Patterns of Heme Oxygenase-1 activities, concentrations and related metabolites in patients with Rheumatoid arthritis

Priya Saini[1], Rashi Garg [2], Sujata Wangkheimayum[3], Varun Dhir [4]

[1]Department of Paramedical, SVFTM, Swami Vivekanand Institute of Engineering and Technology, Banur-140401, Disst – Patiala, Punjab
[2]Department of Immunopathology, PGIMER, Chandigarh, India, 160012
[3]Department of Biochemistry, PGIMER, Chandigarh, India, 160012
[4]Department of Internal Medicine, PGIMER, Chandigarh, India, 160012

***Corresponding Author**: - Priya Saini, priyabiotech222@gmail.com*

Abstract

Methotrexate (Mtx), a drug that acts as a folate antagonist, is commonly used to treat rheumatoid arthritis (RA), which affects nearly 1% of the our world population. Heme oxygenase-1 (HO-1), involved in heme catabolism produces biliverdin (and hence, bilirubin), carbon monoxide (CO) and Fe^{2+}. The primary focus of our study was to investigate the role of Heme oxygenase-1 (HO-1) in Rheumatoid Arthritis (RA).

Materials and Methods

The study included 40 healthy controls, 20 naive RA patients, and 20 methotrexate-treated RA patients. Heme oxygenase-1 (HO-1) activity and concentration were measured using manual chemical methods and ELISA. Additionally, lipid profile and other biochemical parameters were also analyzed using an autoanalyzer.

Conclusion

The concentration of the HO-1 enzyme was lower in RA patients compared to healthy controls, whereas its activity exhibited the opposite trend. HO-1 may be induced by ROS generation; however, further studies are needed to confirm this.

Key words

Rheumatoid Arthritis (RA), Heme oxygenase-1 (HO-1), carbon monoxide (CO), Carboxy haemoglobin (COHB), C-reactive protein (CRP)

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation that primarily affects the joints. It typically results in warm, swollen, and painful joints, most commonly involving the wrists and hands, often symmetrically on both sides of the body. In addition to joint involvement, RA can also affect other organs, leading to complications such as low red blood cell count, inflammation around the lungs, and inflammation of the heart. RA has a global prevalence and occurs more frequently in females. Both genetic and environmental factors are implicated in its development. If left untreated, the persistent inflammation can lead to serious complications, particularly cardiovascular disease, which is reported as a leading cause of death in RA patients [1]. Elevated levels of heme oxygenase-1 (HO-1) have been observed in the synovial fluid of RA patients, and these levels show a correlation with C-reactive protein (CRP), an acute-phase protein that is elevated during inflammatory conditions.

Heme oxygenase (HO) is a microsomal enzyme with three distinct isoforms: HO-1, HO-2, and HO-3. Among these, HO-1 is primarily expressed in the liver. It is an inducible isoform, also known as heat shock protein-32 (Hsp-32), and is typically expressed at low levels under normal conditions. HO-1 catalyzes the oxidative breakdown of heme into biliverdin, free ferrous iron, and carbon monoxide (CO). This reaction takes place in the endoplasmic reticulum, in collaboration with NADPH cytochrome P450 reductase. The biliverdin produced is further reduced to bilirubin by the enzyme biliverdin reductase, which contributes to the formation of bile pigments. Although HO-1 does not directly serve as an antioxidant, its upregulation and the production of its metabolic intermediates have been associated with cytoprotective and anti-inflammatory effects, primarily due to its role in removing free heme. However, limited data are available on HO-1 expression and activity in Rheumatoid Arthritis (RA) patients. Therefore, the present study aims to investigate potential alterations in HO-1 levels and activity in RA patients, along with correlation studies involving related metabolites.

Materials and Methods:

Study Population: A total of 40 control subjects and 40 patients with Rheumatoid Arthritis (RA)—including 20 naive patients and 20 patients undergoing methotrexate treatment for at least 4–6 months—of both genders were recruited for the study after obtaining informed consent. Approximately 4–5 mL of blood was collected from each participant in the morning after an overnight fast. Each RA patient was age- and gender-matched with a healthy control, allowing an age difference of ±3 years within the same gender. A detailed clinical history of RA patients was recorded, including

disease duration, number of joints involved, medication history, family history, and other relevant information.

Anthropometric measurements such as height, weight, waist, hip, and mid-arm circumference were recorded to the nearest centimeter or kilogram. Waist circumference was measured at the midpoint between the lower border of the ribcage and the iliac crest, while hip circumference was measured at the widest point between the hip and buttock. Body Mass Index (BMI) was calculated using height and weight. Subjects were categorized as normal weight (BMI ≥ 18.5 and < 25) and overweight (BMI ≥ 25 and < 30). Both control and RA groups were further classified within each BMI category across two age groups [8].

Biochemical Analyses : In this study, we investigated the patterns of heme oxygenase-1 (HO-1) activity, concentration, and related metabolites across three groups: healthy controls, naive RA patients, and methotrexate-treated RA patients. HO-1 mass were analyzed by gel electrophoresis and quantitated. COHb% (for the activity of HO-1) were measured manually by chemical methods. HO-1 concentration was estimated using a commercial ELISA kit (Human Heme Oxygenase -1 ELISA Kit, Bioassay Technology Laboratory). Other biochemical parameters, lipid profiles, and metabolic markers, were analyzed using automated analyzers.

Statistical Analysis : All data were analyzed using SPSS for Windows. Statistical tests included Student's t-test, Mann-Whitney U test, one-way ANOVA, Kruskal-Wallis test, post hoc tests, correlation, and regression analyses, as appropriate.

Results

Table 1. Demographic Profile of Study Group

Values are expressed as $mean \pm SD$

Parameters	Control (20)	Naïve (20)	Mtx (20)	Significance, P value
Age	23.30 ± 2.02	42.75 ± 10.40	46.55 ± 10.92	<.OO1***
BMI (kg/m^2)	22.47 ± 3.43	23.90 ± 3.76	25.53 ± 4.29	<.05*

Figures in parentheses indicate n, number

P<0.05* is considered as statistically significant and p<0.001*** is considered as highly significant
ns= not significant
n= no. of subjects

Table 2. Comparison between control and diseased

Table 2.1. Non parametric data: Mann-Whitney Test

Parameters	Control (n=20)	RA Patients (n=20)	Significance, P value
%COHB	$.62 \pm .065$	$.85 \pm .33$	<.001***
HO-1 conc.(ng/Ml)	1.84 ± 2.36	$.33 \pm .16$	<.001***

Values are expressed as $mean \pm SD$
Figures in parentheses indicate n, number
P<0.05* is considered as statistically significant and p<0.001*** is considered as highly significant
ns= not significant
n= no. of subjects

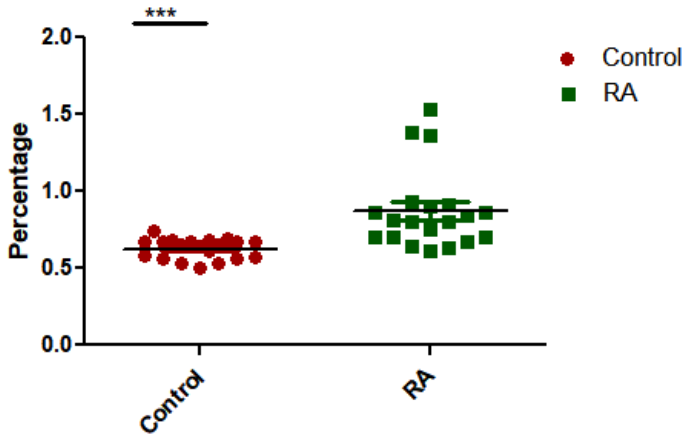

Figure 1: HO – 1 activity shown higher in RA patients as compared to healthy control

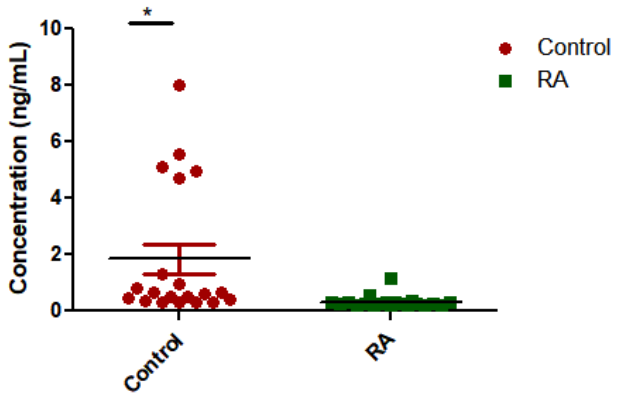

Figure 2: HO - 1 concentration found to be lower in RA patients as compared to control

Table 3. Comparison between control-naïve-Mtx

Table 3.1. Non parametric data: Kruskal-Wallis Test

Comparison of the biochemistry parameters between the 3 study groups

Parameters	Control(20)	Naïve(20)	Mtx (20)	Significance, P value
%COHB	$.62 \pm .06$	$.83 \pm .39$	$.86 \pm .25$	<.001***
HO-1 conc.(ng/Ml)	1.84 ± 2.36	$.32 \pm .20$	$.33 \pm .06$	<.001***

Values are expressed as $mean \pm SD$
Figures in parentheses indicate n, number
P<0.05* is considered as statistically significant and p<0.001*** is considered as highly significant
ns= not significant
n= no. of subjects

Table 4. Comparison between control-naïve, control-mtx, naive-mtx

Table 4.1.1. Non-Parametric data: Mann-Whitney Test

Comparison of biochemistry parameters between control and naïve RA patients

Parameters	Control (20)	Naïve (20)	Significance, P value
%COHB	$.62 \pm .06$	$.83 \pm .39$	<.05*
HO-1 conc.(ng/Ml)	1.84 ± 2.36	$.32 \pm .20$	<.001***

Values are expressed as $mean \pm SD$
Figures in parentheses indicate n, number
P<0.05* is considered as statistically significant and p<0.001*** is considered as highly significant
ns= not significant

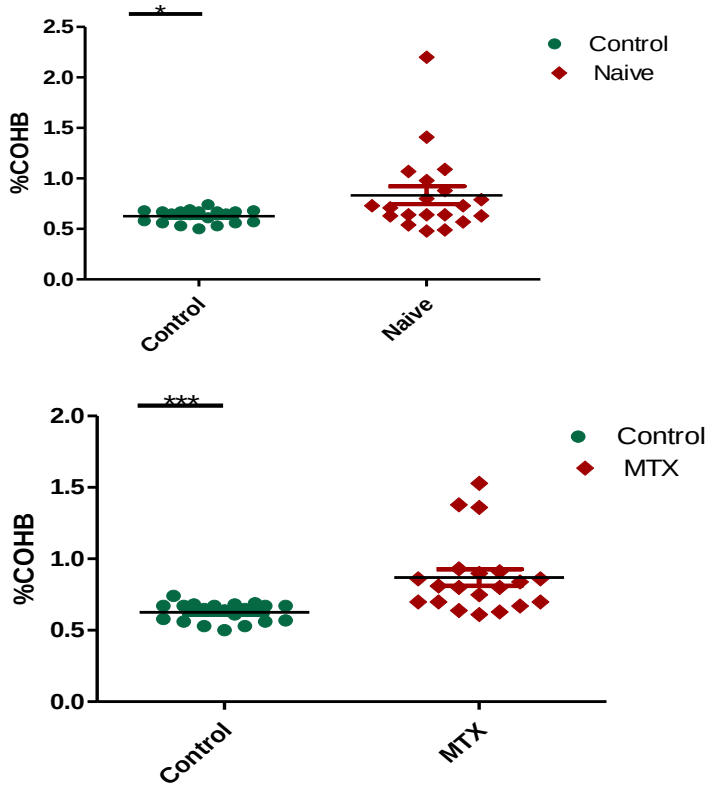

Figure 3: HO – 1 activity shown higher in RA patients (naïve and mtx treated) as compared to healthy control

Table 4.1.2. Non-Parametric data: Mann-Whitney Test

Comparison of the biochemistry parameters between control and treated RA patients

Parameters	Control (20)	Mtx (20)	Significance, P value
%COHB	$.62 \pm .06$	$.86 \pm .25$	<.001***
HO-1 conc.(ng/Ml)	1.84 ± 2.36	$.33 \pm .06$	<.001***

Values are expressed as $mean \pm SD$
Figures in parentheses indicate n, number
P<0.05* is considered as statistically significant and p<0.001*** is considered as highly significant
ns= not significant
n= no. of subjects

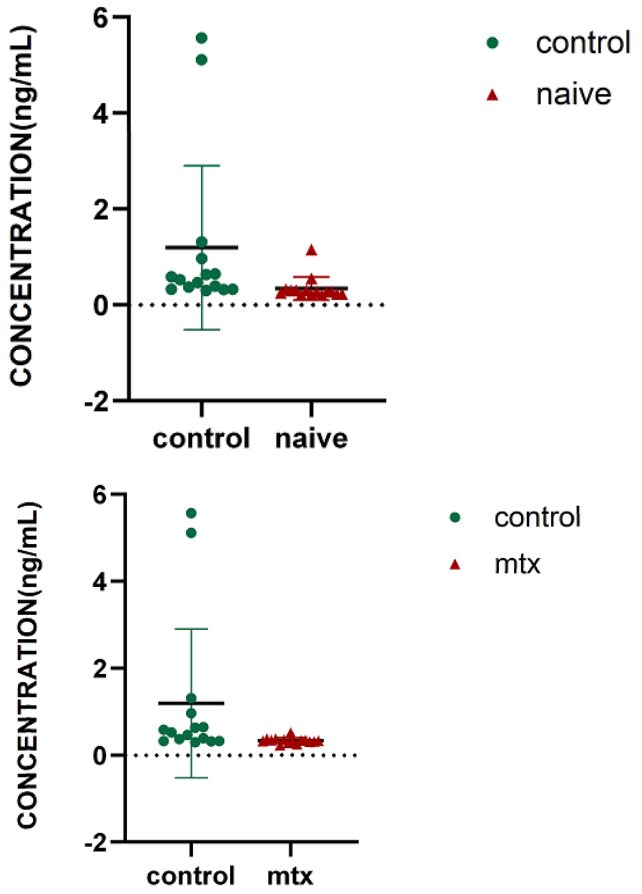

Figure 4: Decrease in HO- 1 concentration in naïve and mtx treated RA patients

Table 4.1.3. Non-Parametric data: Mann-Whitney Test

Comparison of the biochemistry parameters between naïve and treated RA patients

Parameters	**Naïve (20)**	**Mtx (20)**	**Significance, P value**
%COHB	$.83 \pm .39$	$.86 \pm .25$	.198
HO-1 conc.(ng/Ml)	$.32 \pm .20$	$.33 \pm .06$	<.05*

Values are expressed as $mean \pm SD$
Figures in parentheses indicate n, number
P<0.05* is considered as statistically significant and p<0.001*** is considered as highly significant
ns= not significant
n= no. of subjects

Table 5. Correlation between HO-1 and related biochemical parameters of control

Parameters	Correlation Coefficient, r	Significance, P value
HO-1 and ALP	.312	.181
HO-1 and AST	.110	.645
HO-1 and ALT	.331	.154
HO-1 and Total Bilirubin	.493	<.05*
HO-1 and Conjugated Bilirubin	.076	.749

Correlation is significant at the 0.01 level
Correlation is significant at the 0.05 level

Table 6. Correlation between HO-1 and related biochemical parameters of Diseased

Parameters	Correlation Coefficient, r	Significance, P value
HO-1 and ALP	.173	.328
HO-1 and AST	.022	.902
HO-1 and ALT	.180	.308
HO-1 and Total Bilirubin	-.111	.533
HO-1 and Conjugated Bilirubin	-.051	.773

Correlation is significant at the 0.01 level
Correlation is significant at the 0.05 level

Table 7. Correlation between BMI and related biochemical parameters of control

Parameters	Correlation Coefficient, r	Significance, P value
BMI and % COHB	.122	.609
BMI and HO-1	.339	.144

Correlation is significant at the 0.01 level
Correlation is significant at the 0.05 level

Table 8. Correlation between BMI and related biochemical parameters of Diseased

Parameters	Correlation Coefficient, r	Significance, P value
BMI and % COHB	.003	.987
BMI and HO-1	.113	.526

Correlation is significant at the 0.01 level
Correlation is significant at the 0.05 level

Table 9. Correlation between COHb% and related biochemical parameters of control

Parameters	Correlation Coefficient, r	Significance, P value
% COHB and ALP	-.147	.537
% COHB and AST	-.094	.694
% COHB and ALT	-.053	.824
% COHB and Total Bilirubin	-.284	.224
% COHB and Conjugated Bilirubin	-.022	.927

Correlation is significant at the 0.01 level
Correlation is significant at the 0.05 level

Table 10. Correlation between COHb% and related biochemical parameters of Diseased

Parameters	Correlation Coefficient, r	Significance, P value
% COHB and ALP	.098	.548
% COHB and AST	.128	.430
% COHB and ALT	.204	.206
% COHB and Total Bilirubin	.219	.175
% COHB and Conjugated Bilirubin	.446	<.001**

Correlation is significant at the 0.01 level
Correlation is significant at the 0.05 level

Densitometric Qunatification of HO-1 proteins

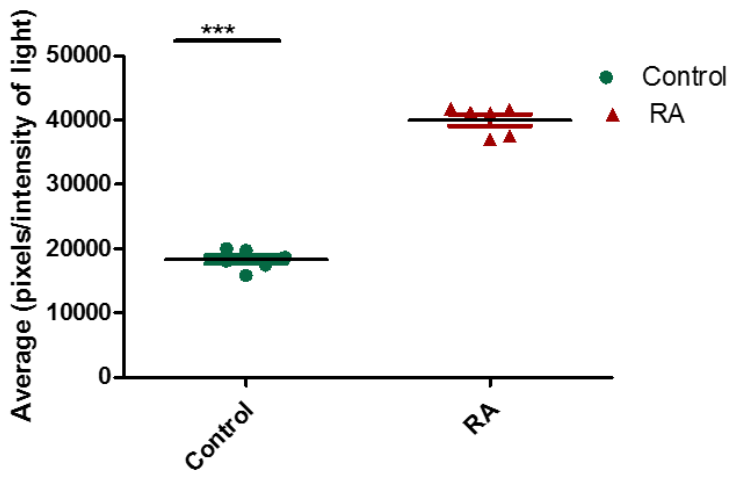

Figure 5: HO-1 concentration in RA and control

Discussion

The demographic characteristics of the study groups are presented in Table 1. One-way ANOVA revealed significant differences in age and BMI among the three groups. Regarding age, RA patients (both naïve and treated) were in the range of 40–50 years, whereas the control subjects had a mean age of slightly less than 25 years.

As shown in Table 2.1, the biochemical parameters of control subjects and RA patients (naïve and treated) were compared using the Student's t-test for parametric data and the Mann–Whitney test for non-parametric data. RA patients showed a significant decrease in HO-1 concentration compared to the control subjects.

However, COHB% are increased in RA patients compared to control subjects. Though COHB% could increase in RA patients as a protected mechanism, it also worth noting that COHB% was performed manually and samples were not processed in duplicates. Graphic picture presented in figure 1 and 2

Table 3.1 shows that, the biochemical parameters differ significantly among the three groups: control subjects, naïve RA patients, and treated RA patients.

Table.4.1.1. shows the comparison of the parameters between control and naïve RA patients. There is a significant decrease in HO- 1 concentration in RA patients. However, HO-1 activity (as COHB%) showed as increase in naïve RA patients. Though decrease in HO-1 concentration with increase in COHB% is conflicting, it is not surprising as COHB% test is sensitive to quite a few factors.

Similar findings were observed between control subjects and methotrexate-treated RA patients, as shown in Table 4.1.2. Additionally, the results are illustrated in graphical form in Figures 3 and 4 . As seen from table 5, there is a significant correlation between HO-1 and total bilirubin in control subjects. However, no correlation was observed between HO-1 and related parameters in RA patients (table 6).

Tables 7 and 8 show that there were no significant correlations between BMI and the parameters—COHb%, and HO-1 concentration—in both control subjects and RA patients.

In table 9 we did not find any significant correlation between COHb % and the parameters AST, ALT, ALP, total bilirubin and direct bilirubin in control groups. In RA patients there was a significant correlation between COHb% and direct bilirubin($p<0.001$) (table 10).

Figure 5 is the densitometric qunatification of HO-1 proteins. It is found HO-1 concentration is higher in RA patients as compared to healthy control.

Though COHB% (HO-1 activity) was significantly higher in MTX treated group compared to naïve RA group, HO-1 concentraion was not so. Some studies have shown increased HO-1 expression in synovial tissue o RA patients, possibly stimulated by inflammatory cytokines (5).

These finding assume significance as COHB% was found to be much higher in both groups of RA patients as compared to control. However, in our own laboratory, healthy younger subjects had a higher COHB % compared to older healthy subjects. The older subjects were in the same age group of RA patients. If more numbers of patients are investigated, it would provided a well validated data.

Conclusion

HO-1 protects against inflammatory and oxidative stress in several conditions, such as ischemia–reperfusion injury, nephropathy, and vascular disease [2]. Recently, several studies reported HO-1 protein expression in synovial tissues in RA patients [3].HO-1 has the ability to downregulate inflammation in RA, indicated that HO-1 might be useful as a marker of joint inflammation in RA patients

HO-1 are important enzymes involved in the metabolism of heme. In the present study the following observation are noted :-

Percent COHb levels are significantly increased in diseased condition as compared to healthy control

COHb% (HO-1 activity) is also observed to be higher in RA patients as compared to healthy controls. Though this finding is not consistent with lower HO-1 concentration, we need to carry out more tests in replicates. However, the increase in COHb% could be a compensatory protective response.

Bibliography

1. Aletaha D, Smolen JS. Diagnosis and management of rheumatoid arthritis. JAMA 2018; 320: 1360-1372
2. Wang XH, Wang K, Zhang F, Li XC, Li J, De W, et al. Heme oxygenase-1 alleviates ischemia/reperfusion injury in aged liver. World J Gastroenterol. 2005; 11: 690–4.
3. Kobayashi H, Takeno M, Saito T, Takeda Y, Kirino Y, Noyori K, et al. Regulatory role of heme oxygenase 1 in inflammation of rheumatoid arthritis. Arthritis Rheum. 2006; 54(4): 1132–42.
4. Keyse SM, Applegate L, Tromvouskis Y, Tyrrell R. Oxidant stress leads to transcriptional activation of the human heme oxygenase gene in cultured skin fibroblasts. Molecular and cellular biology .1990; 10 (9):4967-4969.
5. Kitamura A[1], Nishida K, Komiyama T, Doi H, Kadota Y, Yoshida A, Ozaki T. Increased level of heme oxygenase-1 in rheumatoid arthritis synovial fluid.Mod Rheumatol2011;21(2):150-7.
6. Applegate LA, Luscher P, Tyrrell RM. Induction of heme oxygenase : a general response to oxidant stress in cultured mammalian cells. Cancer Research .1991;51(3):974-978.
7. Abrahm NG, Rezzani R, Rodella L, Kruger A, Taller D, Volti GL, Goodman AI, Kappas A. Overexpression of human heme oxygenase -1 attenuates endothelial cell sloughing in experimental diabetes. American Journal of Physiology - Heart and circulatory Physiology .2004; 287(6): H24-H77.
8. National institute of health national heart,lung and blood institute. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in the adult-evidence report. Obes Res. 1998; 6: 51S-210S
9. Visser k, Van der Heijide D. Optimal dosage and route of administration of methotrexate in rheumatoid arthritis: a systemic review of literature. Ann Rheum Dis 2009; 68: 1094-1099.
10. Visentin M, Zhao R, Goldman ID. The antifoalat. HematolOncol Clin North Am 2012; 26: 629-48.
11. Whittle SL, Hughes RA. Folate supplementation and methotreaxte treatment in rheumatoid arthritis: a review Rheumatology (oxford) 2004; 43:267-71.

12. Budzik GP, Colletti LM, Faltynek CR, Effects of methotrexate on nucleotide pools in normal human T cells and the CEM T cell line. Life Sci 2000; 66: 2297-307.
13. Fairbanks LD, Ruckemann K, QiuY, et al. Methotreaxte inhibits the first commited step of purine biosynthesis in mitogen- stimulated human T-lymphocytes: a metabolic basis for efficacy in rheumatoid arthritis .Biochem J 1999; 342: 143-52.
14. Kikuchi G, Yoshida T, Noguchi M. Heme oxygenase and heme degradation. Biochem Biophys Res Commun. 2005; 338: 558–67.
15. McCoubrey WJ, Huang T, Maines M. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur J Biochem. 1997; 247(2): 725–32.
16. Fernandez P, Guillen MI, Gomar F, Alcaraz MJ. Expression of heme oxygenase-1 and regulation by cytokines in human osteoarthritic chondrocytes. Biochem Pharmacol. 2003; 66:2049–52
17. Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, et al. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. Proc Natl Acad Sci U S A 2001; 98: 8798–803.
18. Otterbein LE, Choi AM. Heme oxygenase: colors of defense against cellular stress. Am J Physiol Lung Cell Mol Physiol 2000; 279: 1029–37.
19. Brouard S, Berberat PO, Tobiasch E, Seldon MP, Bach FH, Soares MP. Heme oxygenase-1-derived carbon monoxide requires the activation of transcription factor NF-κB to protect endothelial cells from tumor necrosis factor-α-mediated apoptosis. J Biol Chem 2002; 277: 17950–61.
20. Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat Med 1996; 2: 87–90.
21. Kirino Y, Takeno M, Iwasaki M, Ueda A, Ohno S, Shirai A, et al. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. Arthritis Res Ther 2005; 7: 616–24.
22. Engel RR, RODKEY FL ,Krill CE Jr. carboxygemoglobin levelas as an index of hemolysis. Pediatrics.1971; 47(4) : 723-30

23. Rodgers PA, VREMAN HJ, Dennery PA, Stevenson DK. Sources of carbon monoxide (CO) in biological systems and applications of CO detection technologies. Seminperinatol 1994; 18(1): 2-10.
24. Norbert W. Tietz, Ermaldinda A. Fiereck. The spectrophotometric measurement of carboxyhemoglobin. Annals of clinical laboratory science, vol. 3, No.1