Research Article

Induction of Heme Oxygenase-١ Delays the Onset of Electrically-Induced Fatigue in Isolated Rat Diaphragm

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Abstract
Background: The role of endogenous heme oxygenase-١ (HO-١) in experimentally-induced muscle fatigue was investigated using isolated rat diaphragm. **Methods:** Rats were randomly divided into the following groups (٦ rats each): control, stimulated non-treated and stimulated pretreated groups with either the HO-١ inducer, hemin (٥٠ mg/kg, i.p.) or the HO-١ inhibitor, zinc protoporphyrin (ZnPP) (٥٠ µmol/kg) before induction of fatigue protocol. Diaphragmatic fatigue was induced by direct low frequency (٥ Hz) electrical stimulation till ٥٠٪ decline of force (٥٠٪ fatigue). The diaphragm was immediately excised, stored at -٨٠ºC for measuring HO-١, glycogen, nitric oxide, reduced glutathione (GSH) and lipid peroxides levels. **Results:** Induction of fatigue produced a significant decrease in glycogen and GSH contents with concomitant increases in HO-١, NO and lipid peroxides levels compared to the control group. Hemin pretreatment delayed the onset of ٥٠٪ fatigue which was associated with significant increases in HO-١ and GSH levels with concomitant decreases in NO and lipid peroxides levels compared to the stimulated non-treated group. On the contrary, ZnPP pretreatment produced the opposite effect and aggravated the condition. **Conclusion:** induction of endogenous HO-١ enzyme during muscle activity is an important mechanism to improve muscle performance partly by overcoming oxidative stress and increasing antioxidant capacity and thus increase the muscle resistance to fatigue. Therefore, Pharmacological induction of HO-١ enzyme could be a promising strategy for preventing ROS-induced diaphragmatic dysfunction, which is involved in many pathological conditions.

Keywords: Heme oxygenase-١, Hemin, Fatigue and Diaphragm

Introduction
The mammalian diaphragm is unique among skeletal muscles as it is chronically active and it is considered to be two distinct muscles based on functional and recruitment differences. The diaphragm is the most important inspiratory muscle, and as such, numerous investigations have explored the relationship between the biochemistry and contractile function of this muscle in both health and disease[(١)].

As respiratory work and metabolic demand on the diaphragm increase, so does production of reactive oxygen species (ROS). ROS are capable of eliciting oxidative stress through oxidation/reduction (redox) reactions with many cellular constituents including proteins, lipid membranes and nucleic acids[(٢)]. ROS production increases during exercise and in several pathological conditions, which could affect the contractile function of the diaphragm, including chronic obstructive pulmonary diseases (COPD), sepsis and inflammation[(٣)].

Respiratory muscle fatigue can be defined as a recoverable loss of the ability to produce muscle force and/or velocity under load, whereas respiratory muscle weakness is characterized by an impaired strength, even in a rested condition[(١)]. The mechanisms under-lying muscle fatigue are poorly understood, but may include alterations within the muscle itself (peripheral fatigue) such as reduced blood flow[(٤)] and increased pro-duction of metabolic by-products (e.g. ROS)[(٥)] and/or diminished activation from the central nervous system (central fatigue)[(٦)].

Diaphragmatic fatigue and weakness have been documented in numerous pathologies including COPD, asthma, respiratory distress syndrome, pulmonary edema and sepsis, which could all, eventually, lead to respiratory failure[٣].

The direct repetitive electrical stimulation (Mstim) allows production of muscle fatigue without any central fatigue. Therefore, this fatigue protocol has been widely used in animals[٧] as well as in humans to study the peripheral mechanisms of skeletal muscle fatigue including the diaphragm[٨].

Heme Oxygenases (HOs) are the rate-limiting enzymes of the initial reaction in the degradation of heme, which yields equimolar quantities of biliverdin, carbon monoxide (CO), and free iron[٩]. Biliverdin is subsequently converted to bilirubin, and free iron is rapidly incorporated into ferritin. So far, three HO isoforms have been identified: HO-٢ and HO-٣ are constitutively expressed in various cells, whereas HO-١ is transcriptionally activated in response to a variety of stimuli including bacterial lipopolysaccharide (LPS), heat stress, hypoxia, and exposure to nitric oxide (NO)[١٠،١١].

HO-١ can be induced in various tissues including skeletal muscle[١٢،١٣]. In this particular tissue, expression of the HO-١ gene is significantly increased after strenuous exercise[١٤], a condition that can lead to muscular oxidative stress and fatigue[١٥،١٦].

There is increasing evidence that HO-١ plays important roles in the cellular defense against oxidative stress and the deleterious effects of proinflammatory cytokines and ROS[١١،١٧]. However, little is known about the functional significance of HO-١ in skeletal muscle contraction and fatigue, especially the diaphragm.

Therefore, the present study was designed to investigate the effect of modulation of HO-١ enzyme on the mechanical and functional properties of rat diaphragmatic muscle to clarify whether the HO pathway is part of a protective mechanism against experimentally-induced diaphragmatic fatigue. Such information could provide new insight into the pathophysiology of diaphragmatic dysfunction which may occur in several pathological conditions such as in sepsis.

Materials and Methods

Animals:

Adult male albino (Sprague dawley strain) rats, about ٤ months old with average weight of ١٨٠-٢٠٠ g were used in the present work. Rats were purchased from the National Research Center, Cairo, Egypt. All animals were housed in stainless steel cages and left freely wandering in their cage for two weeks with normal hour's dark: light cycle for acclimatization. Rats were fed commercial rat chow and water *ad libitum* till the beginning of the experiment. All experimental procedures were in accordance with our institutional guidelines. The ethics protocol was approved by The Laboratory Animals Maintenance and Usage Committee of Faculty of Medicine in Minia University.

Isolated diaphragm muscle preparation

Rats were anesthetized with sodium thiopental (٥٠ mg/kg, intraperitoneal). The diaphragm, with its costal insertions and central tendon, was quickly excised after a combined thoracotomy and laparotomy and was immediately submersed in organ bath containing Kreb's solution (١٣٧ mM NaCl, ٤ mM KCl, ١ mM $MgCl_2$, ١ mM KH_2PO_4, ١٢ mM $NaHCO_3$, ٢ mM $CaCl_2$, and ٦.٥ mM glucose) bubbled with atmospheric air at pH ≈٧.٤ and room temperature[١٨]. Both the control and experimental muscles were submersed in Kreb's solution for ٣٠ minutes before the beginning of the stimulation protocol.

Fatigue protocol:

The diaphragm was mounted vertically with the central tendon positioned superiorly in an organ bath containing Kreb's solution as described above. A silk suture was tied to the central tendon and attached to a force transducer (Bioscience, PALMER, WASHINGTON); while the other end of the diaphragm was fixed to the bath[١٩].The diaphragm was stimulated directly by using platinum plate electrodes placed in close apposition to the muscle as previously adopted by Zhu et al. (٢٠٠٣) [٢٠]. Stimuli were applied with pulse duration of ٠.٥ ms. Muscle preload force was adjusted until optimal fiber length (Lo) for maximal twitch force was achieved. After ١٠ min, thermoequilibration baseline measure-ments

were determined, Single twitches (٠.٥ Hz stimulation) were recorded.

In the experimental muscle, contractions were produced by direct repeated low frequency stimulation (٥ Hz) till ٥٠٪ decline of force (٥٠٪ fatigue) was achieved. Contractile respo-nse was recorded using isotonic transducer and oscillograph (Bio-science; England) on calibrated charts.

A preliminary experiment was done and showed that a current of ٥ Hz is the maximal current producing clonus in case of diaphragm muscle. So, this frequency was selected in the present study.

Drug Preparation:

Hemin chloride, from (Sigma, UT, USA) and zinc protoporphyrin (ZnPP), from (Aldrich, UT, USA), were freshly dissolved in ٠.١ mol/L NaOH adjusted to pH ٧.٤ with ٠.١ mol/L HCl and diluting with saline to required volume (٠.٥ ml of this vehicle was given to non treated rats). Hemin and ZnPP were prepared in darkness and protected from light[٢١].

Preparation of Tissue homogenates:

The diaphragm of each rat was divided into two parts: one part was homogenized in ٣ ml of trichloroacetic acid (TCA) reagent (٥ gm of TCA from Sigma, St. Louis, USA and ١٠٠ mg silver sulphate from NICE Chemicals, Pvt. td. Cochin, India were dissolved in ١٠٠ ml of distilled water) and centrifuged at ٢٠٠٠ g for ١٠ minutes at room temperature. The supernatant was analyzed for determination of glycogen content.

The other part was homogenized on ice with cold potassium phosphate buffer (٠.٠٥ M, pH ٧.٤) and centrifuged at ٢٠٠٠ g for ١٠ minutes at ٤°C. The supernatant was kept at – ٨٠°C for subsequent measurement of lipid peroxides, Nitric oxide (NO), reduced glutathione (GSH) and Hemeoxygenase (HO)-١.

Experimental design:

The rats were classified into the following equal groups (٦ rats each):

١. Control non-stimulated group: in which the isolated diaphragm was immediately submersed in a tissue bath containing Kreb's solution for ٣٠ minutes and served as control.

٢. Fatigue group: in which the isolated diaphragm was directly stimulated by low frequency (٥ Hz) electrical stimulation until ٥٠٪ fatigue was achieved[٢٠] (Zhu et al., ٢٠٠٣).

٣. Fatigue + hemin pretreated group: in which HO-١ was induced by hemin injection (٥٠ mg/kg, i.p.) ٢٤ h prior to the stimulation protocol[٢٢].

٤. Fatigue + ZnPP pretreated group: in which each rat received a single dose of Znpp (HO-١ inhibitor) given intraperitoneally at a dose of ٥٠ µmol/kg ١ h before the stimulation protocol[٢٢].

Immediately after termination of the stimulation protocol, both experimental and control diaphragm muscles were blotted dry, quickly frozen in liquid nitrogen and then stored at -٨٠°C till time for biochemical analysis.

Biochemical analysis:

I. Measurement of muscle HO-١:

HO-١ level was measured in diaphragmatic muscle homogenates by ELISA using Rat HO-١ immunoassay kit (Biovendor, USA) following the manufacturer's instructions.

II. Determination of Glycogen Content:

This was done according to the spectro-photometric method described by Kemp and Adrienne[٢٣], which depends on heating of glycogen solution with concentrated sulphuric acid, induces dehydration of the former and development of pink color, the intensity of which is proportionate to the glycogen concentration present in the sample.

III. Determination of Nitric Oxide (NO) in the Muscle:

The biodiagnostic nitrite assay kit was used according to the method described by Montgomery and Dymock (١٩٦١)[٢٥]. This assay measures endogenous nitrite, the metabolite of NO as indicator of NO level. It depends on the addition of Griess reagent, which forms with nitrite a deep purple azo compound the intensity of which depends on nitrite concentration and is determined by spectrophotometric reading at ٥٤٠ nm against a sample blank and standard.

IV. Determination of Muscle Lipid Peroxides Content:

This was done according to the thiobarbituric acid method described by Ohkawa et al. (١٩٧٩)[٢٦] and depends on measuring the malondialdehyde (MDA) equivalent substances which are breakdown products of lipid peroxides. The thiobarbituric-MDA adducts form colored complexes when extracted with n-butanol/ pyridine; the absorbance of which is read at ٥٣٢ nm using a spectrophotometer. The corres-ponding concentration in nmol/g muscle tissue is calculated from a standard curve.

V. Determination of Muscle GSH Content:

Muscle content of GSH was measured colorimetrically using commercially available kit (Bio-diagnostic, Egypt) as previously described by Beutler et al. (١٩٦٣)[٢٧]. This method is based on the reduction of ٥,٥' dithiobis (٢-nitrobenzoic acid) (DTNB) with GSH to produce a yellow compound. The reduced chro-mogen is directly proportional to GSH concentration and its absorbance can be measured at ٤٠٥ nm.

Statistical analysis:

Data were represented as mean ± standard error of the mean (m ± SEM). Significant difference between groups was analyzed by one-way ANOVA followed by Tukey-Kramar post hoc test for multiple comparisons using Graph pad Prism ٥ software with a value of $P \leq ٠.٠٥$ was considered statistically significant.

Results

The results clearly demonstrated that:

Low frequency (LF) stimulation of rat diaphragm produced a significant increase in HO-١ level (٦.٤٨±٠.٤٣ pg/mg tissue) when compared to the control group (٤.١٣±٠.٣٩ pg/mg tissue). Hemin proved to be an inducer of HO-١ enzyme as evidenced by the further increase in diaphragmatic HO-١ level in stimulated+hemin petreated group (٨.٨٤±٠.٥٧ pg/mg tissue), while ZnPP is an inhibitor of HO-١ enzyme and significantly decreased the HO-١ level (٢.٢٨±٠.٢٩ pg/mg tissue) when compared to the stimulated non-treated group (٦.٤٨±٠.٤٣ pg/mg tissue) (Figure ١).

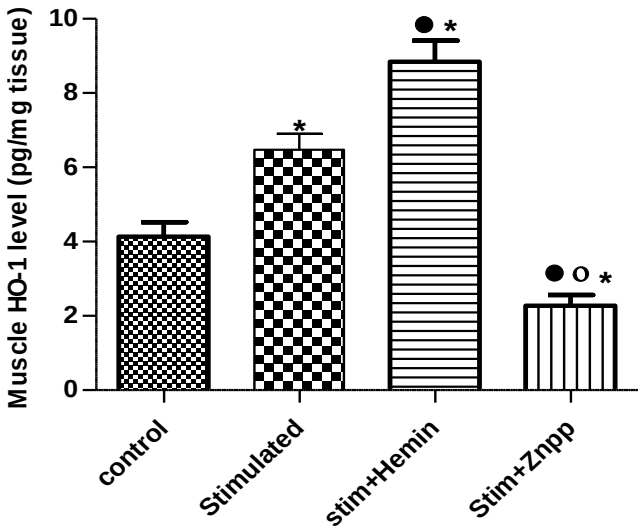

Figure ١: Diaphragmatic heme oxygenase-١ (HO-١) activity level in different experiment groups. *: Significant from control group; ●: Significant from stimulated group; ○: Significant from stim+hemin pretreated group, $P \leq ٠.٠٥$.

LF stimulation of isolated diaphragm produced a gradual progressive decline in muscle power achieving ٥٠٪ decline of force (fatigue) after ٤٦±١.٥٧ sec. pretreatment with the HO-١ inducer, hemin produced a significant delay in the onset of fatigue reaching the ٥٠٪ fatigue after ٧٤.١٧±٢.٩٢ sec. on the other hand, inhibition of HO-١ by ZnPP caused a significant acceleration in the onset of fatigue reaching ٥٠٪ decline of force after ٣٢.٨٣±٢.٣٢ sec (Figure ٢).

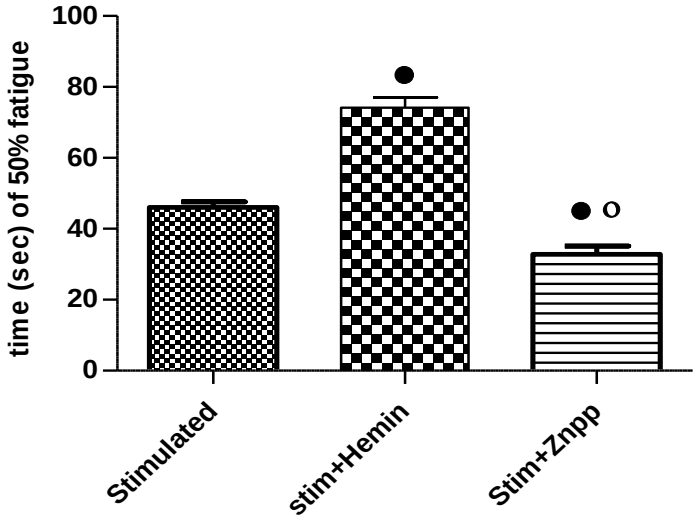

Figure ٢: Time (sec) required to achieve ٥٠٪ fatigue in different experiment groups. ●: Significant from stimulated group; ○: Significant from stim+hemin pretreated group, P ≤ ٠.٠٥. ZnPP: Zinc protoporphyrin

As regards the oxidant/ antioxidant status of the diaphragm, LF stimulation produced a state of oxidative stress in diaphragmatic muscle as evidenced by a significant increase in MDA level (as a marker of lipid peroxidation) reaching (٤٨.١٢± ٢.٠٤ nmol/gm tissue vs. ٣٤.٨٠ ±١.٢٥ nmol/gm tissue for the control group) with concurrent reduction in GSH content (as a marker of antioxidant availability) reaching (٢٤.١١±١.٣١ mg/gm tissue vs. ٣٢.٥٩±٢.٢٩ mg/gm tissue for the control group). Induction of HO-١ by hemin imroved the condition and alleviated the ROS-induced oxidative stress as evidenced by the significant decrease in MDA level (٣١.٧١±١.١٣ nmol/gm tissue) and restored the muscle GSH content (٣٤.٢٧±٢.٣٤ mg/gm tissue) nearly to the control level. On the other hand, inhibition of HO-١ by ZnPP produced the opposite effect recording the highest MDA (٥٧.٧٢±٣.٥١ nmol/gm tissue) and the lowest GSH (١٦.٣٨± ١.٢٤ mg/gm tissue) levels among all experimrntal groups (Figure ٣, ٤).

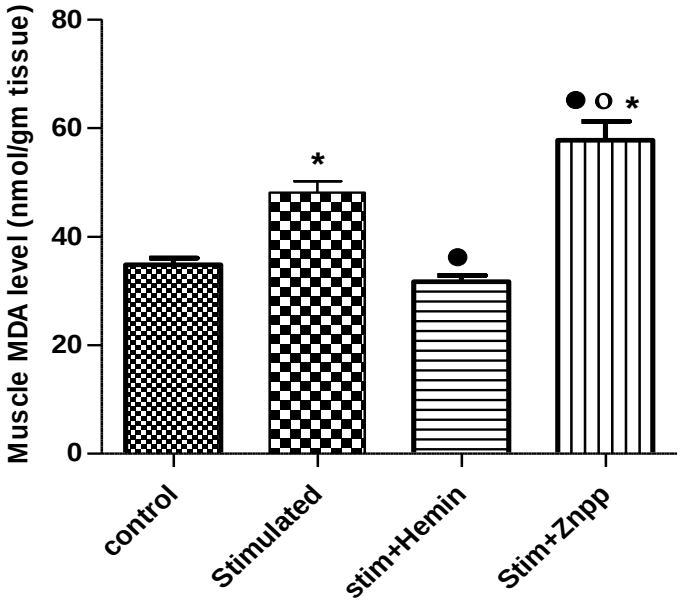

Figure ٣: Diaphragmatic malondialdehyde (MDA) level in different experiment groups. *: Significant from control group; ●: Significant from stimulated group; ○: Significant from stim+hemin pretreated group, P ≤ ٠.٠٥.

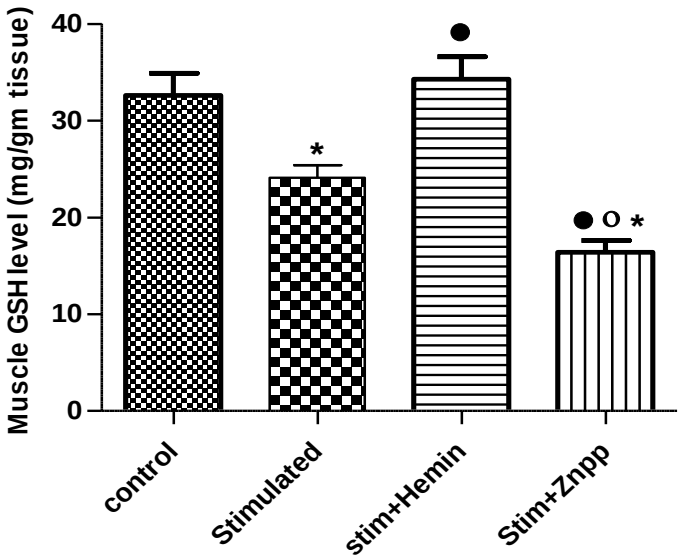

Figure ٤: Diaphragmatic reduced glutathione (GSH) level in different experiment groups. *: Significant from control group; ●: Significant from stimulated group; ○: Significant from stim+hemin pretreated group, P ≤ ٠.٠٥.

Figure (٥) shows the effect LF stimulation of the diaphragm with and without treatments on muscle NO level. LF stimulation of the rat diaphragm produced a significant rise in NO level (٨.٥٥±٠.٨٨ µmol/gm tissue vs. ٣.٢٩±٠.٣٦ µmol/gm tissue for the control group). In the stimulated+ hemin pretreated group, the muscle NO level was significantly decreased (٤.٠٩±٠.٣٣ µmol/gm tissue) while, ZnPP pretreatment to the stimulated group produced the highest increase in muscle NO level (١٤.٣٨±١.٣١ µmol/gm tissue) among all experimetal groups (Figure ٥).

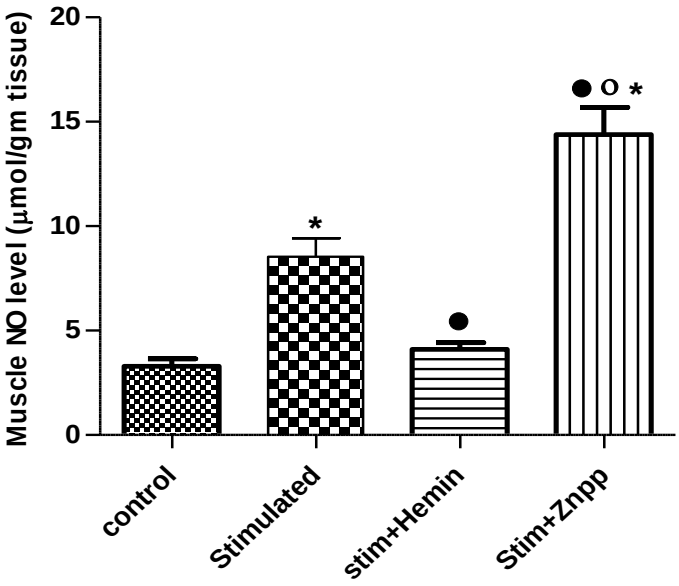

Figure ٥: Diaphragmatic nitric oxide (NO) level in different experiment groups. *: Significant from control group; ●: Significant from stimulated group; ○: Significant from stim+hemin pretreated group, $P \leq ٠.٠٥$.

Finally, as regards the glycogen stores of the muscle, LF stimuation of the diaphragm produced a significant reduction in glycogen stores (٠.٤١± ٠.٠٦ mg/gm tissue vs. ٠.٨٢±٠.٠٥ mg/gm tissue for the control group). Neither hemin nor ZnPP pretreatments to the stimulated groups were able to produce any significant change in muscle glycogen levels (٠.٦٢±٠.٠٧ and ٠.٥٩±٠.٠٤ mg/gm tissue, respedtively) from the corresponding non-treated group as well as the control group (Figure ٦).

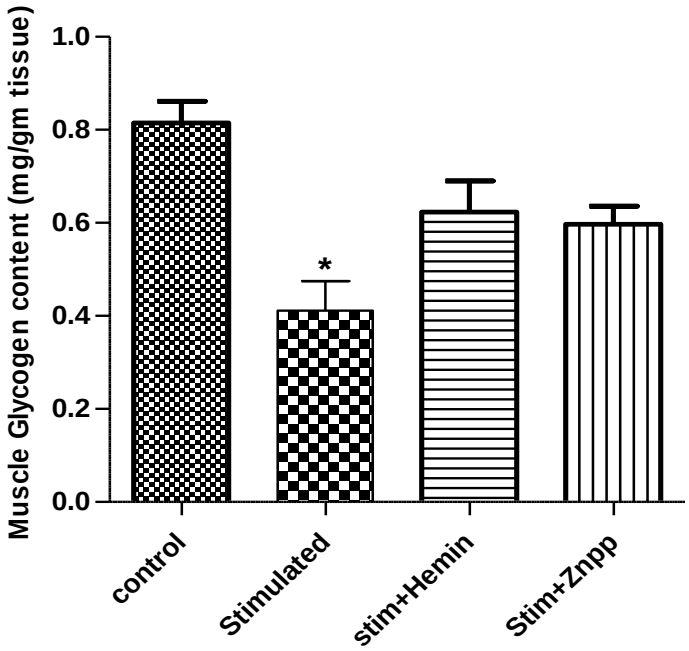

Figure ٦: Glycogen content in diaphragmatic muscle in different experiment groups. *: Significant from control group, $P \leq ٠.٠٥$.

Discussion

The importance of respiratory muscle fatigue, particularly of the diaphragm, has become well recognized in the last decade. If the diaphragm muscle fails, so does effective ventilation and tissue respiration[١]. It has become clear that the process of fatigue is a complex phenomenon with multiple mechanisms requiring different therapeutic approaches for management of such condition.

The present study represents an attempt to investigate the effect of induction of endogenous HO-١ system on the contractile response of isolated rat diaphragm during electrically-induced fatigue in order to evaluate its potential therapeutic value for treatment of diaphragmatic fatigue. Only, low frequency (LF) electrical stimulation was used for induction of diaphragmatic fatigue as it allows the muscle to take its needs of nutrients from the organ bath while being exposed to intermittent hypoxic conditions during subtetanic contra-ctions, in contrast to high frequency stimulation which is expected to expose the muscle to complete anaerobic conditions and deprivation of both blood flow and nutrients.

Basal HO-١ levels were found in the diaphragm muscle of the control group indicating that HO-١ protein is constitutively expressed, at low levels, in ventilatory muscles. This finding is in agreement with previous studies documenting the existence of HO-١ in mammalian muscles and cultured myocytes[١٢, ٢٨].

LF stimulation (٥ Hz) of experimental muscle produced a gradual and progressive decrease in muscle power (i.e. fatigue) that was recorded and stopped at ٥٠٪ decline of force. This progressive loss of power was accompanied with a significant increase in HO-١, NO and lipid peroxides along with a significant decrease in glycogen and GSH levels as copared with the control non-stimulated muscle.

Little is known about the factors that regulate HO expression in skeletal muscle fibers. In non-muscle cells, many conditions such as heat shock, ischemia, hypoxia, endotoxin, prion-flammatory cytokines, and hemin induce HO expression, particularly that of HO-١. In skeletal muscles, Essig et al. (١٩٩٧)[١٤] reported that exhaustive running or artificial stimulation induces muscle HO-١ mRNA expression which could explain the significant increase in HO-١ levels observed in the LF stimulated diaphragm.

Diaphragmatic HO-١ induction during muscle contraction could be mediated via increased production of NO and ROS; which are potent stimulators of HO-١ in skeletal muscle cells and other cell types[١٠,١٢]. In addition, HO-١ transcript is highly induced in skeletal muscle in vivo by strenuous exercise[١٤], a condition known to generate muscular oxidative stress with subsequent ROS production[١٦, ٢٩]. Findings which were confirmed in the present study by the concurrent increases in both NO and Lipid peroxides levels along with HO-١ in the LF stimulated muscle.

It has been shown that LF stimulation produces incomplete tetanic contraction with intermittent circulation, a property which favors ischemic/ reperfusion production of free radicals[٣٠]. In turn, nitric oxide synthase (NOS) activity is stimulated by free radicals according to[٣١] resulting in increased NO production; Findings which are in agreement with the results of the present study.

ROS have biphasic effects on the contractile function of skeletal muscle. Although low basal levels are essential for normal force production, excessive ROS production, such as during strenuous exercise, could lead to serious threat to the cellular antioxidant defense system, such as diminished reserve of antioxidant vitamins and GSH, and increased tissue susceptibility to oxidative stress, which is a major contributor in muscle fatigue[٢].

Yang et al. (٢٠٠٧)[٣٢] reported that HO-١ is one of the most critical cytoprotective mechanisms activated during cellular stress, exerting anti-oxidative and anti-inflammatory functions, modulating the cell cycle and maintaining the microcirculation. Thus, The ROS-induced increase in HO-١ level, during muscle stimulation, could be a physiological compen-satory mechanism to reduce the hazardous effects of these molecules on the contracting diaphragm[٢٢]. Findings which

were confirmed by the higher levels of MDA and lower levels of GSH along with accelerated onset of fatigue, observed in the LF stimulated group pretreated with the HO-١ inhibitor; ZnPP, indicating the important role of HO-١ system in maintaining adequate contractile performance during muscle activity.

Hemin pretreatment proved to be an inducer of HO-١ enzyme as evidenced by increased HO-١ level. These data are consistent with the findings of Vesely et al. (١٩٩٨) and Ndisang et al. (٢٠٠٣)[١٢, ٢١]. On the other hand, zinc proto-porphyrin (ZnPP) pretreatment; the HO-١ inhibitor, in the present study significantly reduced the diaphragmatic HO-١ level. These findings are in accordance with Esther et al. (٢٠٠٢)[٣٨].

The precise mechanism of HO-١ induction is not known. Many inducible genes are expressed in response to activation of various transcript-tional factors by a variety of inducing agents. The binding sites of many transcriptional factors have been identified in the promoter region of the HO-١ gene, and it appears that HO-١ expression is regulated by the activation and binding of such transcriptional factors to these regions. An increase in the binding of a number of transcriptional factors in response to hemin treatment[١٢], most significantly activator protein-٢ and nuclear transcription factor-κB.

In the present study, hemin pretreatment caused a profound improvement in muscle performance and delayed the onset of fatigue as evidenced by the significant increase in the duration required to achieve ٥٠٪ fatigue, and was accompanied with a significant increase in HO-١, GSH levels with a concomitant decrease in NO and MDA levels.

Several mechanisms could be responsible for the protective effect of the HO system against diaphragmatic fatigue. First, this protective role could be related to antioxidant properties of HO. Indeed, HOs are important intracellular antioxi-dant enzymes by virtue of their ability to degrade the prooxidant heme and generate biliverdin and bilirubin, two effective free radical scavengers[١١]. Bilirubin is probably the most abundant endogenous antioxidant in mammalian tissue[٣٣] and has been shown to efficiently scavenge peroxyl radicals in vitro and in vivo[٣٤, ٣٥].

An additional mechanism of protection by the HO system could be related to the down regulation of iNOS expression by degradation of heme, an essential cofactor for iNOS protein assembly and activity[١٣, ٣٦]. This hypothesis could be sustained by our findings showing the significant reduction in NO levels in hemin pretreated group.

Skeletal muscle, including the diaphragm, continually produces NO. At rest, NO production is usually low but it is much enhanced during muscle activity[٣٧]. NO plays a major role in promoting many important processes inside the skeletal muscle fibers such as glucose transport[٣٨], calcium [$Ca^{+٢}$] release from the sarcoplasmic reticulum[٣٩], blood flow[٤٠] and the defense against oxidative stress[٤١]. However, there is also evidence that excessive NO production primarily by the iNOS isoform may have deleterious effects on muscle contractile performance and sarcolemmal integrity[١٨].

The detrimental effects of nitric oxide reactive species, including NO and peroxinitrite, can be partially compensated by the induced expression of HO-١ as it offers strong antioxidant protection[٤٢]. Furthermore, increased CO production, one of the metabolic by products of HO-١, has the potential to inactivate iNOS, reducing the production of nitric oxide reactive species. The endpoints of this feedback loop would be reduced NO trans-formation to reduce oxidative stress and increased CO production to perform NO-equivalent signaling functions, such as stimulation of Guanyl cyclase (GC) and activation of K channels[٤٣]. Finally, increased production of ferritin related to HO induction could also be a contributing factor in the protective role of the HO pathway. Indeed, ferritin, an iron chelator, protects against cellular damage induced by liberation of free iron during oxidative stress[٤٤].

The extent of oxidative damage during muscle contraction is determined not only by the level of free radical generation, but also by the defense capacity of antioxidant system mainly

GSH availability[٤٥]. Laboratory studies have found that GSH supplementation improved muscle performance through inactivation of ROS[٤٦]. Furthermore, it has been reported that antioxidant supple-mentation inhibited fatigue development in human skeletal muscle during prolonged exercise[٤٧] indicating that the antioxidant status of skeletal muscle may be critical for maintaining proper muscle performance and protect muscle against fatigue.

It is possible that increased muscle antioxidant capacity may exert a protective effect on key ion transporting or ion channel proteins in muscle, including the Na^{+}-K^{+} ATPase enzyme, the SR-$Ca^{+٢}$ release channel (ryanodine receptor) and $Ca^{+٢}$-ATPase enzyme, each of which is deleteriously affected by increased ROS production[١٦, ٤٨].

In the present study, a significant reduction in muscle GSH content was observed in LF-stimulated muscle. Hemin pretreatment repleni-shed the muscle GHS stores and delayed the onset of fatigue. On the other hand, ZnPP pretreatment depleted the GSH store which was accompanied with significant acceleration of diaphragmatic fatigue suggesting a strong antioxidant effect of HO-١ induction during muscle contraction.

The antioxidant effect of HO-١ may be medi-ated via an increase in superoxide dismutase and catalase activities[١٧]. In addition, Matsumoto et al., (٢٠٠٦)[٣٥] and Datla et al., (٢٠٠٧)[٤٩] reported that CO and bilirubin from HO-١ have the ability to suppress ROS generation by interfering with NADPH oxidase activity and superoxide production, a major source of oxidative stress with subsequent preservation of GSH stores. The consequences of these former effects will be enhancement in the muscle antioxidant capacity along with improved muscle performance.

Because muscle contraction increases muscle metabolism by an order of magnitude and muscle fatigue is a consequence of muscle contraction, it has long been held that the metabolic cost of muscle activation is a primary factor in fatigue[٥٠]. Proposed mechanisms include alterations of the cellular environment by the buildup of metabolic by-products[٥] and depletion of substrate[٥١]. Muscle glycogen, is an important fuel for contracting skeletal muscle during prolonged exercise and glycogen depletion has been implicated in muscle fatigue[٥٢]

In the present study, LF stimulation caused a significant decrease in muscle glycogen content, as compared with the control non-stimulated group. Neither hemin nor ZnPP pretreatments could produce any significant changes on glycogen content compared to the stimulated non treated muscle. It could be explained that the dose regimen used in this study was acute single dose for both drugs which might not be sufficient to induce any detectable changes in glycogen levels in both groups. Further investingations, using chronic drug regimens, may be required to clarify the relationship between HO-١ and glycogen availability in contracting muscle.

A major finding in our study is that inhibition of HO activity by ZnPP evoked a significant impairment of diaphragmatic contractility and accelerated the onset fatigue which was accompanied with a significant increase in MDA and NO along with a significant reduction in HO-١ and GSH levels, adding more prove on the crucial role of HO-١ system in muscle performance especially during fatiguing contractions.

In conclusion, the present study demonstrates that muscle HO activity plays an important and protective role in attenuating muscle oxidative stress during fatiguing contractions. Pharmacological agents that upregulate HO-١ expression appear therefore as a promising strategy for preventing ROS-induced diaphra-gmatic dysfunction, as well as other pathological situations related to oxidative stress, such as diaphragmatic sepsis.

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