HO-1 PROTECTS SMOKERS EXPOSED TO ARTIFICIAL STONE DUST FOR PULMONARY FUNCTION TESTS DETERIORATION

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ABSTRACT. *Background:* The Heme Oxygenase system, along with its catabolism products, is involved in a variety of crucial physiological functions, including cytoprotection, inflammation, anti-oxidative effects, apoptosis, angiogenesis, and vascular regulation. *Objectives:* To analyze the Heme Oxygenase -1 (HO-1) mediated effect of mild deterioration of pulmonary function testing (PFT) in exposed artificial stone smoking workers. *Methods:* One hundred stone workers divided into current smokers, ex-smokers and never smokers underwent Low Resolution Computed Tomography, PFT, induced sputum (IS) Particle Size Distribution (PSD) and Real Time PCR in IS samples. *Results:* Smoking status had no significant effect on PFT results but it altered the IS differential cell counts. There was significantly less decline in PFT over time for the smokers group. There was a significantly lower fraction of small particles (<2 μm) in the IS of the current smokers group compared to the never- and ex-smokers groups. HO-1 gene expression was higher among smokers compared to never- and ex-smokers groups. A low percentage of small particles (<5 μm) correlated negatively to the percentage of neutrophils and positively to the percentage of macrophages in the sputum of the smokers group. *Conclusions:* We found significantly lower risk for decreased PFT deterioration among smokers workers exposed to artificial stone dust with higher HO-1 gene expression suggesting a possible protective effect of smoking by the involvement of HO-1 mechanism. *(Sarcoidosis Vasc Diffuse Lung Dis 2018; 35: 276-284)*

KEY WORDS: silicosis; artificial stone; occupational disease; lung function; inflammation

INTRODUCTION

Smoking cigarettes causes considerable morbidity and mortality by inducing cancer, chronic lung

and vascular diseases, and oral disease (1). Many studies have demonstrated the effects of smoking on the lungs, including decreased pulmonary function (2), increased oxidative stress (3) and initiation of inflammatory processes which induce biological damage (1). Occupational exposure to hazardous dust also contributes to lung injury. Epidemiological studies have shown that smoking is highest among individuals who are exposed to possible deleterious occupational factors (4), raising the question of the extent to which cigarette smoking contributes to the health injuries observed in occupational diseases. The marble industry in Israel, which manufactures kitchen

and bathroom countertops, is based mainly on artificial stone that contains more than 90% silica (SiO_2). Overexposure of marble workers to high concentrations of silica dust in their workplace may lead to silicosis, a chronic granulomatous disease characterized by inflammatory processes followed by fibrosis of lung tissue (5). Smoking may interact with silica dust to modify the risk of multiple disease outcomes, especially pulmonary diseases, since the lungs are the target organs of hazardous substances (including cigarette smoke) whose route of entering the body is by inhalation. Heme Oxigenase-1 (HO-1) is a cytoprotective enzyme defense against oxidative stress by reduction of oxidation and inflammation. There are several studies that show its involvement in response to hazardous particle exposures such as welding fumes (6), nickel nanoparticles (7), and crystalline silica (8, 9). HO-1 is also affected by smoking due to high levels of reactive components and radicals present in cigarette smoke. These elements trigger cytotoxic effects, leading to increase in its expression (10). Recent years have witnessed accumulating evidence that cigarette smoking, for all of its overwhelming harmful effects, might decrease the incidence and/or severity of several diseases, including ulcerative colitis, endometriosis, uterine fibroids, endometrial cancer, Parkinson's disease, Sjögren's syndrome, farmer's lung, pigeon breeder's disease, and sarcoidosis (11-14). In one study that analyzed the effect of cigarette smoking on pulmonary sarcoidosis, the authors concluded that smoking plays a certain protective role in the occurrence of sarcoidosis (15). Another study supported the idea that smoking reduces the incidence of sarcoidosis, suggesting that the appearance of increased numbers of macrophages in the lower respiratory tract induced by cigarette smoking contributes to the lower incidence of pulmonary sarcoidosis in smokers (16). We recently reported that workers with no history of smoking who were exposed to artificial stone dust displayed airway neutrophilic inflammation and high microparticle content in induced sputum (IS) that correlated with lower pulmonary function parameters (17) but we did not study the smoker population Artificial stone-induced silicosis is a disease which shares characteristics similar to those of sarcoidosis. The aim of the current study, therefore, was to analyze the effect of cigarette smoking on pulmonary function in that population and to investigate the involvement of HO-1 in this process, by means of two noninvasive methods, pulmonary function testing (PFT) and IS. To the best of our knowledge, this is the first time that the effect of smoking on the pulmonary status of this population has been evaluated.

Materials and methods

Study Population

The study was opened in October, 2012 and closed in December, 2014. One-hundred individuals, all males, working with artificial stone were recruited from small enterprises throughout the country. Recruitment was done by physician referral, direct contact with the factories and self-referral. Ethical approval was granted by the Institutional Ethics Committee of Tel Aviv medical center, number 0619-10-TLV. Informed consent was obtained from all individual participants included in the study. All participants paid one visit for clinical assessment by PFT and sputum induction. Workers were divided into three groups: current-smokers, ex-smokers (participants who reported smoking cigarettes in their lifetime and who, at the time of recruitment, no longer smoked) and never smokers.

Occupational Questionnaire and Exposure Assessment

A self-reported occupational questionnaire was completed by all exposed workers. The questionnaire was validated by the Epidemiological and Preventive Medicine Department in the Tel Aviv University School of Public Health. It included demographic and occupational parameters, smoking and alcohol habits, a general and a respiratory health profile and hygiene conditions at the workplace.

Pulmonary Function Tests (PFT)

PFT were performed by a Masterlab spirometer (Masterlab E. Jaeger, Wurzburg, Germany). Measurements were carried out according to standard protocols of the ATS guidelines (18).

Sputum Induction and Processing

Sputum induction and processing were performed with an aerosol of hypertonic saline gener-

ated by an Ultrasonic Nebulizer-Model Omron U1 (Omron Health Care, USA) that has an output of 0.5 ml/min and particles with <5 μm aerodynamic mass median diameters (19).

Particle Analyses (Size and Shape)

Particle size analyses were performed according to a laser technique based on the time of transition theory using a DIPA 2000 Analyzer (Donner Technologies Ltd, Israel) (20). Particle measurement was performed long enough to reach a confidence interval (CI) of 95%.

RNA extraction and real–time polymerase chain reaction (RT-PCR) for HO-1gene expression in IS

RNA was extracted from sputum cells by TRI reagent-chloroform solutions. Quantization of the RNA amount was done by GeneQuant™ 1300 Spectrophotometer (Amersham Biosciences). RT-PCR was carried out with 0.5 μg of total RNA that was extracted from the sputum cells. Quantitative RT-PCR (QRT-PCR) was carried out, using the LightCycler SYBER Green PCR Master Mix (Roche LTD) for HO-1gene expression.

Imaging Score

The study subjects' chest computerized tomographic (CT) scans were evaluated for features indicative of silicosis, including nodular distribution and progressive massive fibrosis in 3 zones (upper, middle and lower) of each lung, using a system for scaling severity described earlier by several groups (21). Each lung zone was graded between 0 and 7

according to the severity of findings. The sum of all the grades from the 6 lung zones yielded the total grade for the subject (ranging from 0-42).

Statistical Analysis

Comparisons between groups were performed by one-way analysis of variance (ANOVA). Correlation between particles <2 μm and continuous variables was done by the Spearman correlation. All statistical analyses were performed using the SPSS software version 22.0 for Windows (SPSS, Chicago, IL, USA). All P values are two-sided, and P values less than 0.05 were considered significant.

Results

One-hundred workers who were exposed to artificial stone dust were included in the present study. They were divided into 3 groups according to smoking status: never smokers (n=36), current smokers (n=32) and ex-smokers (n=32). There were no significant group differences in years of exposure to silica or age of participants (Table 1). Since duration of exposure to silica dust is known to correlate with deterioration of lung function (22), we first looked at its effect in the 3 groups of our cohort (heretofore presented in the order of never, current and ex-smokers). Indeed, the decline in PFT correlated to the duration of exposure, with lower PFT scores being seen with increased years of exposure (FVC 94.7±15.8, 84.8±17.5, and 74.9±17.9; FEV1 92±16.1, 80.3±20.1, and 69.4±21.5, DLCOsb 89.2±16.1, 78.9±14.6, and 70.4±18.7 for <10 years, 10-20 years and >10 years of exposure, respectively, P <0.05) (Fig. 1a). Next,

Table 1. Demographic and Clinical Parameters of the Study Population (n=100)

Smoking status	Never smoker (n=36)	Ex-smoker (n=32)	Current smoker (n=32)	P value*
Pack years, mean (± SD)	0	16.9 (17.5)	20.1 (21.5)	0.054
Age, years, mean (± SD)	46.8 (11.8)	49.9 (10.2)	45.3 (10.7)	0.23
Exposure, years, mean (± SD)	19.6 (9.2)	21.5 (11.0)	19.0 (8.9)	0.57
BMI, mean (± SD)	25.8 (3.8)	26.6 (3.9)	27.9 (4.4)	0.38
Born in Israel, n (%)	27 (75)	21 (65.6)	26 (81.3)	
Formal education (%)†	63.8	62.5	53.1	
Religion (Jewish/Muslim/other)	24/11/1	24/4/4	18/10/4	

SD, standard deviation; BMI, body mass index
*Significance set at P <0.05 (one-way ANOVA)
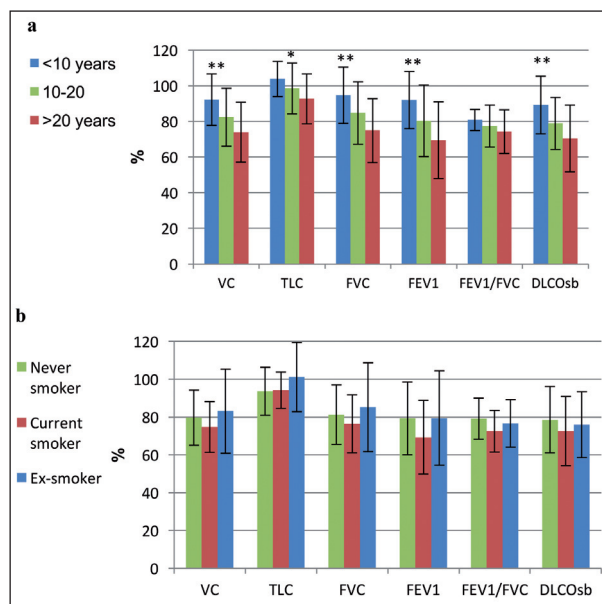†Graduated from high school, professional studies, academic degree

Fig. 1. Pulmonary function tests according to (a) years of exposure (b) smoking status.
*Significant (*P* <0.05, independent t-test), **Significant (*P* <0.01, independent t-test)
VC, vital capacity; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; TLC, total lung capacity; DLCOsb, diffusion lung CO in a single breath

we compared the PFT results between the 3 groups, regardless of the duration of exposure to silica dust, and found no difference between them (Fig. 1b).

In contrast, smoking status altered the inflammatory parameters from the IS differential cell counts, which showed significantly higher levels of eosinophils and lower levels of lymphocytes in the smokers compared to the ex-smokers and the never smokers (Table 2). There were no significant differences in the IS differential cells counts in relation to the length of exposure to silica dust (data not shown).

We then tested the hypothesis that PFT results are affected from both the number of years of exposure and current smoking status and observed different trends of PFT deterioration in each of the 3 groups. The length of exposure to silica dust was not associated with any deterioration of lung functions whatsoever in the current smokers group while there were highly significantly lower scores in VC, TLC, FVC and FEV1 measurements with greater duration of exposure to silica in the ex-smokers group. Moreover, significantly lower values of FEV1 and DLCO were found together with a trend toward significantly lower FVC and VC values as the duration of exposure increased in the never smokers group (Fig. 2 a-e). Finally, smoking showed what appeared to be a "protective" effect in functional parameters in the group with proven silicosis (OR 0.562; CI 0.216-1.464, *P*=0.238) (Table 3).

Using a unique score (see methods) to define the pulmonary involvement of ever smokers in our cohort with the same segmentation (smoking status as well as duration of exposure), we found that the current smoker group had fewer silicosis features (lower scores) than the ex-smokers group (6.7 vs 12.5, respectively, *P*=0.021), in agreement with the PFT findings (Fig. 3).

We had recently found that a larger fraction of small particles in the IS of silica exposed workers

Table 3. Odds Ratio for Smoking And Silicosis

	Silicosis*	Suspected silicosis†
Smokers	19	11
Non-smokers	43	14

OR 0.562 (95% CI 0.216-1.464) *P*=0.238
*Silicosis=established diagnosis of silicosis determined by biopsy and/or HRCT prior to study recruitment.
†Suspected silicosis=without established diagnosis at the time of study recruitment.
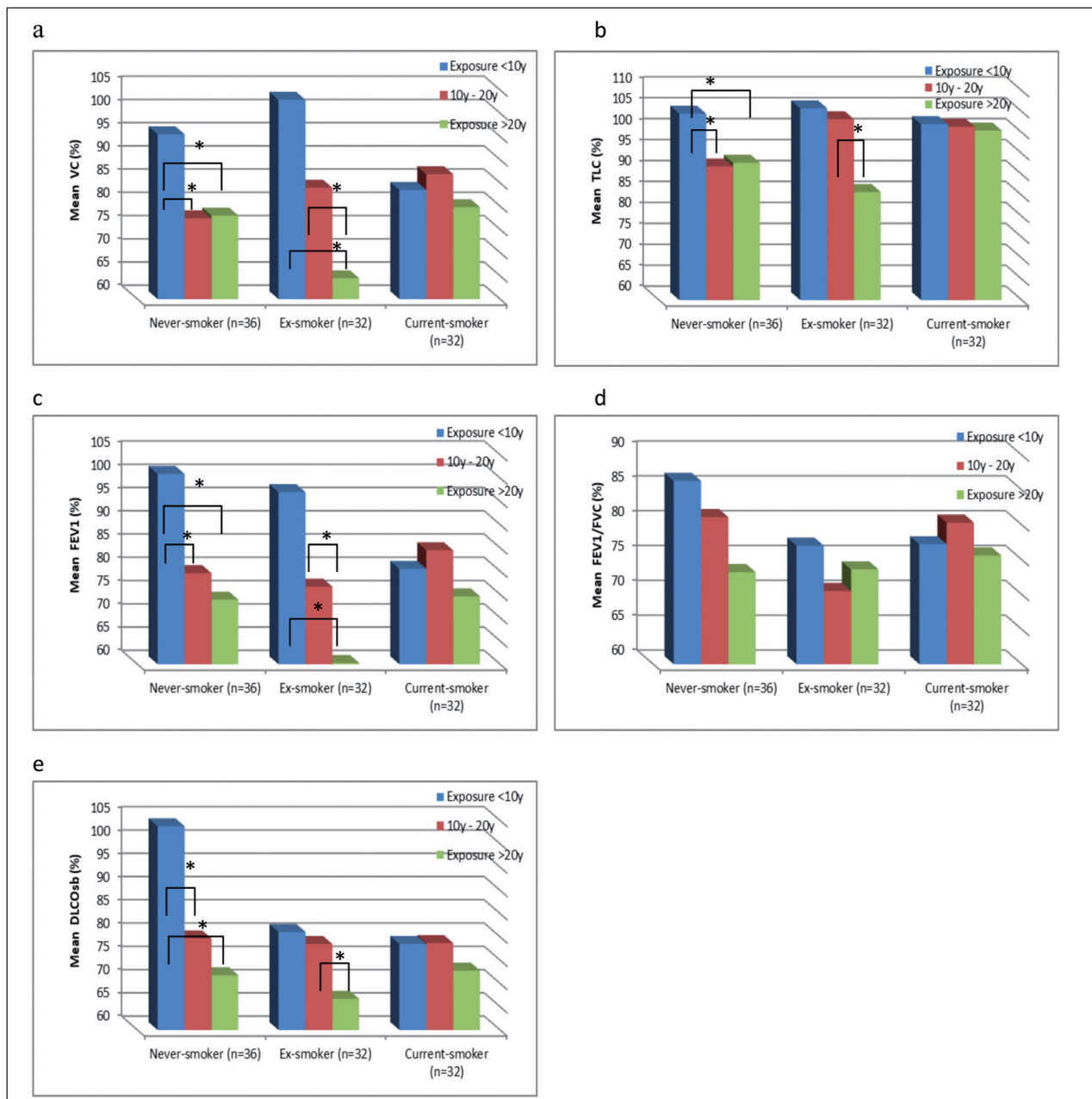CI, confidence intervals; OR, odds ratio

Table 2. Differential Cells Counts* in Induced Sputum

	Never smoker n=30	Ex-smoker n=32	Current smoker n=29	P value†
% Neut	68.0 (24.8)	69.3 (20.4) n=32	67.2 (22.7) n=29	0.94
% Eos	0.7 (1.7)	1.8 (3.8) n=32	3.9 (6.6) n=29	0.02
% Lymph	12.5 (9.8)	12.5 (8.6) n=32	7.9 (5.7) n=29	0.04
% Macro	18.9 (22.3)	14.6 (14.5) n=32	20.1 (22.2) n=29	0.55

DCC, differential cells counts; Neut, neutrophils; Eos, eosinophils; Lymph, lymphocytes; Macro, macrophages
Values are given mean (± standard deviation)
*Adjusted to age.
†*P* <0.05 (one-way ANOVA)

Fig. 2. Pulmonary function tests according to smoking status and years of exposure.
*Significant (*P* <0.05, independent t-test), **Significant (*P* <0.01, independent t-test)
VC, vital capacity; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; TLC, total lung capacity; DLCOsb, diffusion lung CO in a single breath

who had no smoking history correlated with lower PFT scores (23). We therefore looked at the particle size distribution of our current cohort whose smoking status was varied. The particles derived from samples of the current smokers group contained a significantly lower fraction of particles (<5 μm) in the IS samples compared to the ex-smokers and the never smokers groups (91.9±4.9 versus 95.3±3.6 and 94.2±4.6, respectively, *P*=0.038) (Table 4). This low percentage of particles was negatively correlated to the percentage of neutrophils in the IS, suggesting a possible causative link between high neutrophilic
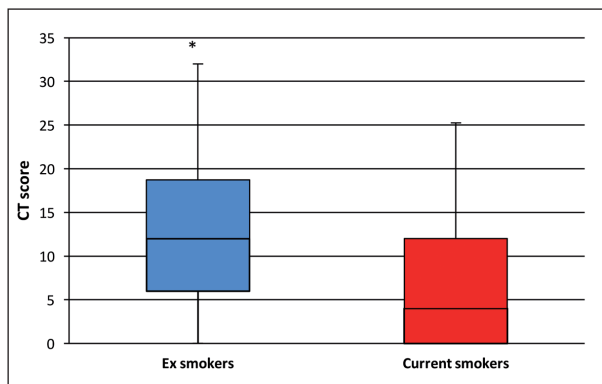
Fig. 3. Chest computerized tomographic (CT) scores among ever smokers.
* Significance set at P <0.05 (Mann-Whitney test)

Table 4. Accumulation of Particles <5 μm in Induced Sputum Cells

	Particles <5 μm	P value*
Never smokers (n = 32)	94.2 (4.6)	0.038
Ex-smokers (n = 26)	95.3 (3.6)	
Current smokers (n = 24)	91.9 (4.9)	

*Current smokers vs. never smokers/ex-smokers; significance set at P <0.05 (one-way ANOVA). Results are presented as mean (SD)
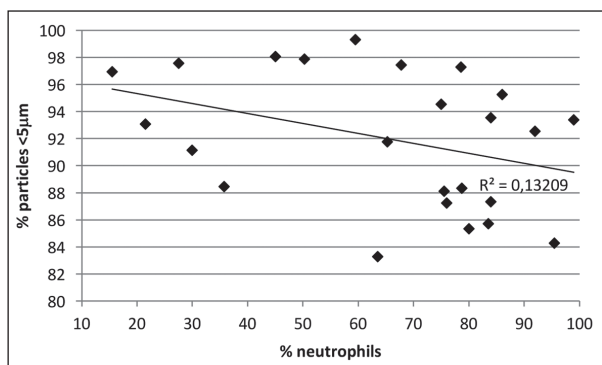

Fig. 4. Correlation between Particle Size Distribution and neutrophils among smokers
PSD were evaluated by DIPA analyzer in IS.
p value of spearman correlation=0.069.

inflammation and lower content of small particles in IS samples (r=-0.377, P=0.069) (Fig. 4).

Looking at the m-RNA expression of HO-1, we observed a significantly higher expression in current smokers compared to ex- and never smokers groups (0.82±0.14; 0.45±0.05; 0.54±0.07, respectively, P <0.05) (Fig. 5). Among non-smokers, HO-1 gene negatively associated with years of exposure to artificial
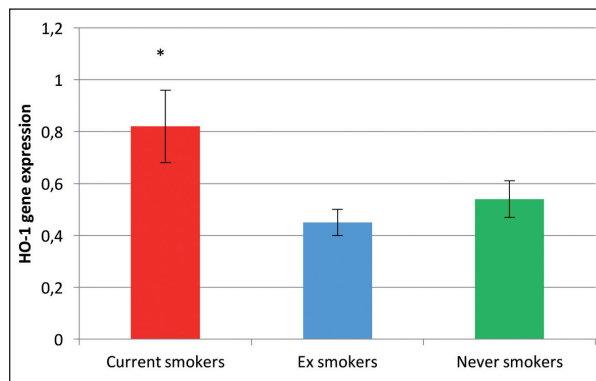

Fig. 5. Evaluation of HO-1 m-RNA expression in IS samples.
HO1 gene expression was done by real time PCR (ΔΔct method) normalized by internal gene (GAPDH). Y axis represents the gene expression relative to GAPDH.
**Significance set at p <0.05 (Kruskal-Wallis test)

Table 5. Correlation between Particle Size Distribution and HO-1 gene expression among non smokers

		Particles <5 μm	Exposure duration
m-RNA of HO-1	r-spearman	0.273	-0.303
	p-value	0.066	0.026
	N	46	54

PSD were evaluated by DIPA analyzer in IS. Exposure duration was evaluated by questionnaires.
HO1 gene expression was done by real time PCR (ΔΔct method) normalized by internal gene (GAPDH).HO-1, hemeoxigenase-1 Statistics. p values are for spearman correlation.
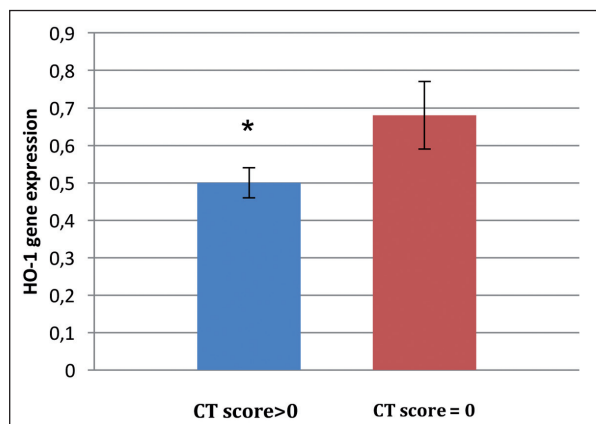

Fig. 6. Ho-1 Expression according to CT score.
CT score was converted to dichotomy parameter.
* p value=0.054 (Mann-Whitney test)

stone dust (Table 5). A marked decreased expression of the gene was observed among the group with a progressive score of lung injury determined by low resolution CT (0.65±0.08; 0.45±0.04. p=0.054) (Fig. 6).

Discussion

The main finding of this study is the unexpected positive effect of smoking among workers exposed to artificial stone dust in correlation to the years of exposure. Current smokers exposed to artificial stone dust had better lung functions and CT imaging findings compared to exposed workers who never or no longer smoked. These findings suggest a possible "protective" effect of cigarette smoking against injury from extended exposure to airborne silicone dust particles. During the last two decades, multiple studies showed a positive effect of smoking on lung function in several granulomatous lung diseases. TLC and DLCO were found to be significantly higher in ever smokers compared to never smokers with sarcoidosis (24), and smokers were less likely to have restrictive spirometry in hypersensitivity pneumonitis (25). Akgun et al. recently showed that pulmonary function loss was positively associated with never smoking among former sandblasters with silicosis, and that the mean number of smoking pack-years in the subjects who died in their cohort was significantly lower (26). We also found that lung functions are more preserved with time among smokers compared to non-smokers and ex smokers as the length of exposure increases. On the other hand, a recently published cohort study among workers exposed to silica in China reported that smoking was one of 6 prognostic risk predictors for the development of silicosis (27), although it should be noted that that study was retrospective and looked solely at prediction models.

The lower prevalence of sarcoidosis, occupational lung diseases and hypersensitivity pneumonitis among smokers is well documented in a number of epidemiological studies (16, 26, 28), however the mechanism by which smoking − unquestionably extremely toxic to the lungs and airways − reduces the pathologic effect in pulmonary diseases is still not well understood. In a murine model of chronic pigeon breeder's lung, cigarette smoke lessened the lymphocyte proliferation and lymphocyte count in the bronchoalveolar fluid (29). That observation supports our finding of lower lymphocyte counts in the sputum of past and present smokers in our cohort. Furthermore, several reports indicated that alveolar macrophages from smokers are functionally impaired and secrete significantly lower levels of proinflammatory cytokines which are crucial for early responses to pathogens and the up-regulation of local host defenses (14). Thus, chronic smoke exposure may induce a decreased inflammatory response to other noxious particles, such as silica particles. We demonstrated a lower level of small particles (<2 μm) in the sputum of smokers exposed to artificial stone dust. These results are in agreement with our earlier findings of lower levels of smaller particles in mice exposed to cigarette smoke and lipopolysaccharides (LPS) and a higher percentage of neutrophils in BAL (30), suggesting that cigarette smoke reduces the amount of smaller particles in the respiratory tract due to neutrophilic-induced inflammation. The lower percentages of small particles among our past and present smokers were correlated to increased neutrophilic inflammation as well. Others have shown that increased penetration of particles into the circulation due to high permeability of the alveolar epithelium was due to LPS-derived inflammation (20). Those inflammatory changes induced leaks and damage to the alveolar barrier, allowing small particles to enter the systemic circulation (30-32).

In this context, we recently reported an outbreak of silica-related autoimmune disease among synthetic stone construction workers diagnosed as having silicosis and referred for lung transplantation. We suggested that the very small silica particles may evade the phagocytic defense and cross the inflamed barrier to enter the systemic circulation (33).

Interestingly, the weak immune response among smokers is not exclusive to the lungs. There are considerable data indicating that smokers have a significantly lower risk for ulcerative colitis (34), sarcoid arthritis (35) and Parkinson's disease (36). One proposed mechanism of action in ulcerative colitis is the heme oxygenase-1 (HO-1) protein level, which is known to be high in smokers (37). Here we also showed higher HO-1 expression among smokers compared to ever smokers, HO-1 augments the anti-inflammatory effects of interleukin-10 and has cytoprotective antioxidant properties (38), thus it may also explain, at least in part, the protective effect of smoking in lung inflammatory states, such as silicosis. The mechanism for the protective effect of smoking seen among our study participants may be associated with the low percentage of particles in the airways of smokers which are known to have a deleterious remodeling and fibrogenic effect (39, 40). On the other hand, among the non-smokers accumula-

tion of particles <5 μm recovered by IS positively associates with HO-1 gene expression, meaning that these particles might provoke oxidative stress, as previously shown in dental technician population and asthmatic children in urban areas (41, 42). We also found that its expression was attenuated as years of exposure were longer, suggest the loss of protection against oxidative stress over time by the chronic exposure to the hazardous dust.

This study has some limitations. First, data on exposure to environmental biomass fumes and other particulate matter that may have a deleterious effect on pulmonary function were not available. Second, our study was designed to include only one clinical visit, thus precluding the possibility of monitoring a tendency of deterioration of pulmonary function among our subjects, although the subgroup analysis on length of exposure did succeed in showing the effect of smoking in a relatively large cohort of workers exposed to silica dust.

Conclusions

Combining data from other diseases may support the hypothesis that cigarette smoking induces an abnormal inflammatory process which diminishes the pathological response to silica particles and thus changes the clinical expression of the natural history of silica exposure. Although the components in cigarette smoking indisputably have detrimental consequences to health, our current results raise the possibility that some of them may be harnessed as potential therapeutic elements to treat pulmonary diseases. Smokers were shown to have a significantly lower risk of ulcerative colitis, endometriosis, sarcoid arthritis, uterine fibroids, endometrial cancer, Parkinson's disease, Sjögren's syndrome, farmers' lung, pigeon breeders' disease, occupational lung diseases, hypersensitivity pneumonitis and sarcoidosis. As absurd as it may sound, smoking may have a similar effect on the lung functions of individuals who are exposed to airborne particles, such as artificial stone dust.

Acknowledgment

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