capacity and FEV_1 by >50%), which are known to occur in single ozone-sensitive subjects.[4] None of the subjects included in the present study had to discontinue the challenge due to a severe decline in lung function, and those who experienced a fall in FEV_1 by more than 10% recovered within 2 hours. Overall, the mean (± SD) change in FEV_1 caused by the ozone challenge (using the 3- to 7-hour minimum postchallenge FEV_1) was –3.62% ± 6.81% (10%; 90% percentiles: –13.1%, 3.75%).

Effect of Ozone on Sputum Composition

Sputum cell differentials could be evaluated for all subjects after all treatment periods. Cytospin quality, as judged by 2 observers, did not result in the exclusion of any sample. The overall geometric mean (95% CI) squamous cell contamination was 5.5% (4.4%, 7.0%). Compared to baseline (visit 1), the screening ozone exposure (visit 2) resulted in a median (quartiles) increase in total cell counts of 128% (46%, 210%). The absolute number of neutrophils increased by 944% (541%, 1348%), and the proportion of neutrophils increased by 404% (174%, 634%), with a reciprocal corresponding decline in the proportion of macrophages by –60% (–49%, –70%). The comparison between screening visit 2 and placebo treatment revealed no statistically significant differences in sputum composition (Figure 2), suggesting that ozone-induced inflammation was reproducible in these subjects.

Effect of Treatment on Sputum Composition

Sputum composition data following placebo and steroid treatment are presented in Table I. Compared to placebo, pretreatment with ICS resulted in a lower total cell count and a lower number of neutrophils (Figure 2). Similar effects were observed after pretreatment with OCS (Figure 2). Compared to placebo, we observed a mean (95% CI) reduction in CXCL-8 of 49% (21%, 67%) after ICS and 34% (0%, 56%) after OCS treatment. Corresponding reductions in MPO concentrations were 55% (41%, 66%) and 42% (25%, 56%), respectively. There was a significant relationship between the absolute number of neutrophils and the concentration of MPO in sputum supernatant ($r = 0.74$, $P < .001$, both log transformed). Total protein levels were not altered by ozone or by steroid treatment. Most of the IL-6 and all TNF-α concentrations were below assay detection limits.

Effects on Exhaled Breath Condensate

The mean (95% CI) volume of air exhaled through the condenser was 126.8 (123.5, 129.0) L, resulting in 2.2 (2.1, 2.4) mL of EBC. No significant differences in the concentrations of CXCL-8, nitrate, 8-isoprostane, or total protein were observed when comparing ICS, OCS,

Table I Sputum Composition (N = 18)

	Adjusted Means			Mean Difference (95% CI)			
	Placebo	ICS	OCS	ICS vs Placebo		OCS vs Placebo	
Macrophages, %	29.9	32.7	45.4	2.8	(–6.4, 12.0)	15.5	(6.2, 24.7)[a]
Neutrophils, %	61.5	53.2	42.9	–8.3	(–18.0, 1.4)	–18.6	(–28.4, –8.9)[a]
Eosinophils, %	0.5	0.5	0.3	–0.04	(–0.5, 0.4)	–0.23	(–0.7, 0.2)
Lymphocytes, %	2.3	2.2	1.4	–0.10	(–1.4, 1.2)	–0.10	(–2.3, 0.4)
	Adjusted Geometric Means			Mean Ratio (95% CI)			
TCC, 10^6/mL	6.33	3.01	3.28	0.48	(0.32, 0.72)[a]	0.52	(0.34, 0.78)[a]
Macrophages, 10^6/mL	1.74	0.86	1.37	0.49	(0.31, 0.78)[a]	0.79	(0.50, 1.25)
Neutrophils, 10^6/mL	3.80	1.49	1.34	0.39	(0.24, 0.65)[a]	0.35	(0.21, 0.58)[a]
Eosinophils, 10^6/mL	0.005	0.005	0.003	1.01	(0.22, 4.64)	0.50	(0.11, 2.30)
Lymphocytes, 10^6/mL	0.04	0.03	0.03	0.67	(0.16, 2.81)	0.59	(0.14, 2.52)

ICS, inhaled corticosteroid; OCS, oral corticosteroid; CI, confidence interval; TCC, total cell count per mL sputum.
a. Indicates that 95% CI does not contain 0 or 1 (log-transformed case), indicating significance at the 5% level.

and placebo. CXCL-1 and IL-6 were not detected in EBC samples.

DISCUSSION

In this study, we demonstrated that single doses of inhaled or oral corticosteroid were capable of significantly attenuating ozone-induced airway inflammation in healthy subjects. We aimed to maximize the likelihood of a positive outcome by including only subjects who were known to be sensitive to ozone in terms of neutrophilia and by ensuring adequate drug levels during ozone exposure through an appropriate timing schedule. Given these prerequisites, the challenge model showed significant effects of both the inhaled and the oral corticosteroids. The challenge as performed in the present study therefore fulfills basic criteria required for profiling novel anti-inflammatory compounds, particularly their efficacy in reducing acute airway neutrophilia. It suggests that taking the marked steroid effects as a reference is a valid approach.

Based on current knowledge, the airflow limitation in chronic obstructive pulmonary disease (COPD) is associated with an abnormal neutrophilic inflammatory response of the lungs to noxious particles or gases.[12] Patients with COPD or smokers with chronic bronchitis and excess mucus production show high numbers of neutrophils in bronchoalveolar lavage or sputum.[13] It is thought that neutrophils promote the development and the progression of the disease by the release of proteases and the production of reactive oxygen species.[12] Therefore, lowering the number of neutrophils within the airways seems a reasonable strategy in the pharmacotherapy of COPD and an indispensable prerequisite for potential future interventions that aim at restoring the normal airway and lung architecture.

To test novel compounds for their ability to attenuate neutrophil recruitment into the airways, human experimental model systems would be of significant value. To ensure acceptability to participants and a rapid turnaround time, acute inflammatory models are preferable. There are several agents known to induce acute neutrophilia, such as endotoxin and ozone. Ozone was selected for this study, as the transient neutrophilia caused by ozone is well documented to be reproducible within subjects,[2,14] it is completely reversible, and its bioreactivity is mainly based on its potent oxidative capacity.[15] It could therefore serve as a model for oxidative stress experienced in asthma or COPD exacerbations.

In line with previous observations,[2] we found increases in the number and percentage of neutrophils that differed between individuals and were, at the same time, reproducible within individuals. This was shown by the correlation between the changes from baseline (visit 1) to ozone screening (visit 2) and from baseline to the ozone challenge following placebo treatment (data not shown). This correlation was significant, despite the fact that the study was not specifically designed to confirm this result. In particular, we did not include a filtered air exposure and thus as-

sessed ozone responses solely by comparison with baseline data.

Overall, there are few studies reporting the effects of drugs on the inflammatory airway response to ozone in human subjects, and the data on corticosteroids are equivocal.[9,10] Nightingale and coworkers[9] enrolled healthy subjects, treated them for 2 weeks with 800 μg budesonide twice daily, exposed them to 400 ppb ozone for 2 hours, and collected sputum 4 hours after inhalation. In contrast, Vagaggini et al[10] enrolled subjects with mild asthma, who were treated with 400 μg budesonide twice daily and exposed to 270 ppb ozone for 2 hours. Sputum was sampled 6 hours after exposure. Only the latter study (ie, the one performed in subjects with asthma) showed a significant treatment effect similar to our results obtained in healthy subjects.[10] The reason for the pronounced effect of steroids in our study is most likely due to the fact that we administered a high dose shortly before ozone exposures and thereby ensured higher drug levels during the challenge. Intersubject variability was also probably reduced because the study included only volunteers who were proven to respond to ozone in terms of airway neutrophilia. Another factor contributing to the positive outcome was likely the avoidance of the high level of ozone, as used by Nightingale et al (400 ppb). This represents a strong stimulus, which caused a marked neutrophil influx into the airways (all subjects showing >75%). In planning the study, we considered this profound stimulus to be less amenable to blockade by therapeutic intervention. Finally, the sampling time of sputum and EBC was chosen earlier compared to the previous studies, although we do not know in detail whether this had a significant effect on the study outcome. Bronchoalveolar lavage data indicate a peak of inflammation about 6 hours after exposure.[8] On the other hand, significant and well-reproducible sputum neutrophilia has been observed as early as 1 hour after the end of exposure.[2]

Corticosteroids are known to attenuate inflammatory processes by binding to steroid receptors and interacting with promoters of genes involved in cell activation.[16] Our study was designed to establish a positive control for potential future studies with experimental drugs. We therefore aimed to maximize the potential for airway protection by a single dose of the drug, without the necessity to reach hard-to-control steady-state conditions. Thus, we used the highest single doses of potent inhaled and oral steroids that had been shown to be safe and well tolerated. Maximum plasma concentrations of prednisolone following 50 mg dosing are reached within 1 hour.[17,18] With a half-life of 4 hours, we expected to cover the duration of the ozone exposure adequately. Similar considerations hold for fluticasone. We would like to point out that our study did not intend to suggest such high-dose regimens as a potential long-term treatment of neutrophilia either in environmental ozone exposures or in patients with airway neutrophilia.

As we aimed to study anti-inflammatory effects, ozone responders were defined on the basis of a more than a 10% increase in sputum neutrophils. Ozone exposures are known to exert effects on lung function, too. We observed only small effects on FEV_1, without significant differences between treatments, with mean changes (preozone to postozone) being –1.4% at visit 2, –4.2% after placebo, –5.0% after ICS, and –3.9% after OCS. It is known that both lung function and neutrophil responses to ozone are reproducible within subjects[2,14] but are independent of each other.[2,4] Thus, the observation of a marked steroid effect on airway neutrophilia, without a concomitant effect on lung function, is compatible with previous findings.

In the present study, airway inflammation was assessed primarily via sputum composition, which is known to be affected by ozone inhalation. To broaden the range of potentially sensitive variables that can be measured noninvasively, we also incorporated EBC sampling and analysis. This required integration of this method into the schedule of a clinical trial involving the collection and intermittent storage of samples prior to the analysis in specialized laboratories. Exhaled breath condensate samples were repeatedly sent every 4 to 6 weeks during the trial duration of approximately 6 months, instead of just 1 shipment for sputum supernatants. We therefore have to consider potential effects of storage on the detection of some of the compounds of EBC.

In conclusion, based on the present findings, we propose an ozone challenge model for the induction of acute neutrophilic airway inflammation that is amenable to short-term therapeutic intervention. The components of the model are a moderate ozone exposure, healthy subjects with a documented response to ozone in terms of airway neutrophilia, sputum induction 3 hours after exposure, and interventions by single high doses of an inhaled or oral corticosteroid shortly before exposures. Using this approach, fluticasone and prednisolone significantly attenuated the neutrophil influx into the upper airways. The design as described in the present study might therefore be suitable for testing novel anti-inflammatory compounds that are tar-

geted to reduce airway neutrophilia, and the steroid effect might be used as a reference for establishing both a scale of responses and comparability between studies.

We thank Arhodula Kratzmann and Kirsten Paasch for excellent technical assistance and their support in assessing differential cell counts, Andrea Ludwig-Sengpiel for her help in clinical assessments, Paul Linacre for supporting biomarker assay validation and analysis, and Ffyona Dawber and Rabia Anselm for clinical operations support.

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