Protective Effect of *Artemisia Asiatica* Extract Against Renal Ischemia-Reperfusion Injury in Mice

Hyuk Jai Jang,[1] Eui Kyun Jeong,[2] Seong Su Kim,[2] Ji Hwan Lee,[1] Mi Young Oh,[1] Ki Sung Kang,[3] Hak Cheol Kwan,[4] Kyung Il Song,[5] Dae Woon Eom,[6] Duck Jong Han[7]

Abstract

Objectives: **An extract of *Artemisia asiatica* was reported to possess antioxidative and cytoprotective actions in various experiments. Ischemia-reperfusion injury remains a major problem in kidney transplant, and the inflammatory response to ischemia-reperfusion injury exacerbates the resultant renal injury. In the present study, we investigated whether an extract of *Artemisia asiatica* exhibits renoprotective effects against ischemia-reperfusion-induced acute kidney injury in mice.**

Materials and Methods: **Renal ischemia-reperfusion injury was induced in male C57BL/6 mice by bilateral renal pedicle occlusion for 30 minutes followed by reperfusion for 48 hours. An extract of *Artemisia asiatica* (100 mg/kg oral) was administered 4 days before ischemia-reperfusion injury. Sham operation and phosphate-buffered saline were used as controls. Blood and renal tissues were evaluated at 48 hours after ischemia-reperfusion injury.**

Results: **Treatment with an extract of *Artemisia asiatica* significantly decreased blood urea nitrogen, serum creatinine levels, and kidney tubular injury ($P \leq .05$). Western blot showed that an extract of *Artemisia asiatica* significantly increased the level of heme oxygenase-1 and B-cell lymphoma 2 at 48 hours after ischemia-reperfusion injury and attenuated the level of inducible nitric oxide synthase.**

Conclusions: **An extract of *Artemisia asiatica* improves acute renal ischemia-reperfusion injury by reducing inflammation and apoptosis. These findings suggest that an extract of *Artemisia asiatica* is a potential therapeutic agent against acute ischemia-induced renal damage.**

Key words: *Heme oxygenase-1, Inducible nitric oxide synthase, Kidney transplant, Renal failure*

Introduction

Renal ischemia-reperfusion injury (IRI) is a major problem in kidney transplant. The pathogenesis of IRI involves complex interactions between biochemical, cellular, vascular endothelial, and tissue-specific factors. Ischemia causes necrosis and apoptosis. Restoration of blood flow, which is important to prevent ongoing injury, paradoxically potentiates the inflammatory response exhausting the previous ischemic damages.[1] Interruption of blood flow to the kidney and subsequent reperfusion causes an acute inflammatory response.[2] Tubular cell apoptosis also contributes to the pathogenesis of renal IRI.[3] Therapeutic approaches aimed at suppressing the inflammatory response and tubular apoptosis may be effective against renal injury and provide better prognosis after IRI.

The plant genus *Artemisia* includes > 300 species, many having medicinal value. Among these plants, *Artemisia asiatica* Nakai (*Artemisia asiatica*) has been used in traditional Oriental medicine for the treatment of microbial infections and inflammatory diseases. Various animal studies revealed that the extract of *Artemisia asiatica* has antioxidative and anti-inflammatory activities that contribute to its

From the Departments of [1]Surgery and [2]Anesthesia, University of Ulsan Gangneung Asan Hospital, Gangneung, Gangwon; [3]College of Korean Medicine, Gachon University, Seongnam, Gyeonggi; [4]Natural Medicine Center, Korea Institute of Science and Technology, Gangneung, Gangwon; Departments of [5]Nephrology and [6]Pathology, University of Ulsan Gangneung Asan Hospital, Gangneung, Gangwon; and the [7]Department of Surgery, University of Ulsan, Asan Medical Center, Seoul, South Korea

Acknowledgements: *This study was supported by a grant from the Gangneung Asan Hospital Biomedical Research Center Promotion Fund. The authors have no conflicts of interest to disclose.*

Corresponding author: *Hyuk Jai Jang, Department of Surgery, Gangneung Asan Hospital, Gangwon-do, Gangneung, Sachean Myeon, Bangdong gil 38, South Korea*
Phone: *+82 33 610 3221* ***Fax:*** *+82 33 641 8120* ***E-mail:*** *jhj@gnah.co.kr*

Experimental and Clinical Transplantation (2015) Suppl 1: 377-382

Copyright © Başkent University 2015
Printed in Turkey. All Rights Reserved.

DOI: 10.6002/ect.mesot2014.P227

protective effects against gastric damage, liver damage, and experimental pancreatitis.[4-6]

Based on these previous findings, it was hypothesized that the extract of *Artemisia asiatica* may suppress inflammatory activation and apoptosis and may confer renoprotection against renal IRI. The aim of the present study was to investigate whether the extract of *Artemisia asiatica* can abolish the harmful effects of renal injury in an animal model of renal IRI.

Materials and Methods

Experimental animals and renal ischemia-reperfusion injury

Male C57BL/6 mice (age, 8 wk; body weight, 22-25 g) were purchased from Dae Han Bio Link Co., Ltd., Eumseong, South Korea. The mice were housed in polycarbonate cages (Makrolon, Bayer Material Science LLC, Pittsburgh, PA, USA) under standard laboratory conditions (temperature, 22°C ± 2°C; relative humidity, 55%). The mice were given standard mouse chow and tap water ad libitum. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication No. 85-23, revised 1985) and was approved by the Ethics Committee of the Korea Institute of Science and Technology.

Mice were subjected to bilateral renal pedicle clamping for 30 minutes with microvascular clamps. Reperfusion commenced after the artery clamps were removed. Occlusion was verified visually by change in the color of the kidneys to a paler shade and reperfusion was verified by a blush appearance. During the procedure, mice were kept well hydrated with warm saline. The mice were maintained at a constant body temperature (37°C) using a warming pad. After the clamps were removed, reperfusion of the kidneys was observed. A similar sham operation was performed in control mice except that the renal pedicles were not clamped. Blood was sampled from the inferior vena cava and renal tissues were removed at 48 hours after reperfusion. Both kidneys were isolated, quick frozen in liquid nitrogen, and stored at -80°C until further analysis.

Artemisia asiatica administration

Isopropanol extracts of *Artemisia asiatica* (Richwood Trading Co., Ltd., Seoul, South Korea) were dissolved in vehicle (5% hydroxypropyl methylcellulose) at a concentration of 100 mg/kg body weight. A total of 30 mice were randomly divided into 3 groups (10 mice per group). The sham and IRI control groups were given a single dose of vehicle only. The drug-treated groups (*Artemisia*-treated) were given pretreatment with *Artemisia* extracts 4 days before bilateral IRI. The mice were given a single intragastric dose of vehicle with *Artemisia* extracts, except the sham and IRI groups.

Biochemical tests

Blood samples were obtained from the inferior vena cava. Serum blood urea nitrogen (BUN) and creatinine levels were measured using an analyzer (Cobas C 702 analyzer, Roche, Basel, Switzerland).

Histologic and immunohistochemical analysis

For histopathologic examination, kidneys were collected, cut coronally, fixed in 10% formaldehyde, and embedded in paraffin. Sections (thickness, 5 μm) were cut, stained with hematoxylin-eosin, and scored with a semiquantitative scale designed to evaluate changes in the kidney at 48 hours after IRI.[7] The percentage of tubules in the corticomedullary junction that displayed cellular necrosis and a loss of brush border were counted and scored in a blinded fashion and graded from 1 to 4. One whole deep coronal section was examined under the microscope and graded according to extent of tubular necrosis, based on percentage of involvement of the kidney. Higher scores represented more severe damage (0, normal kidney; 1 [minimal necrosis], < 5% involvement; 2 [mild necrosis], 5% to 25% involvement; 3 [moderate necrosis], 25% to 75% involvement; and 4 [maximum score, severe], > 75% involvement). Immunochemical staining for monoclonal antibody against heme oxygenase-1 (anti-HO-1 antibody, Cell Signaling, Danvers, MA, USA) was performed for ethanol-fixed, paraffin-embedded tissue sections.

Western blot analysis

Kidneys were crushed in ice-cold 1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5, with protease inhibitors, 25 mM sodium fluoride, 10 mM Sodium Orthovanadate, 0.5 mol/L ethylenediaminetetraacetic acid (EDTA), and surfactant (1% Triton X-100; GenDEPOT, Barker, TX, USA), and centrifuged at

14 000 rpm for 20 minutes. Protein concentration was determined using a protein assay (Bradford Protein Assay, Bio-Rad, Hercules, CA, USA). Aliquots of 200 μg of protein extracts were separated on 10% to 15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE, Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk in buffer (TBST buffer: 10 mM Tris-base, 100 mM sodium chloride, 0.1% Tween-20, pH 8.0), and probed with HO-1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), B-cell lymphoma 2 (Bcl-2) (Cell Signaling, Danvers, MA, USA), and inducible nitric oxide synthase (iNOS) (1:200 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. Membranes were probed with goat antirabbit (1:1000) or goat antimouse (1:1000) horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc). Protein bands were detected using a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA). Images of blots were acquired for quantification and analyzed.

Statistical analyses

All data are presented as mean ± SEM and were evaluated by 1-way analysis of variance with post hoc Bonferroni correction (SPSS for Windows, Version 16.0, SPSS Inc., Chicago, IL, USA). Statistical significance was defined by $P \leq .05$.

Results

Artemisia asiatica attenuated the renal functional deterioration that had been induced by ischemia-reperfusion injury

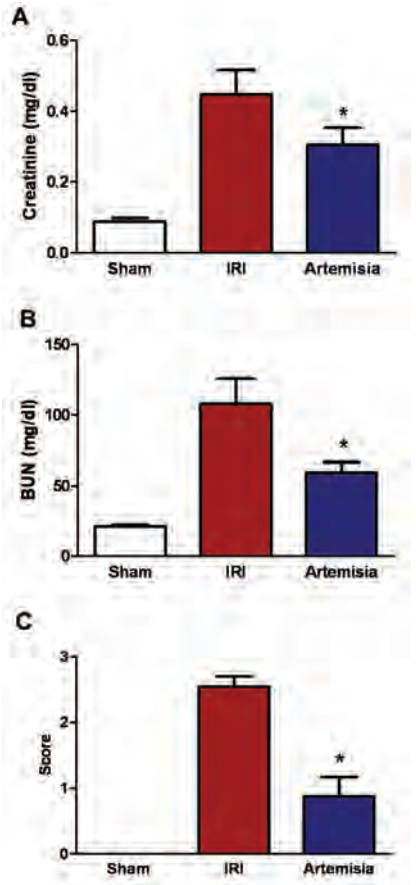
The IRI caused renal dysfunction in the vehicle-treated mice (IRI group), reflected by significant elevation of serum BUN and creatinine levels at 2 days after IRI (Figure 1). Renal dysfunction was attenuated in *Artemisia*-treated mice (*Artemisia* group), with serum BUN and creatinine levels lower than in the control mice at post-IRI day 2 (*Artemisia* vs IRI group, BUN and creatinine; $P \leq .05$) (Figure 1).

Treatment with *Artemisia* also attenuated histologic injury. The IRI group had severe tubular damage, evidenced by widespread tubular necrosis, loss of the brush border, cast formation, and tubular dilation at the corticomedullary junction at 2 days after IRI. However, the tissue injury score was significantly decreased in *Artemisia*-treated mice ($P \leq .05$) (Figure 1). Sham-operated mice incurred no tubular injury.

Artemisia asiatica treatment up-regulated the expression of heme oxygenase-1 proteins in the kidney

To elucidate the mechanism of *Artemisia*-induced cytoprotective effect, we examined protein expression using immunohistochemical staining and Western blot. The enzyme HO-1 has antioxidant and cytoprotective properties against oxidative stress. Expression of HO-1 was observed in the *Artemisia*-treated cells compared with cells from the IRI and sham groups (Figure 2). Treatment with *Artemisia* caused strong HO-1 immunostaining of the kidney compared with the sham and IRI groups (Figure 2).

Figure 1. *Artemisia asiatica* Protects Renal Function in Ischemia-Reperfusion Injury (IRI)



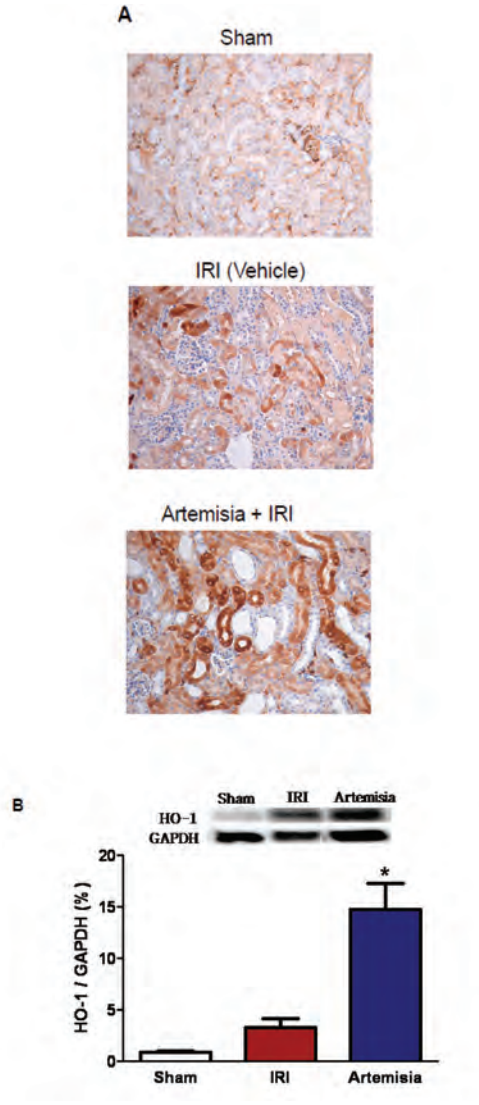
Artemisia asiatica (100 mg/kg) or vehicle was orally administered to mice 4 days before bilateral IRI. Blood samples were collected 2 days after IRI to determine levels of **(A)** serum creatinine and **(B)** blood urea nitrogen. *Artemisia asiatica* treatment markedly attenuated IRI-induced pathologic injury. Mouse kidneys were excised 2 days after IRI and assessed by tubular injury score. **(C)** The kidneys were sectioned and stained with hematoxylin-eosin to evaluate renal tubular damage.
Data were reported as mean ± SEM (*$P \leq .05$, *Artemisia*- vs vehicle-treated IRI groups).

Artemisia treatment significantly up-regulated the expression levels of HO-1 ($P \leq .01$) (Figure 2). This suggested that HO-1 induction by *Artemisia* correlated with its cytoprotective effect against IRI.

Artemisia asiatica treatment up-regulated the expression of B-cell lymphoma 2 protein and down-regulated expression of inducible nitric oxide synthase protein in the kidney

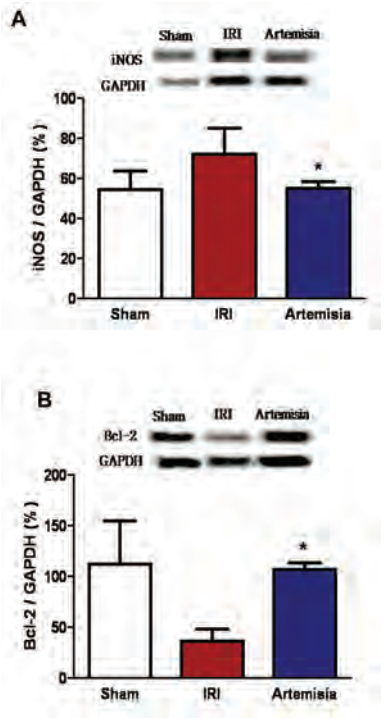
The IRI-induced iNOS protein expression was decreased by *Artemisia asiatica* extract ($P \leq .05$) (Figure 3). Early intracellular events that occur in the apoptotic process comprise mitochondrial changes mediated by protein members of the antiapoptotic Bcl-2 proteins. Therefore, Bcl-2 was evaluated by Western blot analysis to elucidate the mechanism by which *Artemisia asiatica* suppressed apoptosis after IRI. The immunoreactivity for Bcl-2 was markedly increased after treatment with *Artemisia asiatica* ($P \leq .05$) (Figure 3).

Figure 2. *Artemisia asiatica* Treatment Up-Regulated the Expression of Heme Oxygenase-1 (HO-1) in the Kidney



(A, B) Expression of HO-1 was observed in the *Artemisia*-treated cells compared with ischemia-reperfusion injury and sham group. Treatment with *Artemisia* caused stronger HO-1 immunostaining of kidney than in the sham group (hematoxylin-eosin, original magnification ×200). **(B)** *Artemisia asiatica* treatment significantly up-regulated the expression levels of HO-1 ($P \leq .05$). Data were reported as mean ± SEM. (*$P \leq .001$; *Artemisia* vs sham group).

Figure 3. *Artemisia asiatica* Treatment Markedly Attenuates Inducible Nitric Oxide Synthase (iNOS) and Apoptosis Induced by Ischemia-Reperfusion Injury (IRI)



(A) The IRI-induced iNOS protein expression was decreased by *Artemisia asiatica*. **(B)** The expression of Bcl-2 in kidneys was measured at 48 hours after reperfusion by Western blot analysis. Representative Western blots are shown in **(B)**, demonstrating that the expression of B-cell lymphoma 2 (Bcl-2) was up-regulated significantly in *Artemisia*-treated IRI mice. The density of Western blot bands was quantified by software (Fujifilm Multi-Gauge Imaging, Fujifilm, Tokyo, Japan) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
Data were reported as mean ± SEM.
(*$P \leq .05$; *Artemisia*-vs vehicle-treated [IRI] group).

Discussion

In this paper, we showed that therapy with *Artemisia asiatica* extract protected against renal IRI in mice, and the protective effect was associated with suppression of the inflammatory response and tubular apoptosis. It did this by the antiapoptotic property that targets various components of the pathophysiologic pathway involved in IRI.

Artemisia asiatica extracts have been proven to possess anti-inflammatory, antioxidative, and cytoprotective effects and have shown effective protection in various models. In the current experiment, we administered extracts of *Artemisia asiatica*. Plant extracts have been used traditionally as herbal medicines to target inflammatory disease. The antioxidative and cytoprotective actions of *Artemisia asiatica* have been proven in gastric mucosal injury induced by nonsteroidal anti-inflammatory drugs, hepatic fibrosis and inflammation, and fibrosing chronic pancreatic lesions.[4-6] We investigated whether *Artemisia asiatica* can abolish the harmful effects of renal injury in an animal model of renal IRI. The present study showed that treatment of mice with *Artemisia asiatica* resulted in better renal function than in mice not treated with *Artemisia asiatica*. Mice treated with *Artemisia asiatica* had lower plasma levels of BUN and creatinine caused by IRI and lower histopathologic scores. To our knowledge, this is the first study to explore the protective efficacy against renal IRI.

Inflammation after renal IRI is a major contributor to renal cell death. Inflammation is an important mechanism underlying the start and maintenance of renal cell injury because inflammation potentiates necrosis and apoptosis.[8-10] Necrotic cells can potentiate the inflammatory process further via the release of toxic intracellular contents. In the present study, administration of *Artemisia asiatica* reduced IRI-induced expression of inflammatory genes such as iNOS. These data suggest that *Artemisia asiatica* has anti-inflammatory effects in renal IRI.

We investigated the possible mechanisms by our observations. Apoptosis is increasingly recognized as a major form of cell death during IRI and can affect the functional outcome independent of inflammation. There is evidence that renal tubular cell apoptosis plays an essential role in renal IRI and contributes to acute renal failure.[3] Previous animal and human biopsy studies have shown that apoptosis is implicated in cell injury after renal IRI.[9,11] Renal tubular apoptosis is a primary contributor to the pathophysiology of renal IRI.[12] Abolition of early apoptosis improves renal IRI.[9] *Artemisia asiatica* inhibits tubular cell apoptosis after renal ischemia with great efficacy. Our results on the effect of Bcl-2 expression raise the possibility of a direct effect on kidney epithelial cells at the mitochondrial level. Pharmacologic compounds were shown to improve renal injury by diminishing apoptosis.[14-15] Our results provide evidence of the potent cytoprotective effect of *Artemisia asiatica* via the modulation of Bcl-2 gene expression. The Bcl-2 is induced by *Artemisia asiatica*. As a result, mitochondrial activity and cell integrity are maintained.

In the control of oxidative stress and antioxidant defense, HO-1 is an enzyme with antioxidant and cytoprotective properties against oxidative stress. The enzyme HO-1 is a cytoprotective enzyme involved in the response to oxidative stress, and its main function is associated with the degradation of heme to biliverdin, iron, and carbon monoxide.[15] Overexpression of HO-1 in cells resulted in a marked reduction in injury and cytotoxicity induced by oxidative stress.[15-16] The up-regulation of both HO-1 in the *Artemisia* group significantly improved renal IRI, which suggested that HO-1 may be a modulator of *Artemisia asiatica*-associated antioxidant properties.

In summary, *Artemisia asiatica* provided protection for mice against renal IRI, with suppression of inflammatory responses, reduction of tubular cell apoptosis, and cytoprotection. Therefore, *Artemisia asiatica* may be a potential therapeutic agent for renal IRI. The detailed mechanisms of protection should be explored in further studies.

References

1. Aydin Z, van Zonneveld AJ, de Fijter JW, Rabelink TJ. New horizons in prevention and treatment of ischaemic injury to kidney transplants. *Nephrol Dial Transplant*. 2007;22(2):342-346.
2. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? *Kidney Int*. 2004;66(2):480-485.
3. Bonegio R, Lieberthal W. Role of apoptosis in the pathogenesis of acute renal failure. *Curr Opin Nephrol Hypertens*. 2002;11(3):301-308.
4. Oh TY, Ahn GJ, Choi SM, Ahn BO, Kim WB. Increased susceptibility of ethanol-treated gastric mucosa to naproxen and its inhibition by DA-9601, an *Artemisia asiatica* extract. *World J Gastroenterol*. 2005;11(47):7450-7456.
5. Ryu BK, Ahn BO, Oh TY, Kim SH, Kim WB, Lee EB. Studies on protective effect of DA-9601, *Artemisia asiatica* extract, on acetaminophen- and CCl4-induced liver damage in rats. *Arch Pharm Res*. 1998;21(5):508-513.
6. Hahm KB, Kim JH, You BM, et al. Induction of apoptosis with an extract of attenuates the severity of cerulein-induced pancreatitis in rats. *Pancreas*. 1998;17(2):153-157.
7. Rabb H, Mendiola CC, Dietz J, et al. Role of CD11a and CD11b in ischemic acute renal failure in rats. *Am J Physiol*. 1994;267(6 pt 2): F1052-F1058.
8. Lieberthal W, Koh JS, Levine JS. Necrosis and apoptosis in acute renal failure. *Semin Nephrol*. 1998;18(5):505-518.
9. Daemen MA, van 't Veer C, Denecker G, et al. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest*. 1999;104(5):541-549.
10. Friedewald JJ, Rabb H. Inflammatory cells in ischemic acute renal failure. *Kidney Int*. 2004;66(2):486-491.

11. Daemen MA, de Vries B, Buurman WA. Apoptosis and inflammation in renal reperfusion injury. *Transplantation*. 2002;73(11):1693-1700.
12. Devarajan P. Update on mechanisms of ischemic acute kidney injury. *J Am Soc Nephrol.* 2006;17(6):1503-1520.
13. Lee HT, Gallos G, Nasr SH, Emala CW. A1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice. *J Am Soc Nephrol.* 2004;15(1): 102-111.
14. Sharples EJ, Patel N, Brown P, et al. Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. *J Am Soc Nephrol*. 2004;15(8):2115-2124.
15. Soares MP, Usheva A, Brouard S, et al. Modulation of endothelial cell apoptosis by heme oxygenase-1-derived carbon monoxide. *Antioxid Redox Signal*. 2002:4(2):321-329.
16. Taillé C, El-Benna J, Lanone S, et al. Induction of heme oxygenase-1 inhibits NAD(P)H oxidase activity by down-regulating cytochrome b558 expression via the reduction of heme availability. *J Biol Chem*. 2004;279(27):28681-28688.