

# INCREASED SERUM HEME OXYGENASE-1 IN SILICOSIS-SUSPECTED SUBJECTS FROM LIMESTONE CRUSHER FACTORIES

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**ABSTRACT:** Heme Oxygenase-1 (HO-1) is a lung inflammation and oxidative stress biomarker. In this study, it was proposed as a sensitive biomarker to indicate silicosis. The objective of the study was to determine a relationship between serum HO-1 and occupational exposure to silica in limestone crusher workers. Total crystalline silica was quantified by ultraviolet visible spectrometry. A chest radiograph was performed in a General Hospital by a trained radiologist. The serum HO-1 level was determined by sandwich enzyme immunoassay. In the results, the total crystalline silica in the studied factories, ranged from 0.94-27.03 mg/m<sup>3</sup>. The level of serum HO-1 in silica high exposure group was significantly elevated compared with low exposure group. In chest radiography, there were 4 silicosis-suspected subjects and their serum HO-1 levels were significantly higher than non-silicosis subjects after adjustment by mean age and smoking status. A positive relationship between serum HO-1 level and increased employment duration was also observed. The increased serum HO-1 level was specifically related to silica exposure and chest radiograph finding independently from age and smoking status. The serum HO-1 level has a potential to be used as an indicator of silicosis and it could also reflect the oxidative stress caused by silica exposure.

**Keywords:** Silicosis, Heme oxygenase-1, Biomarker, Occupational exposure, Estimated marginal mean, Chest radiograph

## INTRODUCTION

Silicosis is a typical occupational lung disease which is related to long term silica dust exposure in the workplace. It is a fibrotic pulmonary disease with chronic inflammation for which there is no cure [1]. Patients suffer with shallow breathing and have a high risk of lung cancer [1-4]. Silicosis, a form of pneumoconiosis, is still reported worldwide [5]. The economic loss of pneumoconiosis has been calculated as being 2,529 USD per person per year which is a burden to the governments and patients' families [6], although there is no report specifically for silicosis. As such it is a significant public health problem. At present, the diagnosis of silicosis relies on chest radiography showing nodular opacities [1].

This diagnostic method is not sufficiently sensitive to detect early changes in the lung before irreversible pathology develops.

The use of a biomarker has been proposed as a sensitive diagnostic method for silicosis [7]. In clinical practice, a biomarker is recognized as a "surrogate endpoints" which is used to determine a progression of disease before clinical symptom is experienced. At this point, a treatment may be possible and a permanent pathology can be prevented [8, 9].

Heme oxygenase-1 (HO-1) is an inducible form of heme oxygenase, a cytoprotective enzyme that plays a central role in the defense against oxidative and inflammatory insults in the lung. It catabolizes heme to produce carbon monoxide (CO), free iron, and biliverdin [10, 11]. The amount of HO-1 has been shown to be elevated in

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the lung tissues of both human silicosis and silica exposed rats [7, 12, 13]. In addition, serum HO-1 was increased in patients with mild silicosis [7], which could be considered as a silicosis diagnosis biomarker. However, the relationship between occupational exposure of silica and serum HO-1 has not been studied. Therefore, the purpose of this study was to determine a relationship between serum HO-1 and silica exposure and radiologic finding among stone crusher workers from Thailand.

## MATERIALS AND METHODS

Ethics approval was obtained from the Ethical Committee for Research in Human Subjects, Department of Disease Control, Ministry of Public Health, Thailand. The study was conducted as a cross-sectional study, in 5 limestone crusher factories in Northern Thailand. Male workers aged >18 years old were recruited for the study. A dust mask and other personal protective equipment (PPE) were provided to the workers according to Thai regulation and the workers reported that they used PPE regularly. A mobile unit consisted of occupational nurse and industrial hygienist was introduced to each factory to conduct the pulmonary function test, blood sample collection, carrying out interviews and air sampling.

### *Pulmonary function testing and chest radiograph*

Based on the recommendation for standardization of spirometry by the American Thoracic Society (ATS), spirometry was performed while standing, without a nose clip, using a spirometer (Datospir-120, Sibelmed, Spain). Daily calibration was performed prior to testing. Each person performed at least 3 spirometry tests in the standing position. The best values of forced expiratory volume in one second (FEV<sub>1</sub>) were used for analyses. The predicted percentages of FEV<sub>1</sub> (FEV<sub>1</sub> % predicted) and FVC (FVC % predicted) were calculated using the reference value of adult Thais [14].

Each subject had a chest X-ray taken in a local general hospital, the films were interpreted by a trained chest radiologist who is experienced in the reading of pneumoconiosis X-ray according to the 2000 International Labor Organization (ILO) system [15]. The ILO standard radiographs of Pneumoconioses were used as reference to classify the profusion of small opacities of subject's radiograph. There are 4 major categories (0,1,2,3) and each category is subcategorized into 12 (0/-, 0/0, 0/1, 1/0, 1/1, 1/2, 2/1, 2/2, 2/3, 3/2, 3/3 and 3/+). Category 0 refers to the absence of any

opacities and category 3 refers to the most profuse. Our results fell into 0/0 and 0/1 subcategories, 0/0 means no visible opacity and 0/1 has some opacity but not sufficient to be called 1/0. According to Chen et al. 2001 guideline [16], 0/1 category was defined as a silicosis-suspected subject.

### *Serum heme oxygenase-1 (HO-1) determination*

Venipuncture blood was used to prepare serum. After collection of the whole blood in plastic tube, allowed the blood to clot by leaving at room temperature for 30 minutes. The clot was removed by centrifuging at 1,000 x g for 10 minutes in a refrigerated centrifuge. Serum was immediately transferred to Cryo tube and kept in freezer (-20 °C) for further analysis.

Sandwich Enzyme Immunoassay Kit (Cusabio Biotech, China) was used to determine HO-1 with Ultraviolet Visible Spectrometry. Detection limit of this assay was 15.6 - 1,000 ng/ml. The microtiter plate provided in a kit was pre-coated with an antibody specific to HO-1. One hundred microliters of serum sample was added to the plate with biotin-conjugated HO-1 polyclonal antibody. After washed out unbound antibody and HO-1, Avidin conjugated to horseradish peroxidase (HRP) was added to each micro plate well and incubated for 1 hour at 37 °C. Then a TMB (3,3',5,5' tetramethylbenzidine) substrate solution was added to each well. The enzyme (HRP) and substrate were allowed to react for 30 minutes. Only those wells that contain HO-1 biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a stop solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm.

### *Crystalline silica determination*

We selected 3 spots in working area as a sampling site in each factory. A full shift of ambient dust sampling was performed using constant-flow personal sampling pumps with PVC filters. Total crystalline silica was determined by ultraviolet visible spectrometry according to NIOSH Method 7601 [17] at the Department of Medical Sciences, Ministry of Public Health.

### *Data Analysis*

Data were analyzed with the SPSS statistical package. We analyzed data for the following statistical parameters: means for ambient silica (low exposure ≤12 mg/m<sup>3</sup>, high exposure >12 mg/m<sup>3</sup>), and cigarettes smoked per day (low ≤8 cigarettes per day and high >8 cigarettes per day.), quartiles for age (Q1=32.0, Q2=37.0, Q3=49.5 year)

**Table 1** Mean comparisons of age, employment duration, BMI, HO-1 and pulmonary function parameters between radiographic findings

Variables	Radiographic findings				Total (85)		t-test
	0/0 (81)		0/1 (4)				p-value
	Mean	S.E.	Mean	S.E.	Mean	S.E.	-
Age (year)	39.84	1.16	47.50	3.57	40.20	1.13	0.153
Employment duration (years)	6.72	0.59	9.97	2.39	6.88	0.58	0.234
BMI (kg/m <sup>2</sup> )	25.02	1.04	23.90	1.21	24.96	0.99	0.813
Silica level (mg/m <sup>3</sup> )	12.40	0.61	15.29	0.21	12.53	0.58	<0.001
HO-1 (ng/ml)	383.97	23.68	656.49	76.67	396.79	23.63	0.014
FVC (% predicted)	91.24	1.28	97.76	3.19	91.54	1.24	0.266
FEV <sub>1</sub> (% predicted)	100.58	1.61	109.77	5.79	101.01	1.57	0.217
Cigarettes smokes per day	n=42		n=2		n=44		
-	7.93	0.79	13.00	7.00	8.16	0.80	0.190

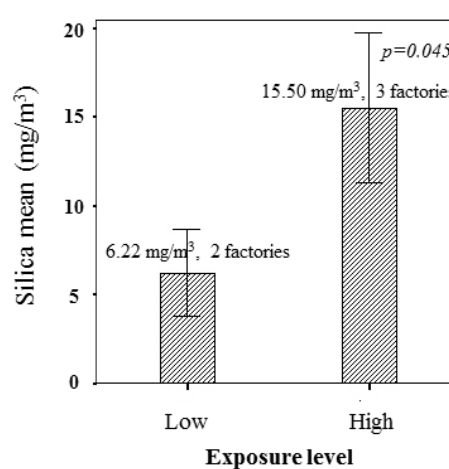
The non-silicosis subject (0/0) and silicosis-suspected subject (0/1) were defined base on the guideline of Chen et al. [16]

and employment duration (Q<sub>1</sub>=3.0, Q<sub>2</sub>=5.5, Q<sub>3</sub>=10.0 year), percent of predicted value for Thais(%) for FVC, FEV<sub>1</sub> (low <80%, normal ≥80%) [14]. Mann-Whitney statistical test was used to compare ambient mean silica level between exposure groups. The mean comparisons between radiographic findings, exposure group, smoking status, cigarettes smokes per day, FVC % predicted and FEV<sub>1</sub> % predicted groups were done using student t-test. ANOVA test was used to determine HO-1 means distribution according to age and employment duration groups. Estimated marginal mean of HO-1 was calculated by generalized linear model after adjustment by mean age and smoking status. The level of statistical significant was set at  $p=0.05$ .

## RESULTS

There were 5 stone crusher factories with 85 workers enrolled to our study. Silica level mean was 12.53 mg/m<sup>3</sup> and it ranged from 0.94 -27.03 mg/m<sup>3</sup>. There were 2 factories in low exposure group (n=21) and 3 factories in high exposure group (n=64). The mean silica between low exposure and high exposure groups was significantly different (6.22 vs 15.50 mg/m<sup>3</sup>,  $p=0.045$ ) (Figure 1).

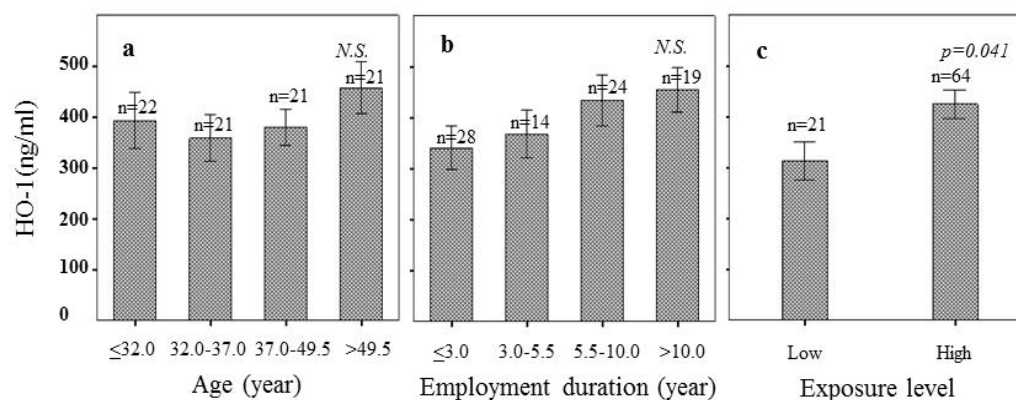
The subjects age and employment duration means were 40.20 and 6.88 year, respectively. Their FVC % predicted, and FEV<sub>1</sub> % predicted means were within normal range (80-120%) for Thais [14]. They were free from silicosis by radiographic finding but 4 subjects had 0/1 profusion score, called silicosis-suspected subject [16]. Means of silica and HO-1 in silicosis-suspected group was significantly higher than non-silicosis group (15.40 vs 12.40,  $p<0.001$  and 656.49 vs 383.97,  $p=0.014$ , respectively (Table 1). Pulmonary function test for both FVC % predicted

**Figure 1** Mean comparison of silica according to the silica exposure group.

The Mann Whitney  $p$ -value was shown.

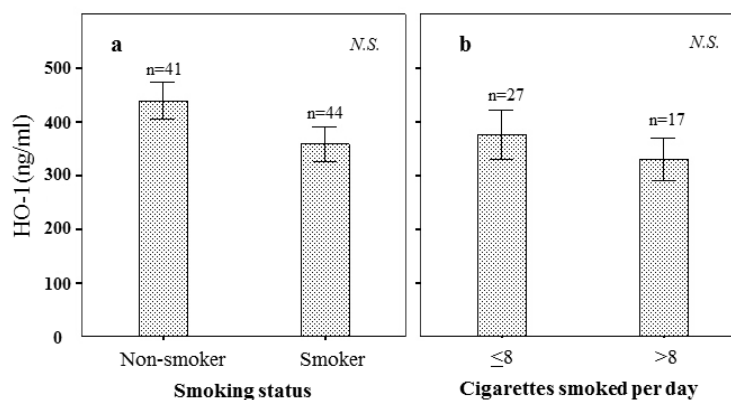
and FEV<sub>1</sub> % predicted were not significantly different between silicosis-suspected and non-silicosis groups ( $p=0.266$  and  $p=0.217$ , respectively (Table 1). All of silicosis-suspected subjects were in silica high exposure group.

Even the serum HO-1 mean of age group >49.50 was the highest but it was not significantly different from other age groups and the relationship between serum HO-1 mean and age group was not observed, analyzed by ANOVA (Figure 2a). Serum HO-1 mean in employment duration >10.0 year group was the highest and serum HO-1 trended to increase as employment duration increased but this relationship was not significant (Figure 2b). On the other hand, mean of serum HO-1 in silica high exposure group was significantly higher than low exposure group (t-test  $p=0.041$ ) (Figure 2c). The mean of serum HO-1 according to FVC % predicted and FEV<sub>1</sub> % predicted groups were not significantly different (Table 2).



**Figure 2** Mean comparison of HO-1 according to age, employment duration and silica exposure level.

ANOVA was used to determine mean distribution according to age and employment duration group whereas t-test was used to compare mean between exposure level groups. N.S. Not significant.



**Figure 3** Mean comparison of HO-1 level according to smoking status and cigarettes smoked per day groups. The mean comparison was done by student t-test. N.S. Not significant

**Table 2** Mean comparison of HO-1 level according to FVC % predicted and FEV<sub>1</sub> % predicted

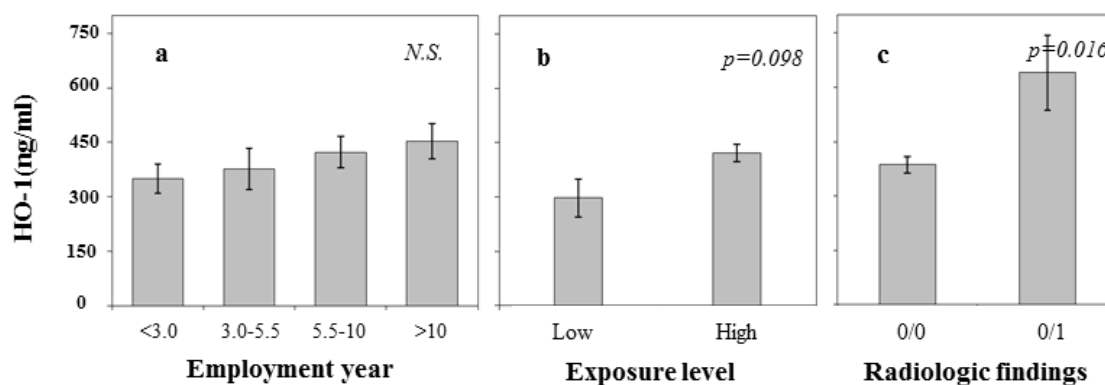
Pulmonary function parameter	N	Mean	S.E.	t-test p-value
<b>FVC % predicted</b>				
<80	13	312.32	58.67	0.130
>80	72	412.05	25.56	
<b>FEV<sub>1</sub> % predicted</b>				
<80	8	267.05	63.81	0.077
>80	77	410.27	24.84	

The mean comparison was done by student t-test.

According to smoking status, there were 41 non-smokers and 44 smokers and their serum HO-1 means were not significantly different (Figure 3a). In addition, when we classified smokers according to cigarettes smoked per day, the mean difference of serum HO-1 level between cigarettes smoked per day groups ( $\leq 8$  and  $> 8$  cigarettes per day) was not observed (Figure 3b).

Because the escalation of serum HO-1 in human was related to age and smoking status, we then

calculated the estimated marginal mean by generalized linear model after adjustment by mean age (40.20 years) and smoking status (Non-smoker=0, Smoker=1). The positive relation between employment duration and serum HO-1 was still observed (Figure 4a). The border line significant relationship between silica level and serum HO-1 mean was shown with  $p=0.098$  (Figure 4b). On the other hand, the estimated marginal mean of serum HO-1 in silicosis-suspected



**Figure 4** Distribution of HO-1 estimated marginal mean according to after adjustment by mean age and smoking status. The estimated marginal mean was calculated using generalized linear model after adjustment by mean age (40.20 years) and smoking status (non-smoker=0, smoker=1). N.S. Not significant. 0/0=non-silicosis subject, 0/1=silicosis-suspected subject

group was significantly higher than non-silicosis group ( $p=0.016$ ) (Figure 4c).

## DISCUSSION

According to a guideline of ILO, the diagnosis of silicosis is done by chest radiograph with a previous silica exposure history. They categorized profusion of small opacities in 12 categories from 0/- to 3/+ and the category 1/0 or greater is recognized as silicosis [16, 18]. Once a patient is recognized to have silicosis by chest radiograph, an effective treatment is not available [2]. In addition, a pulmonary function is not specific to silicosis and un-changed pulmonary function in early stage of silicosis is common [1]. As such, there is a need to identify a sensitive method to identify high risk group from contracting silicosis.

Biomarkers have been developed in order to use as an indicator of disease progression and to identify a high risk population [19]. Because biomarker determination is quick and easy to get a result, it is applied in screening of many diseases such as Febrile neutropenia, cancer, cardiovascular disease, and hyperthyroidism [8]. In this study, the advantage of having a biomarker in silicosis screening was determined.

We proposed serum HO-1 as a silicosis biomarker. The lung is a major source of circulating HO-1. It is one of lung inflammation and oxidative stress biomarkers [7, 20]. A relationship between serum HO-1 and silica exposure was determined in workers from limestone crusher factories in Northern Thailand. The main process of crusher factory was the breaking down of a limestone into small pieces mechanically which caused distribution of silica particles to the air. The workers were at risk of silica exposure and silicosis. The ambient silica in

selected factories ranged 0.94-27.03 mg/m<sup>3</sup> and the factories were then grouped into high exposure and low exposure group. The silica mean was significantly different between these groups ( $p=0.045$ ) (Figure 1).

The level of HO-1 in high exposure group was significantly higher than low exposure group (Figure 2c). Because smoking and age were found to be a confounding factor of lung inflammation [7], we then verified a relationship between silica level and increase HO-1 using estimated marginal mean. After the relationship was adjusted by mean age and smoking status, the HO-1 level in high exposure group was higher than low exposure group (Figure 4b). This result implied that increased HO-1 among these workers was mainly related to silica exposure independent from smoking status and age. In addition, the mean distributions of HO-1 according to age group (Figure 2a) and smoking status (Figure 3a) were not significantly different.

There were 4 subjects who showed profusion score with 0/1 which is recognized as silicosis-suspected subject [16]. Their serum HO-1 was significantly higher than normal group (656.49 vs 383.97 ng/ml (Table 1). Even after adjustment by mean age and smoking status, the serum HO-1 in silicosis-suspected group was significantly higher than non-silicosis group (Figure 4c). This result supported the observation of Sato et al. [7, 12] in which the increase HO-1 in simple silicosis patients was shown. These patients showed profusion score  $\geq 1/0$  with mildly impaired respiratory function which implied low severity or early stage of silicosis. The increase serum HO-1 in simple silicosis and silicosis-suspected subjects denoted that serum HO-1 was increased in the beginning of silicosis.

Our subjects employment duration mean was 6.88 year which was lower than the duration to develop chronic silicosis (> 10 years) [18]. However, the relationship between increased serum HO-1 and increased employment duration was observed (Figure 2b, Figure 4a). This result indicated that the longer exposure period the higher serum HO-1 level. The respirable silica was gradually accumulated in the lungs which increased oxidative stress and lung inflammation [1,18]. The longer employment duration implied the longer exposure time which increased accumulated silica in the lung. Then, oxidative stress was increased which caused increased serum HO-1 [13].

The specificity of HO-1 in silicosis indication was also evidenced in previous studies [7, 12, 13]. The level of HO-1 in silicosis patients were significantly elevated compared to the age-matched control subjects or chronic obstructive pulmonary disease patient (COPD) [7]. The COPD patients showed impaired pulmonary function and increased oxidative in the same level of silicosis patients. But their serum HO-1 did not increase and were significantly lower than silicosis patients. This information supported the specificity of serum HO-1 in silicosis indication.

In this study, we showed the evidence to support the specificity of serum HO-1 to silica exposure and early stage of silicosis. This biomarker together with silica exposure history should be very useful for occupational health surveillance program in a high risk population. We suggest that future surveys should be carried out in patient with various stages of silicosis, and patient with other respiratory disease such as COPD, asthma and emphysema to clarify the specificity of HO-1 to silicosis.

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