

## Effect of Modulation of Nitric Oxide on Rat Diaphragm Isometric Contraction and Fatigue Resistance in Hyperoxic and Hypoxic Conditions

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### ABSTRACT

The role of nitric oxide (NO) in ventilatory muscle contractile function has been under debate for several years. Moreover little is known about NO role under hypoxic conditions and the contribution of inducible nitric oxide synthetase (iNOS) in its generation. The aim of this study was to investigate the effect of NO on the force generation and fatigue resistance of the rat diaphragm muscle under acute in vitro hypoxia and to compare these effects to those under hyperoxic conditions. The effects of the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA), the NO scavenger hemoglobin, and the NO donor Na nitroprusside on the maximal twitch force ( $F_t$ ), submaximal tetanic force ( $F_{30}$ ), maximal tetanic force ( $F_0$ ) and isotonic endurance time under hyperoxic and hypoxic conditions were evaluated. Also diaphragm iNOS activity and nitrotyrosine level as a marker of peroxynitrite were measured. Inhibition of NO production and its scavenging using L-NMMA and Hb respectively had no effect on the diaphragm isometric contraction, the recruitment of its muscle fibers and fatigue resistance under hyperoxic condition. Hypoxia significantly reduced  $F_t$ ,  $F_{30}$ ,  $F_0$  and fatigue resistance with increased diaphragm iNOS activity and nitrotyrosine level. Hypoxia significantly reduced  $F_t$  and  $F_0$  in L-NMMA group compared to hyperoxic control one. While L-NMMA significantly increased  $F_{30}$  and decreased isotonic fatigability during hypoxia concomitant with reduction of iNOS activity and nitrotyrosine level compared to hypoxic control group. Moreover Hb induced similar results with additional significant improvement of  $F_t$  and  $F_0$ . The effects of L-NMMA were prevented by co-administration with the NOS substrate L-arginine. On the other hand, Excessive exogenous NO production by Na nitroprusside markedly decreased isometric contractile and fatigue properties during both hyperoxia and hypoxia. In conclusion the results of this study showed that the iNOS is activated in the diaphragm under hypoxia and may contribute partially to NO generation in hypoxia. Also they indicate that NO has a more prominent role in rat diaphragm under in vitro hypoxia compared to hyperoxic condition and that it contributes to the depression of force generation in the hypoxic diaphragm in vitro. Inhibition of NO generation during hypoxia may have a protective effect which could be a target in clinical conditions.

**Key words:** NO- diaphragm- iNOS-fatigue.

### INTRODUCTION

Hypoxia is a common feature in several respiratory diseases, including

chronic obstructive pulmonary disease, acute respiratory distress syndrome, and pneumonia. Moreover during any exacerbation of these

diseases, acute hypoxia may occur. Under hypoxic conditions both in vitro and in vivo, the diaphragm is tonic and isometric force generation is impaired and fatigability is increased<sup>1</sup>. Heunks et al<sup>2</sup> showed that under resting conditions, hypoxia mainly affects isometric contractile properties with no effect on shortening velocity. Loss of the diaphragm isometric contractile properties may lead to loss of approximately 70 % of its respiratory function since its curvature will be lost.

However, the cellular mechanisms of hypoxia-induced impairment of contractile performance are incompletely understood, but oxidative and nitrosative stress could be at play<sup>3</sup>. Nitric oxide (NO), a highly reactive second messenger, is produced by NO synthetases (NOSs). The activity of the constitutive NOS isoforms: neuronal and endothelial nitric oxide synthetases (nNOS and eNOS respectively) is primarily regulated by intracellular Ca<sup>2+</sup> concentration<sup>4</sup>. Hypoxia may impair intracellular Ca<sup>2+</sup> homeostasis, which in turn could increase NO production by NOS<sup>5</sup>. There are now several other studies demonstrating increased iNOS expression under hypoxic conditions in myocytes, in pulmonary endothelial cells<sup>6</sup> and in macrophages<sup>7</sup>. Many studies argued the increase of iNOS in diaphragm hypoxia and stated that it is only induced after exposure to bacterial lipopolysaccharides (LPS) or inflammatory cytokines<sup>8</sup>. However the excessive NO production by these agents primarily by the iNOS isoform had deleterious effects on muscle contractile performance.

It has been proposed that many of the deleterious effects of NO are mediated by the formation of peroxynitrite<sup>9</sup>, the reaction product of NO and superoxide anions (O<sub>2</sub><sup>-</sup>)<sup>10</sup>. It can result in cellular injury and cell death by causing oxidation of sulphydryls, lipid peroxidation, and nitration of tyrosine residues in protein to form nitrotyrosine<sup>11</sup>. The contribution of peroxynitrite; the potent nitrating and oxidizing agent in muscle impaired force generation induced by hypoxia is still under investigation.

The aim of the present study was to investigate: 1) the effect of NO modulation on force generation and fatigue resistance of the rat diaphragm under in vitro hyperoxic and hypoxic conditions. 2) The stimulatory effect of hypoxia on iNOS activity as one of the NOS isoforms. 3) The hypothesis that the decrease of NO generation and subsequently of nitrotyrosine as a marker for peroxynitrite formation will improve the hypoxic outcome.

## MATERIAL & METHODS

60 Male rats (150-180 g) were used. The rats were anesthetized with pentobarbital sodium (70 mg/kg body weight) injected intra peritoneally. Diaphragm bundles were prepared as previously described<sup>12</sup>. Briefly, after a combined thoracotomy and laparotomy, the diaphragm and adherent lower ribs were quickly excised and were immediately submersed in a cooled oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>) Krebs solution at pH = 7.4. One rectangular strip (about 2.5 mm wide) was dissected from the central costal region of the right and

left hemidiaphragms parallel to the long axis of the muscle fibers. Silk sutures were tied firmly to both ends and were mounted vertically in a separate tissue bath containing Krebs solution, maintained at 26°C.

The muscle origin was tied to a glass hook fixed to the bottom of the tissue bath. The insertion of the muscle bundles at the costal margin was attached to a metal clamp. The suture attached to the central tendon was connected to the lever arm and an isometric force transducer. The muscle was stimulated directly using platinum plate electrodes placed in close apposition to either side of the muscle.

Stimuli were applied using a pulse rate of 0.2ms and train duration of 400ms and were delivered from an electronic square wave stimulator (CF-Palmer-London). Data acquisition and storage of the amplified signal were performed using the computer (Intracept TSC-Panasonic- Japan). Muscle preload force was adjusted until the optimal fiber length ( $L_0$ ) for the maximal twitch force ( $F_t$ ) was achieved.

**Contractile Measurements:** After 15 min of thermoequilibration during hyperoxia (95% O<sub>2</sub> and 5% CO<sub>2</sub>), baseline  $F_t$ , submaximal tetanic force ( $F_{30}$ ) and maximal tetanic force ( $F_0$ ) measurements were determined (at 1, 30 and 100 Hz respectively in trains of 250ms duration) with 2 min intervals. Subsequently, the perfusion of the tissue bath was either maintained with hyperoxia or switched to hypoxia (95% N<sub>2</sub> and 5% CO<sub>2</sub>; 26°C); the Krebs solution was changed to the experimental conditions (according to treatment

groups below). After 60 min of incubation, the previous forces were remeasured and presented as percentages of initial values.

The transducer and computer systems were then set for force control (isotonic mode) to determine isotonic fatigue as described by Zhu et al <sup>1</sup>. The load clamp was set for maximal power output (~33.3%  $P_o$ ), and the muscle was stimulated at 100 Hz (330 ms train duration) every 2 s. Stimulations continued until no muscle shortening could be observed, and this period was defined as the isotonic endurance time.

According to the either hyperoxic or hypoxic incubation as well as to the differently used incubation medium, the rats' diaphragms were divided into the following groups (n= 6/group):

- **Hyperoxic control group:** Diaphragm muscle strip was incubated in oxygenated Krebs solution (95% O<sub>2</sub>-5% CO<sub>2</sub>) for 60 min.
  - **Hypoxic control group:** Diaphragm muscle strip was incubated in hypoxic Krebs solution (95% N<sub>2</sub> and 5% CO<sub>2</sub>) for 60 min.
  - **Hyperoxic L-NMMA group:**
  - **Hypoxic L-NMMA group:**
- In the last two groups,  $N^G$ -monomethyl-L-arginine (L-NMMA) (AcOH, Calbiochem, Breda, The Netherlands) in a concentration of 10  $\mu$ M was added to Krebs solution and the diaphragm was incubated for 60 min<sup>13</sup> under hyperoxic and hypoxic conditions respectively. This concentration has been shown to decrease the release of NO by 68% in rat extensor digitorum longus (EDL) muscle<sup>13</sup>

and the NOS activity in diaphragm is lower than that in EDL muscle (5.2 compared to 13.5 pmol/ min/mg protein respectively)<sup>14</sup>.

- Hyperoxic L-NMMA+ L-arginine group
- Hypoxic L-NMMA+ L-arginine group:  
30 µM of L-arginine; the NOS substrate (Sigma Chemical) were used to exclude the NOS-independent effects of L-NMMA, because L-arginine in a threefold concentration completely reverses the effect of L-NMMA on endothelium-dependent contractions *in vitro*<sup>15</sup>.
- Hyperoxic Hb group
- Hypoxic Hb group

Hemoglobin (Hb) in a dose of 150 µM (bovine, Sigma Chemical, Zwijndrecht, the Netherlands)<sup>14</sup>; the NO scavenger was used to ascertain that the effects of L-NMMA were indeed mediated by endogenous NO. Hb was incubated with the diaphragm for 60 min under either hyperoxic or hypoxic conditions respectively.

- Hyperoxic Na nitroprusside group
- Hypoxic Na nitroprusside group  
1mM of Na nitroprusside the NO donor; (Calbiochem, Breda, The Netherlands), was incubated with the diaphragm for 60 min under either hyperoxic or hypoxic conditions<sup>14</sup>. These groups were performed to determine whether the contractile properties were also sensitive to exogenous NO.

At the end of the experiment, the pH and the partial pressure of oxygen ( $P_{O_2}$ ) of the Krebs solution in the tissue baths were measured. Then, the length and dry mass of each

diaphragm strip were measured. Cross sectional area was calculated by dividing diaphragm strip mass (grams) by strip length (centimeters) times specific density.

Part from each diaphragm strip was taken to measure diaphragm iNOS activity and nitrotyrosine level.

#### **Measurement of diaphragm iNOS activity:**

Materials were purchased from Sigma Chemical (St. Louis, MO). Frozen diaphragm muscle tissues from each animal were homogenized in six volumes (wt/vol) of homogenization buffer (pH 7.4) composed of 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml phenylmethylsulfonyl fluoride, 0.32 mM sucrose, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 10 µg/ml pepstatin A. The crude homogenates were centrifuged at 4°C for 15 min at 10,000 rpm. 50 µl of the supernatant was added to 10 ml prewarmed (37°C) tubes containing 100 µl of reaction buffer of the following composition: 50 mM KH<sub>2</sub>PO<sub>4</sub>, 60 mM valine, 1.5 mM NADPH, 10 mM FAD, 1.2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1 mg/ml bovine serum albumin, 1 µg/ml calmodulin, 10 µM tetrahydrobiopterin, and 25 µl of 120 µM stock L-[2,3-<sup>3</sup>H] arginine (150-200 cpm/pmol). The samples were incubated for 30 min at 37°C, and the reaction was terminated by the addition of 500 µl of cold (4°C) stop buffer (pH 5.5, 100 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 12 mM EDTA). To obtain free L-[<sup>3</sup>H] citrulline for the determination of enzyme activity, 2 ml of Dowex 50w resin (8% cross-

linked,  $\text{Na}^+$  form) were added to eliminate excess L-[2,3- $^3\text{H}$ ]arginine. The supernatant was removed and examined for the presence of L-[ $^3\text{H}$ ]citrulline by liquid scintillation counting. Enzyme activity was expressed in  $\mu\text{mol}$  of L-citrulline produced per minute per milligram of total protein. Protein was measured by the Bradford technique with bovine serum albumin as standard. To differentiate between iNOS activity, which is independent of  $\text{Ca}^{2+}$  and calmodulin, and constitutive NOS isoform activity ( $\text{Ca}^{2+}$  and calmodulin dependent), NOS activity was also measured in the presence of 1.5 mM each ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and EDTA, which replaced  $\text{CaCl}_2$  and calmodulin in the reaction buffer, and in the presence of 1 mM  $N^{\text{G}}$ -nitro-L-arginine methyl ester (NOS inhibitor).  $\text{Ca}^{2+}$ /calmodulin-independent iNOS activity was calculated as the difference between samples assayed in the presence of EGTA/EDTA and in the presence of  $N^{\text{G}}$ -nitro-L-arginine methyl ester<sup>16</sup>.

#### **Nitrotyrosine Measurement:**

Peroxynitrite formation in the diaphragm was evaluated by detection of nitrotyrosine residues using Western immunoblotting with a monoclonal anti-nitrotyrosine antibody of high specificity (StressGen Biotechnologies, Victoria Canada), as discussed previously<sup>17</sup>. Crude muscle homogenate proteins (10  $\mu\text{g}$ ) were heated for 5 min at 95°C, then briefly about 30  $\mu\text{l}$  of homogenate supernatant was separated on 10% sodium dodecylsulfate- SDS- polyacrylamide gel electrophoresis at (200 V for 1 h).

High- and low-molecular-weight standards of nitrotyrosine (Upstate Biotechnology) were run in parallel as positive controls and Proteins were transferred to nitrocellulose membrane using a semi-dry transfer apparatus. Membranes were subsequently incubated with primary monoclonal antibodies raised against nitrotyrosine in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline with 1% Tween 20 (PBST). After three 10-min washes with PBST on a rotating shaker, the membranes were incubated with a secondary antibody (polyclonal anti-mouse IgG horseradish peroxidase conjugated) for 1h at room temperature. The membranes were finally washed twice for 10 min with PBST. Afterward, protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences Europe). The blots were scanned with an imaging densitometer, and optical densities (OD) of positive nitrotyrosine protein bands were quantified using documentation system supplied by (Biometra, Germany). Total nitrotyrosine OD was calculated for each samples by adding the OD of individual positive protein bands.

#### **Statistical Analysis:**

The results were analyzed using SPSS computer software package, version 10.0 (Chicago-IL, USA)<sup>18</sup>. Data were presented as mean  $\pm$  S.D. Differences among the parameters of the different groups were compared by one-way ANOVA. The results were considered statistically significant at  $P \leq 0.05$ .

## RESULTS

### Verification of tissue bath hypoxia and strip dimensions:

Perfusion of the tissue baths with the hypoxic gas mixture significantly reduced  $P_0_2$  in the Krebs solution to about 54 mmHg compared to 639 mmHg in the hyperoxia groups ( $P < 0.005$ ). The pH was 7.3 in the hypoxic groups and in the hyperoxic groups was 7.42 ( $P > 0.05$ ). Diaphragm muscle strip dimensions were not significantly different among the experimental groups ( $P > 0.05$ ). Average muscle strip weight was  $27.2 \pm 0.6$  mg and strip length at  $L_0$  was  $18.3 \pm 0.6$  mm.

### Effect of NO modulation in the hyperoxic groups:

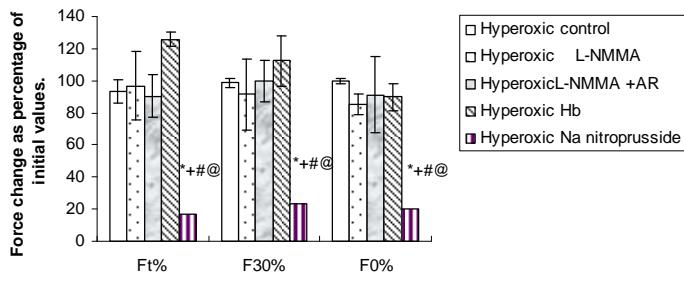
As revealed in figure 1, there was no significant differences in the means of maximal twitch force ( $F_t$ ), submaximal tetanic force ( $F_{30}$ ) and maximal tetanic force ( $F_0$ ), (expressed as percentages of initial values)

between the hyperoxic control group, that incubated with L-NMMA, that incubated with L-NMMA + L-Arginine and that incubated with Hb. These data show that inhibition of Nitric Oxide production and action has no effect on isometric diaphragm contraction and the recruitment of its muscle fibers under hyperoxic conditions.

While incubation of the hyperoxic diaphragm with Na nitroprusside, the NO donor induced a significant decrease in its  $F_t$ ,  $F_{30}$  and  $F_0$  percentages compared to similar parameters respectively in the other previous hyperoxic groups (figure 1).

This indicates that excessive exogenous NO production can reduce the diaphragm isometric force contraction and can inhibit the recruitment of its muscle fibers in response to different current frequencies under hyperoxic conditions.

**Figure 1: Changes in maximal diaphragm twitch force ( $F_t$ ), submaximal tetanic force ( $F_{30}$ ) and maximal tetanic force ( $F_0$ ) expressed as percentage of initial values after 1 hour of incubation in different hyperoxic media.**



\*: Significant compared to hyperoxic control group.

+: Significant compared to hyperoxic L-NMMA group.

#: Significant compared to hyperoxic L-NMMA+L-arginine (AR) group.

@: Significant compared to hyperoxic Hb group.

As shown in table 1, the endurance time showed no significant difference upon incubation of the diaphragm with L-NMMA, L-NMMA + L-arginine and with Hb under hyperoxic conditions as compared to the hyperoxic control group and as compared to each others. This indicates that inhibition of NO production and action has no effect on the diaphragm fatigue resistance under hyperoxic conditions.

While incubation of the hyperoxic diaphragm with Na nitroprusside decreased its endurance time significantly compared to that incubated with L-NMMA and to that incubated with Hb and insignificantly compared to that of the hyperoxic control diaphragm and to that incubated with L-NMMA+ L-arginine. These data indicate that excessive exogenous NO production can accelerate diaphragm fatigue under hyperoxic conditions.

Measuring the diaphragm nitrotyrosine level at the end of the experiment revealed insignificant differences between the different hyperoxic groups.

The iNOS activity was significantly decreased in the hyperoxic group incubated with L-NMMA compared to the other hyperoxic groups. Incubation of the diaphragm with Hb increased significantly its iNOS expression compared to the hyperoxic control group, to the L-NMMA group and to that incubated with L-NMMA+ L-arginine groups and insignificantly to the Na nitroprusside incubated group. These results indicate that scavenging NO is accompanied by upregulation of diaphragm iNOS activity under hyperoxic conditions.

Incubation with Na nitroprusside insignificantly changed its iNOS activity compared to the other hyperoxic groups.

**Table 1: Endurance time, nitrotyrosine level and inducible Nitric Oxide synthase (iNOS) activity in the diaphragm of the different hyperoxic groups**

|   | Hyperoxic control group | Hyperoxic L-NMMA group | Hyperoxic L-NMMA + L Arginine group | Hyperoxic Hb     | Hyperoxic Na nitroprusside |
|---|-------------------------|------------------------|-------------------------------------|------------------|----------------------------|
| <b>Endurance time (seconds).</b>                  | 59.33±2.06              | 74.6± 21.8             | 54± 1                               | 78.66± 14.6      | 43.8± 6.9+ @               |
| <b>Nitrotyrosine level (ng/mg tissue protein)</b> | 0.76±0.16               | 0.66±0.3               | 0.85±0.4                            | 1.17±0.47        | 0.81±0.51                  |
| <b>iNOS activity (μmol/mg tissue protein/min)</b> | 103.9±10.7              | 80.98±2.9*             | 104.8±9.1+                          | 163.8±26.9 * + # | 125.7±10.7+                |

\*: Significant compared to hyperoxic control group at P≤0.05.

+: Significant compared to hyperoxic L-NMMA group at P≤0.05.

#: Significant compared to hyperoxic L-NMMA+L-arginine group at P≤0.05.

@: Significant compared to hyperoxic Hb group at P≤0.05.

*Effect of hypoxia on the different groups:*

The effect of hypoxic incubation of the diaphragm is revealed by table 2 as the hypoxic control group showed a significant decrease in its force generation at all current frequencies (i.e:  $F_t\%$ ,  $F_{30}\%$  and  $F_0\%$ ) and in its endurance time compared to the hyperoxic control group highlighting the deleterious effects of hypoxia.

Also it significantly increased the diaphragm nitrotyrosine level and iNOS activity compared to the hyperoxic group indicating increased NO production.

Hypoxic incubation of the diaphragm concomitant with L-NMMA significantly decreased its  $F_t\%$  and  $F_0\%$ , with insignificant change in its  $F_{30}\%$ , endurance time, and diaphragm nitrotyrosine level and iNOS activity as compared to the hyperoxic L-NMMA group.

The effect of hypoxia was apparent again after inhibition of the L-NMMA effect with L-arginine with significant decrease of  $F_t\%$ ,  $F_{30}\%$ ,  $F_0\%$  and endurance time with a significant increase in diaphragm nitrotyrosine level and iNOS activity

compared to hyperoxic L-NMMA+L-arginine group as revealed in table 2.

To prove the effects mediated by NO, the hypoxic L-NMMA group was compared to hypoxic control group as revealed in table 2 also in figures 2, 3, 4 and 5. Incubation of the diaphragm with L-NMMA had no significant effect on its  $F_t$  and  $F_0$  percentages of initial values during hypoxia when compared to those of the hypoxic control and L-NMMA+L-Arginine groups.

While incubation with L-NMMA increased significantly the diaphragm  $F_{30}\%$ , the endurance time and decreased both the diaphragm nitrotyrosine level and iNOS activity during hypoxia compared to that of the hypoxic control and hypoxic L-NMMA+L-Arginine incubated groups.

These results demonstrate that the diaphragm submaximal contraction and fatigue can be more resistant to hypoxia under NO synthetase inhibition and that the upregulating effect of hypoxia on diaphragm nitrotyrosine level and iNOS activity is abolished by inhibition of NO production.

**Table 2: Changes in diaphragm isometric contraction forces expressed as percentages of initial values, endurance time, diaphragm nitrotyrosine level and iNOS activity in hypoxic control, hypoxic L-NMMA and hypoxic L-NMMA +L-arginine groups compared to their hyperoxic control groups**

|   | Hyperoxic control | Hypoxic control | Hyperoxic L-NMMA | Hypoxic L-NMMA | Hyperoxic L-NMMA +L-arginine | Hypoxic L-NMMA+L-arginine |
|---|-------------------|-----------------|------------------|----------------|------------------------------|---------------------------|
| <b>Ft%</b>  | 93.28±7.3         | 47.1±18.8●      | 96.93±21         | 64.4±11.9●     | 90.5±13.4                    | 41.9±13.9●                |
| <b>F30%</b>   | 98.76±2.8         | 57.81±17●       | 91.56±22         | 95±21.2*       | 99.8±13                      | 50.7±13.5● +              |
| <b>F0%</b>  | 99.67±1.9         | 47.53±8.1●      | 84.93±6.4        | 45.1±5.9●      | 90.9±23.8                    | 43.89±16.9●               |
| <b>Endurance time.<br/>(seconds)</b>                  | 59.3±2.06         | 42.7±2.2●       | 74.6±21.8        | 62±1.6*        | 54±1                         | 41.33±6.02●+              |
| <b>nitrotyrosine<br/>(ng/mg tissue protein)</b>       | 0.76± 0.16        | 2.27± 0.63●     | 0.66± 0.3        | 0.77± 0.3*     | 0.85± 0.4                    | 1.63± 0.32●+              |
| <b>iNOS activity<br/>(μmol/mg tissue protein/min)</b> | 103.9±10.7        | 186.2±10.14●    | 80.98±2.9        | 93.5± 23.4*    | 104.8±9.1                    | 185.6± 9.8● +             |

●: Significant compared to its hyperoxic group at P≤0.05.

\*: Significant compared to hypoxic control group.

+: Significant compared to hypoxic LNMMMA group.

Table 3 shows that hypoxia in the presence of Hb incubation of the diaphragm did not significantly change its F<sub>t</sub>, F<sub>30</sub>, F<sub>0</sub> percentages of initial values, its endurance time as well as its nitrotyrosine level and its

iNOS activity compared to similar parameters in the Hb hyperoxic group. These results highlight again the beneficial effect of decreasing NO level during hypoxia.

**Table 3: Changes in diaphragm isometric contraction forces, endurance time, and diaphragm nitrotyrosine level and iNOS activity in hypoxic groups incubated with hemoglobin (Hb) and with Na nitroprusside compared to their hyperoxic groups.**

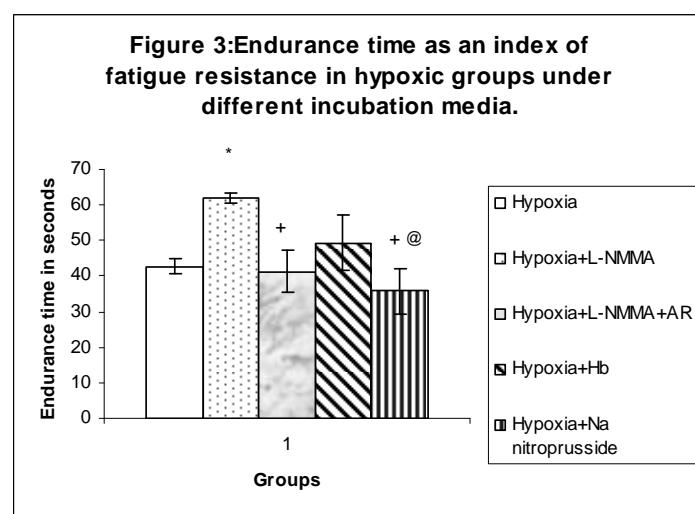
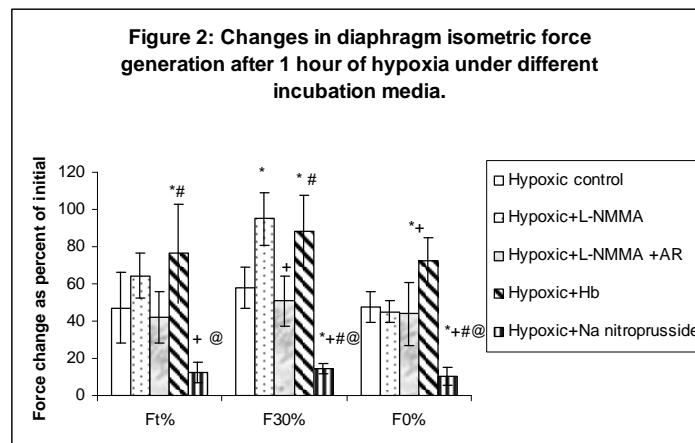
|   | Hyperoxic Hb group | Hypoxic Hb group | Hyperoxic Na nitroprusside group | Hypoxic Na nitroprusside group |
|---|--------------------|------------------|----------------------------------|--------------------------------|
| <b>F<sub>t</sub>%</b>                             | 125.72 ±54.7       | 76.4±26.5        | 16.03 ±3.3                       | 11.8±5                         |
| <b>F<sub>30</sub>%</b>                            | 112.35 ±39.2       | 88.39 ±19.3      | 26.4 ±15.1                       | 14.6 ±2.7                      |
| <b>F<sub>0</sub>%</b>                             | 89.75 ±20.4        | 72.23 ±12.8      | 19.8 ±9 .6                       | 10.39 ±4.6●                    |
| <b>Endurance time (seconds).</b>                  | 78.66±14.6         | 49.33 ±7.8       | 43.16 ±6.4                       | 35.6 ±6.3●                     |
| <b>nitrotyrosine (ng/mg tissue protein)</b>       | 1.17± 0.47         | 0.77± 0.4        | 0.81± 0.1                        | 1.61± 0.3●                     |
| <b>iNOS activity (μmol/mg tissue protein/min)</b> | 163.8± 26.93       | 195.4± 23.03     | 130.6± 15.81                     | 146.3± 26.1                    |

●: Significant compared to its hyperoxic group at P≤0.05.

To prove that these beneficial effects were mediated by scavenging NO, the hypoxic Hb incubated group was compared to other hypoxic groups. Figure 2 reveal a significantly increased F<sub>t</sub>% and F<sub>30</sub>% of the hypoxic Hb group compared to control hypoxic and to hypoxic L-NMMA+ L-arginine groups and F<sub>0</sub>% compared to both the control hypoxic and to hypoxic +L-NMMA groups. These data indicate that scavenging of NO can improve the diaphragm maximal twitch force, the submaximal and the maximal recruitment of its muscle fibers under hypoxic conditions.

Incubation of the diaphragm with Hb increased its endurance time during hypoxia compared to the

hypoxic Na nitroprusside incubated group however insignificantly compared to all other groups including hypoxic control group as observed in figure 3. While it significantly decreased its nitrotyrosine level in response to hypoxia compared hypoxic control and hypoxic L-NMMA + L-arginine groups (figure 4). The diaphragm iNOS activity was still significantly increased in Hb incubated group following hypoxia compared to hypoxic L-NMMA and Na nitroprusside groups and insignificantly changed from the hypoxic control and hypoxic L-NMMA + L-arginine groups (figure 5).



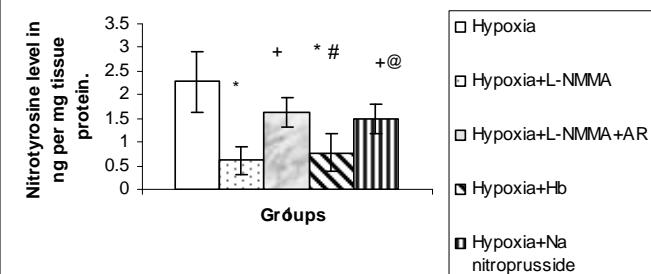
\*: Significant compared to hypoxic control group.

+: Significant compared to hypoxic L-NMMA group.

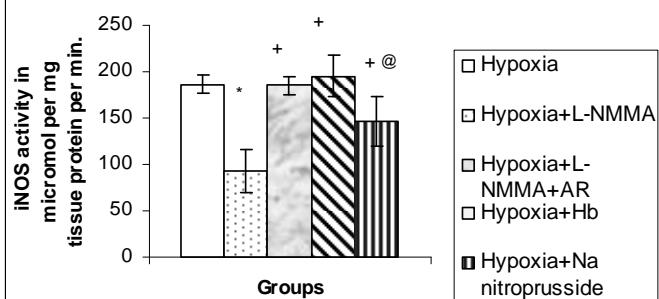
#: Significant compared to hypoxic L-NMMA+L-arginine group.

@: Significant compared to hypoxic Hb group.

**Figure 4 : Nitrotyrosine level as a marker of peroxynitrite in hypoxic diaphragm tissue under different incubation media**



**Figure 5: Diaphragm iNOS activity in hypoxic diaphragm tissue under different incubation media.**



\*: Significant compared to hypoxic control group.

+: Significant compared to hypoxic L-NMMA group.

#: Significant compared to hypoxic L-NMMA+L-arginine group.

@: Significant compared to hypoxic Hb group.

On the other hand, hypoxic exposure of the Na nitroprusside incubated diaphragm decreased significantly its  $F_t\%$  and its endurance time, while it increased significantly its nitrotyrosine level and insignificantly iNOS activity compared to Na nitroprusside hyperoxic group. Both  $F_t\%$  and  $F_{30}\%$  were also decreased by hypoxia however insignificantly as they were already reduced by Na nitroprusside in the hyperoxic group.

Compared to hypoxic groups, Na nitroprusside, induced a significant decrease in hypoxic diaphragm  $F_t\%$  compared to that of hypoxic L-NMMA and hypoxic Hb group, however, insignificantly to hypoxic control and hypoxic+ L-NMMA+ L-arginine groups. Moreover there was a significant decrease in its  $F_{30}\%$  and  $F_0$  percentages compared to similar parameters in all the other hypoxic groups including the hypoxic control group as shown in figure 2. These

results indicate that excessive exogenous NO production aggravates the hypoxia-induced impairment of the diaphragm force generation.

Also Na nitroprusside significantly reduced endurance time and significantly increased diaphragm nitrotyrosine level compared to L-NMMA and Hb hypoxic groups (figure 3, 4, 5).

## DISCUSSION

The role of NO in the physiology of skeletal muscle has been controversial for several years. The present study revealed that inhibition of NO production (as in L-NMMA group) had no effect on the diaphragm isometric contraction, the recruitment of its muscle fibers and fatigue resistance under hyperoxic condition which may indicate minimal role of the endogenously produced NO under hyperoxic conditions in the rat diaphragm.

These results are similar to those detected by Zhu et al<sup>19</sup>, although these authors did not measure NOS activity, but they found similar results on diaphragm isometric contraction under hyperoxic incubation. However, these authors reported an L-NMMA dose-dependent reduction of the rat diaphragm contractile force and suggested that some amount of NO is needed to maintain diaphragm muscle function under hyperoxic conditions.

On the other hand they are in odd with Reid et al<sup>20</sup>, who found an increase in diaphragm F<sub>30%</sub> by low concentration of L-NMMA (10 µMol similar to that in the present study) during hyperoxia and suggested that

NO inhibits excitation-contraction coupling in unfatigued muscle.

Another study showed that normal muscle contraction induces a decline in muscle NOS activity, which if localized to the mitochondria might represent a compensatory mechanism through which muscle contractility and mitochondrial function are protected from the inhibitory influence of NO<sup>21</sup>.

The L-NMMA used in this study is an amino acid analogue of L-arginine and an established inhibitor of NO generation in various tissues, including skeletal muscle cells in vitro<sup>22</sup>. It competes with arginine for binding on NOS molecules and equally inhibits all three isoforms of NOS (eNOS, nNOS and iNOS)<sup>23</sup>. The L-NAME also inhibits L-arginine transport into cultured cells<sup>24</sup>. The decrease in iNOS activity in the hyperoxic L-NMMA group of this study was not accompanied by a significant decrease in NO production as indicated by the nitrotyrosine level compared to the other hyperoxic group denoting that iNOS may not play an important role under hyperoxic conditions.

These results are in line with Hirschfield et al<sup>25</sup>, who found that iNOS deficiency had no effect on NO production and contractile function of hyperoxic rat skeletal muscle in vitro, and suggested that this process is not regulated by iNOS isoform. These authors suggested that the association of the iNOS isoform with muscle mitochondria and the capacity of NO to inhibit mitochondrial respiration might denote that iNOS may regulate mitochondrial function. They also suggested that iNOS might primarily

regulate other aspects of muscle function as muscle development, vascular tone, glucose uptake and creatine kinase activity.

Hemoglobin is known to bind avidly and to nullify rapidly the effects of NO and acts as a scavenger<sup>26</sup>. Thus it was used as further evidence that the observed responses were NO mediated. Similarly it had no effect on the diaphragm isometric contraction, the recruitment of its muscle fibers and fatigue resistance under hyperoxic condition. However it significantly increased iNOS activity which is related to its action. Hb does not act directly on iNOS activity but since it does not enter cells and scavenges NO extracellularly, it subsequently, scavenges the intracellular muscle cells generated NO by creating a diffusion gradient of NO out of the cell "sink"<sup>26</sup>. This may stimulate iNOS activity to compensate for the scavenged NO since NO usually adjust its own synthesis by feedback inhibition to restrict its production<sup>27</sup>. Thus it is suggested that diaphragm muscle cells might be able to adjust their endogenous NO concentration and that some amount of NO may be important for maintaining muscle function under hyperoxic condition.

On the other hand, excessive exogenous NO production (as evident in Na nitroprusside incubation group) reduced the diaphragm  $F_t$ ,  $F_{30}$  and  $F_0$  percentages under hyperoxic conditions compared to all the other groups. Also it decreased its fatigue resistance compared to the L-NMMA and Hb groups. However, since in normal skeletal muscle fibers reactive oxygen species (ROS) are produced at

relatively low levels, thus excessive exogenous NO production was not accompanied by increased nitrotyrosine production under hyperoxic conditions. The released NO from Na nitroprusside was not scavenged by  $O_2$  to produce nitrogen dioxide<sup>28</sup>, but it is likely that it diffused rapidly in solution through the cells and tissues to impair the diaphragm muscle contraction. Since the submaximal tetanic contraction ( $F_{30}$ ) is related to sarcoplasmic reticulum (SR)  $Ca^{2+}$  release and  $Ca^{2+}$  sensitivity of cross-bridge recruitment, while the maximal tetanic contraction ( $F_0$ ) is correlated with the number of active cross-bridges formed by myosin heavy chain heads that attach to actin<sup>29</sup>; thus, the reduction of both by excessive exogenous NO production may denote its involvement in these processes.

Hypoxic incubation of the rat diaphragm in this work markedly impaired its force generation, including  $F_t$ ,  $F_{30}$  and  $F_0$  compared to the hyperoxic group. These data are in line with previous studies of hypoxia on isometric diaphragm contractility in vitro and imply that hypoxia impairs cross-bridge recruitment or reduce force per cross bridge<sup>30</sup>. Such effect may occur for several reasons, including, down regulation of mitochondrial enzymes, decrease  $Ca^{2+}$  release from the SR and reduced sarcolemmal excitability<sup>31</sup>.

Furthermore, hypoxia had detrimental effects on the isotonic fatigue as indicated by a decline in isotonic endurance time which is dependent on the generated force and the shortening velocity. Since the force was clamped at 33.3% of  $P_o$ ,

force generation was constant during the entire fatigue protocol; thus, the reduction in endurance time is the result of a reduction in the shortening velocity. As previously mentioned, the shortening velocity is correlated with myosin ATPase activity<sup>32</sup>; thus it is likely that ATPase activity is also decreased during hypoxia.

The causative agents underlying these changes during hypoxia may be related to increased production of free radicals<sup>33</sup>. The involvement of NO in these changes is also at play. Increasing evidence show that biological activities of NO and ROS are strongly interdependent, since their redox- derivatives compete for the same metal centre and thiol groups on target proteins<sup>34</sup>. Moreover NO can react with mitochondria-derived superoxide anions generated during hypoxia to form peroxynitrite<sup>35</sup>, which reacts with all major classes of molecules and can cause toxicity independent of NO or superoxide<sup>36</sup>. Studies on exogenous peroxynitrite has shown to decrease  $F_0$  of rat diaphragm single fibers without alterations in submaximal force generation<sup>36</sup>. Therefore, increased levels of peroxynitrite might act as a mediator in hypoxia-induced contractile dysfunction. In this study, there was a significant increase in both diaphragm iNOS activity and nitrotyrosine as a marker of peroxynitrite level in hypoxia denoting increased production of both NO and superoxide and a more prominent role of iNOS in hypoxia compared to hyperoxia.

The mechanism underlying the increase in NO production under hypoxia is not known. Previous

studies showed that hypoxia impairs the SR Ca<sup>2+</sup> reuptake from the intracellular space. This could result in an increase in Ca<sup>2+</sup> under hypoxic conditions serving as a second messenger to activate eNOS and nNOS<sup>8</sup>. Melillo et al<sup>37</sup> found that a hypoxia- responsive enhancer activity was inducible by hypoxia which can activate the iNOS in macrophages and provided the first evidence that iNOS is a hypoxia-inducible gene. Also Jung et al<sup>38</sup>, showed that increased expression of hypoxia-inducible factor-1 (HIF-1) resulted in the transcriptional activation of iNOS gene expression under hypoxic conditions in myocardial cells. They have also demonstrated that interleukin-1 $\beta$  was further augmented under hypoxic conditions via the transcription factor HIF-1 and induced iNOS gene and protein expression in cardiac myocytes.

Induction of iNOS by hypoxia appears to vary between different tissues. Palmer and colleagues<sup>6</sup> have reported that 3 weeks of hypoxia in conscious rats was associated with the induction of iNOS mRNA in bronchial epithelial, pulmonary endothelial, and smooth muscle cells. While no significant iNOS induction occurred in the brain in response to 12-48 h of hypoxia in rats<sup>39</sup>. Another study by Javeshghani et al<sup>40</sup> indicated that prolonged periods of hypobaric hypoxia had no influence on iNOS induction in the diaphragm and suggested that despite the presence of a putative hypoxia-induced element in iNOS promoter, induction of the iNOS gene in response to in vivo hypoxia is regulated at the local level. However, these authors could not

exclude that the link between iNOS induction and recycling of L-citrulline to L-arginine, the main supplier of iNOS is particularly strong during *in vivo* hypoxia.

Moreover, hypoxia might affect the NO scavenging properties of the muscle, which could result in an elevated NO generation in the hypoxic muscle<sup>24</sup>. It is also important to note that the half-life of NO is increased during the decrease in PO<sub>2</sub><sup>41</sup>.

In this study, exposure of the rat diaphragm to hypoxia in the presence of L-NMMA and Hb unaltered contractility during submaximal activation with complete prevention of hypoxia-induced elevated nitrotyrosine formation compared to hyperoxic groups. The contribution of NO to this changes during hypoxia was evident when these two groups were compared to hypoxic control one and similar results were obtained. In addition, hypoxia did not affect both F<sub>t</sub> and F<sub>0</sub> force generation in the Hb incubated diaphragm. Moreover the beneficial effect of inhibition of NOS in hypoxia was completely reversed by addition of L-arginine simultaneously with L-NMMA which denotes that NO plays a prominent role in hypoxia-induced contractile dysfunction.

The affinity of NO for hemoglobin is about 1,500 times that of carbon monoxide in the absence of oxygen and combines with Hb to form nitrosyl-hemoglobin during hypoxia<sup>28</sup>. However, the fatigue resistance during hypoxia was improved by L-NMMA and not by Hb compared to hypoxic control group; thus, it is likely that higher concentrations of Hb would be

needed to scavenge the large amount of NO generated during hypoxia.

These results agree with the findings of Ottenheijm et al<sup>42</sup> who found that inhibition of NO formation during hypoxia by L-NMMA improved the submaximal and not the maximal tetanic contraction concomitant with reduced peroxynitrite formation. They stated that the effects of NO on diaphragm maximal activation is modest, in contrast to its greater effects during submaximal activation.

Also Zhu et al<sup>43</sup> found impaired *in vitro* rat diaphragm isometric contraction under 60 min and 90 min hypoxic incubation and its improvement under L-NMMA and Hb incubation. However they showed that L-NMMA had a dose-dependent effect on force generation of the rat diaphragm during hypoxia. A dose of 10 µM improved submaximal tetanic contraction (F<sub>30</sub>) evident after 60 min of incubation during hypoxia while a dose of 30 µM improved also its maximal tetanic contraction (F<sub>0</sub>). Thus providing another plausible explanation for the results obtained in this work.

These authors in another study<sup>43</sup>, showed that L-NMMA decreased diaphragm nitrotyrosine formation under hypoxic conditions without affecting diaphragm bundle contractility. This is an apparent discrepancy. However, differences in the phenotype of the muscle under investigation could provide an explanation.

On the other hand, Zhu et al<sup>1</sup> found that either inhibition of NOS by L-NMMA or scavenging NO by hemoglobin slowed velocity of

shortening and increased the isotonic fatigability of the rat diaphragm *in vitro* during hypoxia. They suggested that endogenous NO has a protective role in hypoxia-mediated modulation of muscle contraction *in vitro* by its antioxidant effect against ROS and that NO can protect cells against toxicity mediated by hydrogen peroxide. However, they mentioned that the net effect of NO depends not only on its relative concentration but also on the balance between the levels of NO and ROS.

Moreover the results of this study indicated that excessive exogenous NO production aggravated the hypoxia-induced impairment of the diaphragm force generation compared to hypoxic control. It was expected that it will lead to further increase in nitrotyrosine level during hypoxia. However, it was only significantly increased compared to L-NAME and Hb hypoxic groups. Thus it is likely that the excessive NO production and hypoxia reached their maximum inhibitory effect and ROS released during hypoxia was probably saturated with the endogenously produced NO.

The mechanism by which the NO produced by skeletal muscle NOS and NO donor may impair diaphragmatic force generation can be by means of the cyclic guanosine monophosphate (cGMP) signaling pathway which can negatively modulate skeletal muscle contractile function<sup>14</sup>. Also it can be by cGMP-independent one<sup>20</sup>. The calcium-release ryanodine receptor (RYR1) channels of the SR are likely sites for such an interaction, especially the thiol groups presented on these channels<sup>44</sup>. NO prevent oxidant-

induced channel opening reducing the rate of calcium release<sup>45</sup>. Also studies in permeabilized rabbit psoas muscle fibres demonstrated that the NO donors reduced myofibrillar Ca<sup>2+</sup>sensitivity<sup>46</sup>, which would contribute to the reduction of force generation observed in the Na nitroprusside incubated group of this study.

NO also binds to cytochrome c oxidase and lowers its affinity for O<sub>2</sub>, and this is a main mechanism for inhibition of mitochondrial respiration<sup>47</sup>. This is likely to decrease cellular ATP and increases ADP, AMP, guanosine 5'-diphosphate, and P<sub>i</sub>. These metabolites are known to regulate diverse cellular processes such as ion transport, protein synthesis, and muscle contraction; thus contributing to the hypoxia impaired force generation. Another potential target for NO is the reactive thiols presented on the myosin head reducing its ATPase activity<sup>48</sup>, this would reduce the shortening velocity and therefore decrease fatigue resistance during hypoxia which is consistent with the current study.

In addition to the increased NO generation in hypoxia, the formed peroxy nitrite might act as a mediator in hypoxia-induced contractile protein dysfunction. Potential targets for peroxy nitrite in the muscle include myosin, actin and troponin I, which have tyrosine groups that can be nitrated<sup>43</sup>. Peroxy nitrite can also affect calcium homeostasis by inactivating Ca<sup>2+</sup>ATPase<sup>49</sup>, although other data are conflicting<sup>50</sup>. Studies with cultured myocytes revealed that plasma membrane proteins and myofibrillar creatine kinase are potential targets for peroxy nitrite as well<sup>51</sup>. In addition,

mitochondria potentially provide an abundant source of superoxide and high concentrations of NOS are localized to mitochondria in skeletal muscle; consequently, peroxynitrite may reach high concentrations in mitochondria, affecting nitration of mitochondrial proteins and thereby impair mitochondrial function<sup>52</sup>. Other studies also suggested that peroxynitrite, through its interaction with glucose or reduced glutathione, could also generate NO donors and thus had indirect effects on mitochondrial respiration<sup>53</sup>.

**In conclusion**, the present study indicates increased NO generation under hypoxia and that its inhibition and subsequently of peroxynitrite formation increases the force generation and fatigue resistance of the rat diaphragm during in vitro hypoxia. Also it showed that the iNOS is activated in the diaphragm under hypoxia and may contribute partially to NO generation in hypoxia. Whether this protective effect by a NOS inhibitor or a NO scavenger during hypoxia could be realized in clinical conditions is too early to speculate. Future investigations are needed to determine the physiological role of iNOS and NO to develop successful strategies for improving muscle function under hypoxic conditions.

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### تأثير تعديل أوكسيد النايتريك على الإنقباض الأيزومترى للحجاب الحاجز للفأر تحت ظروف نقص وجود الأوكسجين

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دور أوكسيد النايتريك في إنقباض عضلات التنفس كان محور جدال لأعوام كثيرة، كما أن ما يعرف عن دوره تحت ظروف نقص الأوكسجين ودور إنزيم الأنديوسيل نايتريك أوكسيد سنسبيتز في تكوين أوكسيد النايتريك قليل. يهدف هذا البحث إلى معرفة تأثير أوكسيد النايتريك على توليد القوة ومقاومة الإجهاد في عضلة الحجاب الحاجز للفأر تحت ظروف نقص الأوكسجين الحاد خارج الجسم ومقارنة هذا التأثير بغيره تحت ظروف توافر الأوكسجين. تم في هذا البحث تقييم تأثير مانع النايتريك أوكسيد سنسبيتز: إن مونوميثيل أل ارجينين وتأثير قابض أوكسيد النايتريك الهيموجلوبين ومانح أوكسيد النايتريك الصوديوم نايتروبروسيد على قوة الإنقباض الأعلى ، قوة التيتانك المتوسطة وقوة التيتانك الأعلى كذلك على وقت قدرة إحتمال الإجهاد الأيزومترى تحت ظروف توافر الأوكسجين ونقصه. كذلك تم قياس نشاط الأنديوسيل نايتراك أوكسيد سنسبيتز ومستوى النيتروتيروزين كمؤشر للبروكسي نايتريت في الحجاب الحاجز للفأر.

منع تكوين أوكسيد النايتريك بإستخدام إن مونوميثيل أل ارجينين وتنبيطيه بإستخدام الهيموجلوبين لم يكن له تأثير على الإنقباض الأيزومترى للحجاب الحاجز وقدره على إحتمال الإجهاد تحت ظروف توافر أوكسجين. نقص الأوكسجين أدى إلى نقص له دلالة إحصائية في كل من قوة الإنقباض الأعلى ، قوة التيتانك المتوسطة وقوة التيتانك الأعلى وفي قدرة الحجاب الحاجز على إحتمال الإجهاد مع زيادة نشاط الأنديوسيل نايتراك أوكسيد سنسبيتز ومستوى النيتروتيروزين في المجموعة الضابطة. بينما أدى إن مونوميثيل أل ارجينين إلى زيادة لها دلالة إحصائية في قوة التيتانك المتوسطة وفي إحتمال الإجهاد خلال فترة نقص الأوكسجين مع نقص في نشاط الأنديوسيل نايتراك أوكسيد سنسبيتز وفي مستوى النايتروتيروزين مقارنة بمجموعة نقص الأوكسجين الضابطة. إضافة الهيموجلوبين أدى إلى نفس النتائج مع زيادة لها دلالة إحصائية في قوة الإنقباض الأعلى وقوة التيتانك الأعلى. تأثير إن مونوميثيل أل ارجينين منع عند اضافة الارجينين. زيادة أوكسيد النايتريك الخارجي عن طريق الصوديوم نايتروبروسيد أدى إلى نقص له دلالة إحصائية في الإنقباض الأيزومترى وفي إحتمال الإجهاد للحجاب الحاجز تحت ظروف نقص الأوكسجين وتوافره. خاتماً: نتائج هذا البحث تدل على أن الأنديوسيل نايتراك أوكسيد سنسبيتز يزيد نشاطه في الحجاب الحاجز للفأر عند تعرضه لنقص الأوكسجين ويشارك جزئياً في توليد أوكسيد النايتريك . كما أن أوكسيد النايتريك له دور بارز في الحجاب الحاجز للفأر عند تعرضه لنقص الأوكسجين خارج الجسم ويشارك في تنبيط قوة إنقباضه مقارنه بدوره الأقل أهمية تحت ظروف توافر الأوكسجين. تنبيط أوكسيد النايتريك أثناء نقص الأوكسجين قد يكون له دور في حماية الحجاب الحاجز وقد يكون هدف في الأوساط الإكلينيكية.