Egyptian Journal of Basic and Clinical Pharmacology
December 2013, Vol. 3 No.2: 1-9
http://www.ejbcp.eg.net/

Original Article

Hemeoxygenase and Nitric Oxide: a Cross Talk in Cold Stress-Induced Injury

Adel Saad[a] and Mohamed Ibrahim[b]
[a] Department of Physiology, Faculty of Medicine, Minia University, Egypt
[b] Department of Pharmacology, Faculty of Medicine, Minia University, Egypt

A B S T R A C T

Background and aim: Hemeoxygenase-1 (HO-1) is a stress responsive protein induced by various oxidative stress agents. It has protective effects in several organs including liver and kidney against oxidative stress injury however, the mechanisms underlying the effects of HO-1 remains poorly defined. This study investigated the effect of HO-1 inducer (Hemin) on liver and kidney tissues in rats exposed to cold restraint stress (CRS) and the possible contribution of nitric oxide in this effect. **Methods:** Male Wistar rats were divided into 5 groups, normal control group, CRS non treated group, CRS + hemin (50 mg/kg i.p.) group, CRS + aminioguanidine (inhibitor of inducible nitric oxide synthase (iNOS)) (50 mg/kg s.c.) group and CRS + hemin + aminoguanidine group. Liver and kidney tissues injuries were assessed biochemically and histopathologically. Samples from liver and kidney were used for estimation of oxidative stress markers**. Results:** Exposure to CRS caused injury of liver and kidney manifested by elevated serum alanine aminotransferase (ALT), and serum urea and creatinine, and confirmed by histopathological changes. Liver and kidney injuries were associated with elevation of oxidative stress markers. Pre-treatment with either hemin or aminoguanidine restored the levels of oxidative stress to near normal values with improvement of the hisopathological changes. No further protection was noticed when hemin coadministered with iNOS inhibitor (aminoguandine). **Conclusion:** The results of this study suggest that release of HO-1 in CRS tissue injury exhibits a protective antioxidant effect probably via inhibition of iNOS.

Key Words: Hemin-Cold Stress, Hemin-iNOS, iNOS-Cold Stress

Corresponding Author: Adel Hussein Saad **Email:** *adelhazem2@yahoo.com*

1. INTRODUCTION

Stress is an aversive stimulus capable of altering physiological homeostasis and the balance of body. It is considered to be risk factor in several diseases, including digestive tract, liver, renal, circulatory, and neuroendocrine diseases (Chrousos et al. 1992). Exposure to stress situations has been suggested to impair antioxidant defenses, leading to oxidative damage by changing the balance between oxidant and antioxidant factors (Alptekin et al. 1996, Seckin et al. 1997, Sahin et al. 2007). Exposure to low temperatures is an important stressing physical agent (Yuksel et al. 2008, Gallo et al. 2009). Previous studies have reported that acute exposure to cold stress produced cardiac injury (Meneghini et al. 2009) lipid depletion in the adrenal gland and glycogen depletion in the liver (Ferriera et al. 2010), and altered renal function in rats (Sabharwal et al. 2004).

Hemeoxygenase (HO) is the rate-limiting enzyme in heme catabolism, a process which leads to the generation of equimolar amounts of biliverdin, free iron and carbon monoxide (CO) (Maines, 1997). Hemeoxygenase-1 (HO-1) is induced by a vast array of stimuli, including oxidative stress, heat shock, ultraviolet radiation, ischemia-reperfusion, heavy metals, bacterial lipopolysaccharide (LPS), cytokines, nitric oxide (NO), and its substrate, heme (Shibahara, 1988). HO-1, once expressed under various pathological conditions, it has the ability to metabolize high amounts of free heme to produce high concentrations of its enzymatic by-products that can influence various biological events and can confer cytoprotection and anti-inflammatory effects in several disease models (Abraham et al. 2008).

Nitric oxide (NO) which is a ubiquitous messenger molecule in many different organ systems plays an important role in the regulation of various cellular functions (Moncada et al. 1991, Berdeaux, 1993, Knowles et al. 1994). NO is produced by the enzyme NO synthase (NOS), which exists in three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). The overproduction of NO under oxidative stress conditions by iNOS generates reactive nitrogen species that may amplify the inflammatory response

(Kato et al. 2009). This study was designed to investigate the effect of HO-1 inducer, hemin, on oxidative stress parameters in liver, and kidney tissues of rats exposed to cold restraint stress and the possible contribution of NO in this effect.

2. MATERIALS AND METHODS

2.1. Chemicals

Hemin and aminoguanidine were supplied as powders from Sigma (St. Louis, MO, USA). Hemin was dissolved in a freshly prepared solution of NaOH (0.1N) while aminoguanidine was dissolved in phosphate buffered saline (PBS) (pH 7.4).

2.2. Animals

Adult male Wistar rats weighing 150 – 250 g were obtained from the National Center of Research, El-Giza, Egypt. Rats were housed in a light and temperature controlled room on a 12/12 h light/dark cycle and fed with a standard pellet lab chow and left to acclimatize for one week before inclusion in the experiment. The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of Faculty of Medicine, Minia University.

2.3. Experimental protocol

Rats were divided randomly into five groups: Normal control group (n=12) that received vehicles either NaOH (0.1N) or PBS (pH 7.4) and kept at room temperature, Cold restraint stress group (CRS) (n=6) in which the rats were placed on a wooden board with the four limbs extended and fixed to the 4 corners of the wooden board so that the animal was unable to move, and then the wooden board carrying the animal was placed in a refrigerator (4°C) for 4 hours (Murkami et al, 1985) after receiving the vehicles (NaOH and PBS) to serve as positive control, CRS + hemin group (n=6) that treated with hemin as a single dose (50 mg/kg i.p.) 24 h before cold restraint stress was performed (Matsuka et al, 2007), CRS + aminoguanidine group (n=6) that pretreated with aminoguanidine (50mg/kg s.c.) 18 h and 1 h before CRS (Kato et al, 2009), and CRS + hemin + aminoguanidine group (n=6) that co-treated with hemin and aminoquanidine as indicated above. The rats were deprived of food and water during stress exposure. The stress procedure was performed between 09:00 – 1:00.

At the end of the experimental protocol, rats were anesthetized with sodium thiopental anesthesia (50 mg/kg, i.p.) and sacrificed by collecting the blood into dry tubes by cardiac puncture. Serum was obtained by centrifugation at1500 × g for 10 min. and stored at −80°C until they were analyzed. Liver and kidneys were rapidly removed, washed with ice-cold saline and divided into 2 parts. One part was kept in 10% formalin for histopathological examination and second part was stored at −80°C for measurement of lipid peroxidation products, catalase, reduced glutathione (GSH) and NO content.

2.4. Biochemical measurements

2.4.1. Determination of serum liver transaminases

Serum ALT level was determined by using commercially available kit (Biodiagnostic, Egypt) according to the manufacturer protocol (Reitman and Frankel, 1957).

2.4.2. Determination of serum urea and creatinine

Serum urea and creatinine were determined by using commercially available kit (Biodiagnostic, Egypt) according to the manufacturer protocol (Fawcett et al, 1960).

2.4.3. Determination of lipid peroxides

Tissues were homogenized in ice-cold 0.15 M KCl (10%, w/v). MDA was measured by the thiobarbituric acid test (Ohkawa et al, 1979). The breakdown product of 1,1,3,3-tetraethoxypropane was used as a standard.

2.4.5. Determination of catalase content

Liver and kidney tissue homogenate were assayed for its content of catalase using commercially available kit (Biodiagnostic, Cairo, Egypt) according to the manufacturer protocol (Aebi, 1984).

2.4.6. Determination of GSH level

GSH content in liver and kidney was determined using commercially available kit (Biodiagnostic, Egypt) according to the manufacturer instructions. The procedure is based on the reduction of 2-nitrobenzoic acid by glutathione to produce a yellow compound which was measured spectrophotometrically at 405nm using Shimadzu Spectrophotometer UV 1201 (Japan) (Beutler, et al., 1963).

2.4.7. Determination of NO level

The hepatic and kidney tissue content of NO was detected by measuring the nitrate/nitrite (NOx), the stable degradation products of NO. Thus, the stable end products of NO, nitrite (NO_2^-) and nitrate (NO_3^-) were used as indicators of NO production. NOx was measured after the reduction of nitrate to nitrite by copperized cadmium granules (Cd) in glycine buffer at pH 9.7. Quantitation of NO_2^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 540 *n*m is formed by reaction of nitrite with a mixture of naphthylethylenediamine and sulphanilamide using

Shimadzu Spectrophotometer UV 1201 (Japan) (Green et al., 1982)

2.5. Histopathological examination

For microscopic evaluation of histopathological changes, tissues were embedded in paraffin, then sectioned and stained with haematoxylin and eosin (H&E).

2.6. Statistical Analysis of the Data

Data are shown as means ± SEM. The results were analyzed by one way analysis of variant (ANOVA) followed by t-test with $P \leq 0.05$ selected as the criterion for statistical significance using Software GraphPad Prism Version 5 (GraphPad Software Inc, La Jolla, CA, USA).

3. RESULTS

3.1. Serum ALT level

There was significant elevation of serum ALT in CRS group compared with normal control group. Administration of Hemin caused significant decrease (by 30%) in serum ALT values compared with CRS. Also, administration of aminoguanidine resulted in a significant decrease (by about 30%) in serum ALT compared to cold restraint group. Coadministration of hemin with aminoguanidine produced no further decrease in serum ALT level compared with either drug (Figure.1A).

3.2. Serum levels of urea and creatinine

Figure 1 (B & C) shows the effects of CRS on serum urea and creatinine levels respectively, There was significant elevation of serum urea and creatinine (about 60% and 150%, respectively) in CRS group compared with normal control group. Administration of hemin caused significant decrease in serum urea and creatinine values (by about 26% and 45%, respectively) compared with cold restraint stress. Also, administration of aminoguanidine resulted in a significant decrease (by about 28% and 50% respectively) in serum urea and creatinine, the coadministration of hemin with aminoguanidine produce no further decrease in serum urea and creatinine levels compared with either hemin or aminoguanidne.

3.3. Hepatic tissue content of oxidative stress markers (catalase, GSH, MDA and NOx)

The results in Table (1) summarises the effects of CRS and different treatments on hepatic content of catalase, GSH, MDA and NOx, In CRS group, catalse significantly decreased (by about 40%) compared to control group. While treatment with either hemin or aminoguanidine increased catalase significantly (by about 45%) compared with CRS group. Coadministration of hemin plus aminoguanidine produces no significant increase in catalase level compared to either hemin or aminoguanidine.

GSH contents in hepatic tissue were significantly decreased in CRS rats compared with normal control group. Administration of hemin caused significant increase in GSH content (by about 70%) compared with cold stressed group. Administration of aminoguanidine also resulted in significant increase in GSH compared with CRS rats. There was no further increase in GSH level when hemin coadministered with aminoguanidine (about 60%).

There was significant elevation in hepatic tissue MDA content in cold restraint group compared with normal control group. Administration of Hemin caused significant decrease (by about 30%) in MDA content compared with cold stressed group. Administration of aminoguanidine resulted in significant decrease in MDA (by about 34%) compared with cold restraint stress. There was no significant difference in the effect of combined hemin and aminoguanidine compared to hemin or aminoguanidine alone (about 34%).

There was significant elevation in hepatic tissue NOx content in cod stress group (about 100%) compared with normal control group. Administration of Hemin caused significant decrease in NOx content (about 50%) compared with cold stressed group. Administration of aminoguanidine resulted also in significant decrease in NOx compared with cold restraint stress. There was no further decrease in NOx level when hemin coadministered with aminoguanidine. No significant difference in the effect of hemin and aminoguanidine compared to hemin or aminoguanidine alone.

3.4. Renal tissue content of Catalase, GSH, MDA and NOx

Table (2) shows the results obtained for measurement of catalase, GSH, MDA and NOx in kidney tissue. Measurement of catalase as one of the antioxidant enzymes showed that cold stress decreased its level significantly (about 35%) compared to control group while Hemin treatment increased catalase significantly and the same effect was noticed with aminoguanidine (about 35%), the coadministration of hemin plus aminoguanidine produce no further increase in catalase level which was nearly similar to the effect of either of these drugs separately.

GSH contents in renal tissue were significantly decreased in cold stressed rats (about 40%) compared with normal control group. Administration of Hemin caused significant increase in GSH content (about 66%) compared with cold stressed group.

Administration of aminoguanidine resulted in significant increase in GSH (about 77%) compared with cold restraint stress. Also there was no further increase in GSH level when hemin coadministered with aminoguanidine (about 60%).

There was significant elevation in renal tissue MDA content in cold restraint group (about 130%) compared with normal control group. Administration of Hemin caused significant decrease in MDA content (about 31%) compared with cold stressed group. Administration of aminoguanidine resulted in significant decrease in MDA (about 34%) compared with cold restraint stress. The coadministration of hemin and aminiguanidine produce no further decrease in MDA level (about 34%) compared to either of both drugs separately.

There was significant elevation in renal tissue NOx in cold stress group (about 50%) compared with normal control group. Administration of Hemin caused significant decrease in NOx content (about 37%) compared with cold stressed group. Administration of aminoguanidine resulted also in significant decrease in NOx (about 39%) compared with cold restraint stress. Also there was no further decrease in NOx level when hemin coadministered with aminoguanidine (about 38%).

3.5. Histopathological Findings

Figure (2) shows liver histopathological findings in different groups. A represents normal hepatic architecture. In B, CRS, there was marked congestion of the central vein, with dilatation of sinusoidal spaces and inflammatory cell infiltration. In hemin-treated rats (C), there was improvement in hepatic architecture, but still showed mild sinusoidal dilatation. Rats treated with aminoguanidine (D) or hemin + aminoguanidine (E) showed hepatic structure comparable to that of control.

Figure (3) shows renal slides, CRS group showed marked degeneration in glomeruli, with dilatation in Bowmans capsule, as well as inflammatory infiltration with necrotic debris in most of the renal tubules. Hemin alone in cold stress-subjected rats improved glomerular architecture, but still showed inflammatory cellular infiltration. Rats subjected to cold stress and treated with aminoguanidine or hemin together with aminoguanidine showed renal structure comparable to that of control.

Table 1: Effect of cold restraint stress on different oxidative biomarkers in hepatic tissue and its modulation by different treatments

	CAT (U/gm)	*GSH (mg/gm)*	*MDA (nmol/gm)*	*NOx (nmol/gm)*
Control	1.27 ± 0.09	6.40 ± 0.55	0.42 ± 0.03	119.72 ± 9.63
CRS	0.76 ± 0.04*	3.21 ± 0.25*	1.07 ± 0.08*	236.74 ± 36.95*
Hem	1.11 ± 0.05†	6.35 ± 0.30†	0.53 ± 0.05†	115.67 ± 18.87†
Amino	1.24 ± 0.1†	5.50 ± 0.13†	0.52 ± 0.02†	111.43 ± 33.91†
Hem/Amino	1.16 ± 0.05†	6.21 ± 0.08†	0.62 ± 0.05†	139.79 ± 6.70†

Values presented as mean ± SEM (6 rats)
CRS: cold restraint stress, Hem: Hemin treated, Amino: aminoguanidine treated, Hem+Amino: Hemin plus aminoguanidine treated, CAT:catalase, GSH: reduced glutathione, MDA: malondialdehyde, NOx: total nitrite.
One-way analysis of variance (ANOVA) followed by the t-test was used to analyze the results for statistically significant difference.
* $P < 0.05$ compared with control group.
† $P < 0.05$ compared with cold restraint group.

Table 2: Effect of cold restraint stress on different oxidative biomarkers in renal tissue and its modulation by different treatments

	CAT (U/gm)	*GSH (mg/gm)*	*MDA (nmol/gm)*	*NOx(nmol/gm)*
Control	1.29 ± 0.05	5.76 ± 0.66	0.41 ± 0.01	135.27 ± 10.56
CRS	0.82 ± 0.04*	3.36 ± 0.22*	0.96 ± 0.05*	202.06 ± 17.50*
Hem	1.06 ± 0.09†	5.58 ± 0.43†	0.66 ± 0.09†	127.94 ± 19.84†
Amino	1.13 ± 0.08†	5.97 ± 0.56†	0.63 ± 0.06†	123.81 ± 16.55†
Hem/Amino	1.04 ± 0.06†	5.27 ± 0.49†	0.63 ± 0.07†	125.66 ± 7.29†

Values presented as mean ± SEM (6 rats).
CRS: cold restraint stress, Hem: Hemin treated, Amino: aminoguanidine treated, Hem+Amino: Hemin plus aminoguanidine treated, CAT:catalase, GSH: reduced glutathione, MDA: malondialdehyde, NOx: total nitrite.
One-way analysis of variance (ANOVA) followed by the t-test was used to analyze the results for statistically significant difference.
* $P < 0.05$ compared with control group.
† $P < 0.05$ compared with cold restraint group.

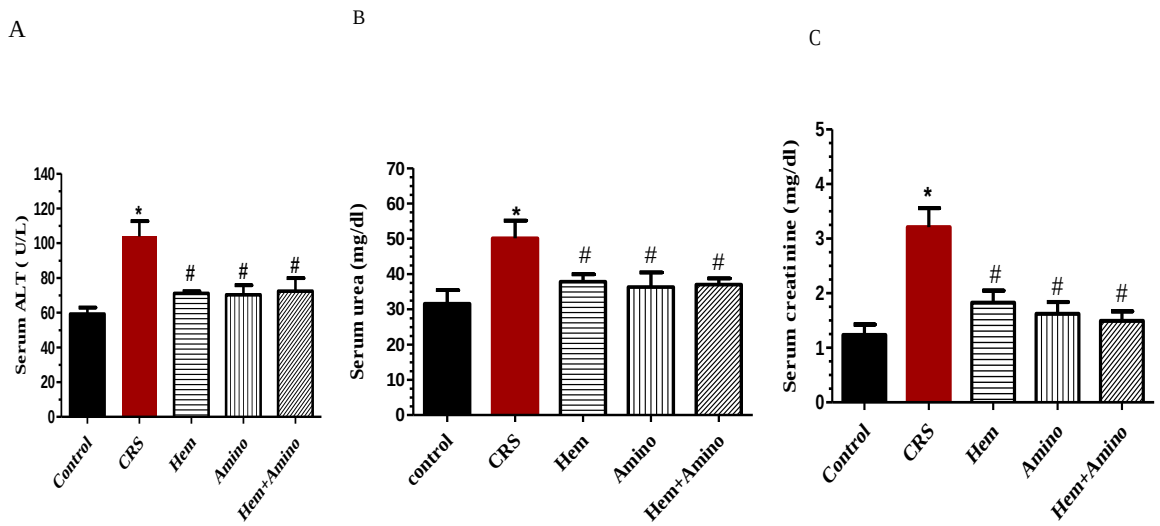

Figure 1 : Effect of cold restraint stress on serum ALT (A), serum urea (B) and serum creatinine (C) and its modulation by different treatments

Values presented as mean ± SEM (6 rats)

CRS: cold restraint stress, Hem: Hemin treated, Amino: aminoguanidine treated, Hem+Amino: Hemin plus aminoguanidine treated, CAT:catalase, GSH: reduced glutathione, MDA: malondialdehyde, NOx: total nitrite.

One-way analysis of variance (ANOVA) followed by the t-test was used to analyze the results for statistically significant difference.

* $P < 0.05$ compared with control group.

# $P < 0.05$ compared with cold restraint group.

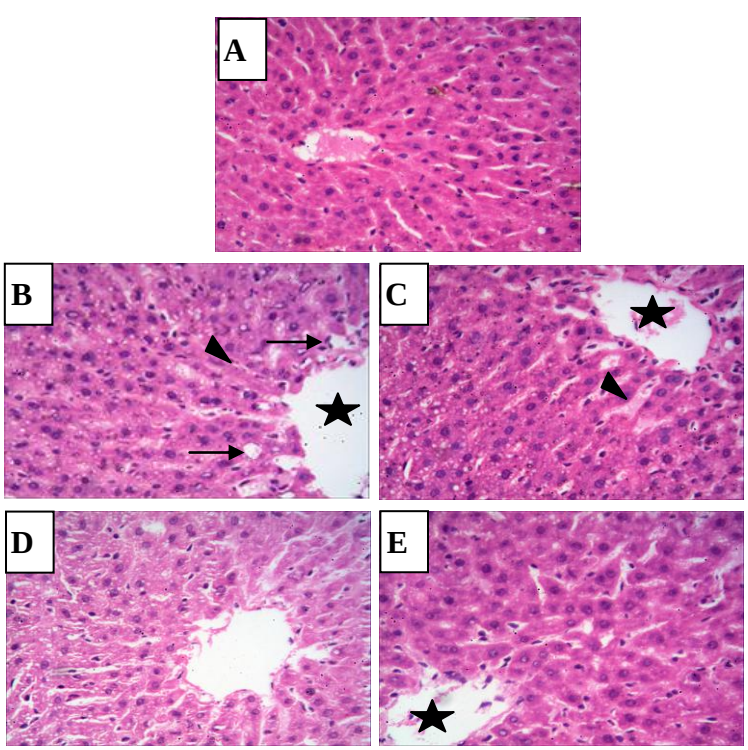

Figure 2: Photomicrograph of liver stained with hematoxylin and eosin (×200). A: control B: cold stress C: cold stress/Hemin-treated D: cold stress/Aminoguanidine-treated and E: cold stress/Hemin+aminoguanidine-treated groups. Central vein (star) dilated sinusoidal spaces (arrow) inflammatory cells (arrow head).

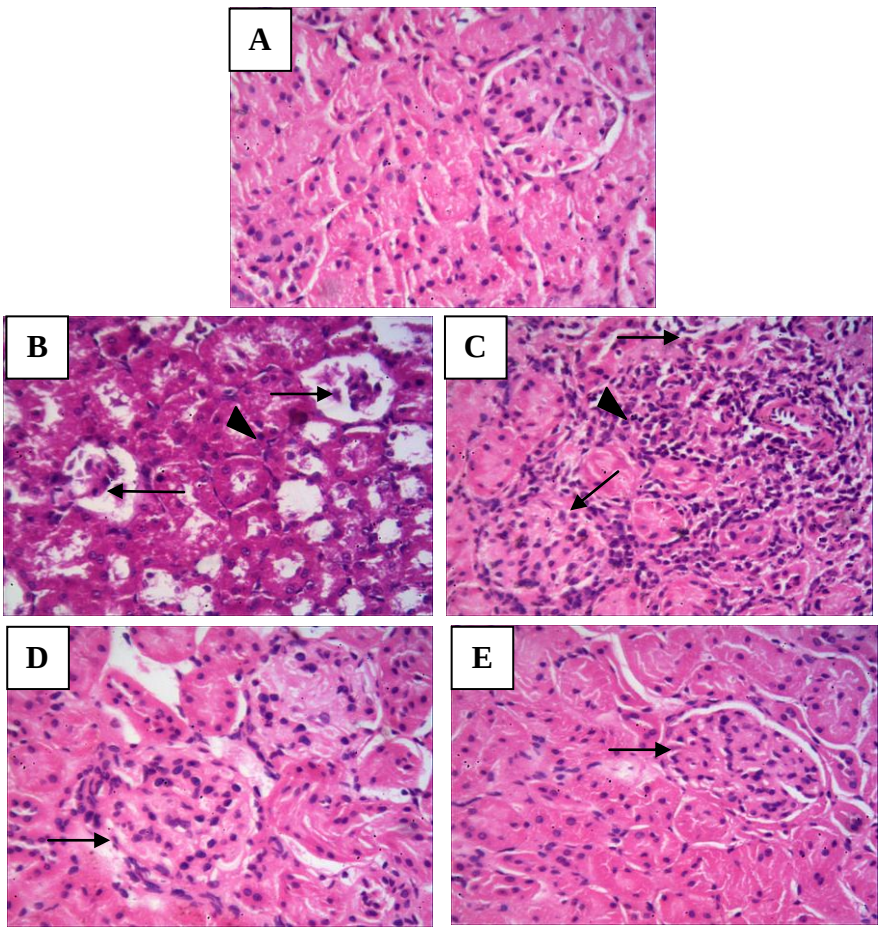

Figure 3: Photomicrograph of kidney stained with hematoxylin and eosin (×200). A: control B: cold stress C: cold stress/Hemin-treated D: cold stress/Aminoguanidine-treated and E: cold stress/Hemin+aminoguanidine-treated groups. Glomeruli (arrows) inflammatory cells (arrow head).

4. DISCUSSION

All living organisms respond to stress changes in the environment in various ways. Exposure to stress leads to behavioral and organic changes to improve the ability of the organism to adjust homeostasis and increase its chances for survival (Chrousos, 2000). Exposure to an extreme environment such as heat and cold is a form of stress experienced by all organisms (Kanayama et al., 1999).

Induction of endogenous pathways that are selectively engaged in restoring cellular homeostasis represents an important cell defense mechanism against cellular damage. The induction of HO-1, which is triggered by a range of oxidant-related stimuli, appears to act as a ubiquitous defensive system in a variety of cell types (Matsuoka et al., 2007).

This study aimed at investigating the effect of HO-1 in CRS tissue injury and the possible contribution of iNOS in such effect.

The results of this study showed that cold stress induced oxidative injury in liver and kidney was manifested by increased serum levels of liver enzyme (ALT) as well as by increased serum urea and creatinine, respectively. Tissue injuries were confirmed by histopathological changes. These findings are consistent with previous studies (Sabharwal et al. 2004, Ferriera et al. 2010).

The data of the present study showed that liver and kidney injuries were associated with increased lipid peroxides and NO levels in addition to decreased level of GSH and catalase. Similar findings had been reported by Sahin et al. 2007.

CRS was found to enhance catabolic and suppress anabolic processes and cause elevated metabolic rate leading to generation of free radicals.

Increased formation of these radicals resulted in increased oxidative stress. Therefore, the imbalance between free radical formation and elimination might be responsible for the oxidative stress effects of CRS (Filipovic et al., 2010).

Administration of hemin attenuated hepatic and renal injuries in CRS rats as evidenced biochemically and confirmed by histopathology. Such protective effect was associated with improved tissue oxidative markers. These protective effects were also confirmed in other reports (Kpitulnik, 2004, Ryter et al., 2006 and Datla et al., 2007). It has been reported that induction of HO-1 prevented CRS tissue injuries via antioxidants effects (Lesuy et al., 1994). Induction of HO-1 scavenges free radicals as effectively as α-tocopherol, which is regarded as the most potent antioxidant against lipid peroxidation. (Stocker et al., 1987)

Nitric oxide (NO) is a crucial signaling molecule in vertebrates produced in several cell types by NOS. Inducible NOS is expressed in macrophages and some other cell types upon their activation by a wide range of proinflammatory stimuli and stressful conditions (Jan et al., 2012). Despite the fact that NO can play beneficial roles, it can also be involved in numerous pathological situations such as hypotension accompanying septic shock, essential hypertension, and atherosclerosis (Loscalzo et al., 1995). The increased formation of NO was found to have neurotoxic effects and can contribute to the pathogenesis of stroke and other neurodegenerative disorders (Jaffrey et al., 1995). In general, the overwhelming production of NO contributes to the pathogenesis of both acute and chronic inflammatory processes and NO has been recognized as one of the main signaling molecules involved in these processes (Bogdan, 2001).

The relation between NO and HO-1 is complex and far from understood. A few reports indicate that NO itself, is responsible for the induction of HO-1 and that NO can increase CO production via binding to the heme moiety of HO (Motterlini et al., 1996). In addition to CO, equimolar concentrations of biliverdin are produced during the HO-mediated heme degradation. Biliverdin is then degraded to bilirubin, also known as a strong antioxidant. All these data indicate that HO-1 pathway within the tissues might constitute an important defense mechanism against oxidative stress and against the deleterious effects of NO (Henningsson et al., 1999).

The results of the experiment demonstrated that exposure to CRS elevated the level of NO in hepatic and renal tissues which was accompanied by increased level of lipid peroxidation, decreased level of reduced glutathione and catalase. This is consistent with previous reports by Radi et al. (2002) who reported that NO, at physiological concentration, is relatively unreactive, but when there is excessive production, it may be converted to a number of more reactive derivatives, collectively known as reactive nitrogen species (RNS). These, in turn, will react with superoxide and NO to produce peroxynitrite (ONOO). The later is a powerful oxidant, able to damage many biological molecules and may decompose to release small amounts of OH radicals, resulting in increased oxidative stress leading to decrease reduced glutathione and catalase (Şahin et al., 2007, Tijana et al., 2010).

The mechanism by which HO-1 confers protection against oxidative stress is not completely elucidated. It has been suggested that the catalytic by-products of heme degradation by HO-1 provide antioxidant functions either directly or indirectly (Petrache et al., 2000). In a trial to explore the possible contribution of iNOS in the protective effect of HO-1 in our experiment we investigated the effect of one the selective iNOS inhibitor (aminoguanidine) on oxidative damage induced by cold restraint stress. Aminoguanidine treatment exerted protective effects against cold restraint stress induced oxidative damage manifested by decreased NO production, decreased lipid peroxides, and elevated reduced glutathione and catalase. The protective effects of aminoguanidine were nearly similar to levels observed in hemin treated group. The addition of aminoguanidine to hemin did not produce further enhancement of the protective effect of hemin which was nearly similar suggesting that hemin may produce its antioxidant and anti-inflammatory effects via inhibition of iNOS with subsequent decreasing NO overproduction.

5. CONCLUSION

In conclusion, CRS is observed to affect especially the prooxidant-antioxidant status in the liver and kidney tissues of rats. In addition, in stress-exposed rats, hemin treatment was found to efficiently ameliorate oxidative stress, at least in part, via suppression of iNOS induction and NO production. These findings raise the possibility that there is a cross talk between HO-1 and NO and the modulation of this cross talk might be a potential therapeutic target in the prevention of oxidative stress conditions.

6. ACKNOWLEDGEMENT

We thank Dr. Dalia Abdel-Rahim assistant professor of Pathology, Faculty of Medicine, Minia University for her kind help in the part of the histopathological examination.

7. REFERENCES

Abraham, N.G., Kappas, A. (2008). Pharmacological and clinical aspects of hemeoxygenase. Pharmacol Rev 60: 79–127.

Aebi, H. (1984). Methods Enzymol 105: 121-126.

Alptekin, N., Seçkin, Ş., Doğru-Abbasoğlu, S., Yelkenci, F., Koçak, N., Toker, G., et al. (1996). Lipid peroxides, glutathione, γ-glutamyl cysteine synthetase and γ-glutamyl transpeptidase activities in several tissues of rats following water-immersion stress. Pharmacol Res. 34:167–169.

Berdeaux, A. (1993). Nitric oxide: an ubiquitous messenger. Fundam Clin Pharmacol 7: 401–411.

Bombig, M.T., Ferreira, C., Mora, O., Soares, J.D., Póvoa, R., Filho, B., et al. (2003). Pravastatin protection from cold stress in myocardium of rats. Jpn Heart J. 44:243-55.

Beutler, E., Duron, O., Kelly, M.B. (1963). J. Lab Clin. Med. 61, 882.

Bogdan, C. (2001). "Nitric oxide and the immune response," Nature Immunology, 2 (10): 907–916.

Chrousos, G.P, Gold, P.W. (1992). The concepts of stress system disorders: overview of physical and behavioral homeostasis. JAMA. 267:1244–1252.

Chrousos, GP. (2000). The role of stress and the hypothalamic pituitary adrenal axis in the pathogenesis of the metabolic syndrome: neuroendocrine and target tissue-related causes. Int J Obes Relat Metab Disord 24 (2):S50–5.

Dalta, S.R., Dusting, G.J., Mori, T.A., Taylor, C.J., Croft, K.D., Jiang, F. (2007). Induction of hemeoxygenase-1 in vivo suppresses NADPH oxidase derived oxidative stress. Hypertension 50: 636-642.

Fawcett, J.K., and Soctt, J.E. (1960). J. Clin. Path. 13: 156-15.

Ferreira, M., de Abreu, L.C., Valenti, V.E., Meneghini, A., Murad, N., Ferreira, C. (2010). Effects of electrical counter shock and cold stress on the liver and adrenal gland. Clinics. 65(3):291-6.

Filipovic, D., Mandic, L.M., Kanazir, D., Pajovic, S.B. (2010). Acute and/or chronic stress models modulate CuZnSOD and MnSOD protein expression in rat liver. Mol Cell Biochem. 338:167–174.

Gallo, C.B., Mimura, M.A., Sugaya, N.N. (2009). Psychological stress and recurrent aphthous stomatitis. Clinics. 64:645-8.

Green, L.C., Wagner, D.A., Glagowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R. (1982). Analysis of nitrate, nitrite and N nitrate in biological fluids. Anal Biochem 126:131–8.

Henningsson, R., Alm, P., Ekstro¨m, P., and Lundquist, I. (1999). Heme-oxygenase and carbon monoxide: regulatory roles in islet hormone release. A biochemical, immunohistochemical and confocal microscopical study. *Diabetes* 48: 66–76.

Jaffrey, S.R., and Snyder, S.H. (1995). "Nitric oxide: a neural messenger," Annual Review of Cell and Developmental Biology, 11: 417–440.

Jan, V., Antonın, L., and Lukas, K. (2012). Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges. Hindawi Publishing Corporation Mediators of Inflammation Volume 2012, Article ID 318087, 22 pages?????.

Kanayama, N., Khatun, S., Belayet, H., She, L., Terao, T. (1999). Chronic local cold stress to the soles induces hypertension in rats. Am J Hypertens 12:1124 – 9.

Kapitulnik, J. (2004). Bilirubin: an endogenous product of heme degradation with both cytotoxic and cytoprotective properties, Mol Pharmacol 66: 773-779.

Kato, S., Ito, Y., Ohkawa, F., Takeuchi, K. (2009). Pathogenic importance of endothelial isozyme of nitric oxide synthase (eNOS) in aggravation of gastric lesions induced by indomethacin and cold restraint stress in adjuvant-induced arthritic rats. Gastroenterology. 136 (1):W1559.

Kato, S., Kawahara, R., Yasuda, M., Amagase, K., and Takeuchi, K. (2009). Aggravation of Cold-Restraint Stress–Induced Gastric Lesions in Adjuvant Arthritic Rats: Pathogenic Role of Inducible and Endothelial Nitric Oxide. J Pharmacol Sci 111:244 – 252.

Knowles, R.G., and Moncada, S. (1994). Nitric oxide synthases in mammals. Biochem J 298: 249–258.

Lesuy, S.F., Tomaro, M.L. (1994). Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. Biochim Biophys Acta 1223: 9–14.

Loscalzo, J., and Welch, G. (1995). "Nitric oxide and its role in the cardiovascular system," Progress in Cardiovascular Diseases, 38(2): 87–104.

Maines, M.D. (1997). The heme oxygenase system: a regulator of second messenger gases. Ann Rev Pharmacol Toxicol 37: 517–554.

Matsuoka, Y., Masuda, H., Yokoyama, M., and Kihara, K. (2007). Protective effects of heme oxygenase-1 against cyclophosphamide-induced haemorrhagic cystitis in rats. B J U International 100:1402 - 1408

Meneghini, A., Ferreira, C., Abreu, L.C., Valenti, V.E., Ferreira, M., Filho, C.F., et al. (2009). Memantine prevents cardiomyocytes nuclear size reduction in the left ventricle of rats exposed to cold stress. Clinics. 64:921-6.

Moncada, S., Palmer, M.J., Higgs, E.A. (1991). Nitric oxide: Physiology, pathology and pharmacology. Pharmacol Rev. 43:109–142.

Motterlini, R., Foresti, R., Intaglietta, M., and Winslow, RB. (1996). NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am J Physiol Heart Circ Physiol* 270: H107–H114.

Murkami, M., Lam, S.K., Inada, M., and Miyakes, T. (1985). Pathophysiology and pathogenesis of acute gastric mucosal lesions after hypothermic-restraint stress in rats. Gastroenterology 88: 660-665.

Ohkawa, H., Ohishi, N., Yagi, K. (1979). Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Anal Biochem. 95:351–358.

Petrache, I., Otterbein, L.E., Alam, J., Wiegand, G.W., Choi, A.M. (2000). Hemeoxygenase-1 inhibits TNF-α-induced apoptosis in cultured fibroblasts. Am J Physiol Lung Cell Mol Physiol 278: L312–9

Radi, R., Cassina, A., Hodara, R., Quijana, C. and Castro, L. (2002). Peroxynitrite reactions and formation in mitochondria. Free Radic. Biol. Med. 33:1451-1464.

Reitman, A., and Frankel, S. (1957). Amer. J. Clin. Path. 28: 56. where is the title?????

Ryter, S.W., Alam, J., Choi, A.M. (2006). Hemeoxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev 86:583-650.

Sabharwal, R., Johns, E.J., and Eggenton, S. (2004). The influence of acute hypothermia on renal function of anaesthetized euthermic and acclimatized rats. Exp Physiol 89 (4): 455-463.

Şahin, E., Gümüşlü, S. (2007). Immobilization stress in rat tissues: alterations in protein oxidation, lipid peroxidation and antioxidant system. Comp Biochem Physiol C. 144:342–347.

Seçkin, Ş., Alptekin, N., Doğru-Abbasoğlu, S., Koçak-Toker, N., Toker, G., Uysal, M. (1997). The effect of chronic stress on hepatic and gastric lipid peroxidation in long-term depletion of glutathione rats. Pharmacol Res. 36:55–58.

Shibahara, S. (1988). Regulation of heme oxygenase gene expression. Semin Hematol 25: 370–376.

Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N., Ames, B.N. (1987). Bilirubin is an antioxidant of possible physiological importance. Science 235:1043–1046.

Tijana, B.R., Slavica, S.B., Branka, R.P., Svetlana, G.D., Pavlovici, S.Z., Cakic, P.D., and Zorica, S.S. (2010). Superoxide dismutase and catalase activities in the liver and muscle of barbell (barbus barus) and its intestinal parasite (pomphoryinchus laevis) from the Danube river, Serbia Arch. Biol. Sci., Belgrade, 62 (1):97-105.

Yuksel, S., Asma, D., Yesilada, O. (2008). Antioxidative and metabolic responses to extended cold exposure in rats. Acta Biol Hung. 59:57-66