ROLE OF NITRIC OXIDE ON DIAPHRAGMATIC MUSCLE CONTRACTION UNDER DIFFERENT FREQUENCIES OF STIMULATION

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ABSTRACT

Background: Nitric oxide (NO) exhibits diverse vital roles in the body functions. It is clearly recognized that NO participates in the control of the vascular tone as an antagonist of adrenergic system. Neural NO-synthes is present in the sarcolemma of type II skeletal muscle fibers. Objective: Evaluating the role of nitric oxide on diaphragmatic muscle contraction under different frequencies of stimulation. Materials and methods: Thirty adult male albino rats weighing 140 - 155 g were chosen to be the model of the present study. They were divided into two equal groups: Group I (Low frequency stimulated group), and Group II (High frequency stimulated group). Results: NO significantly decreased maximal twitch force (ΔY) and contraction time (ΔX), while increased half relaxation time (1/2 Rt) when the rat diaphragm preparation was stimulated directly at either low (0.5 Hz) or high (100 Hz) frequencies. However, when the preparation was stimulated indirectly at low (0.5 Hz) or high (100 Hz) frequencies, a significant increase in ΔY and ΔX associated with decrease in (1/2 Rt) were recorded. Conclusion: NO has dual action on the pre and postsynaptic levels which was antagonized by bovine hemoglobin that acts as scavenger for NO. Keywords: Nitric oxide, diaphragm, skeletal muscle.

INTRODUCTION

NO regulates vascular tone through neuronal NO synthase (Seddon et al., 2009). It plays an important role during adaptation of the organism to variable environments including the changes in motor activity. The force of diaphragmatic contraction decreases on exposure to NO, while the force of contraction in mouse hind limb increases after 15 minutes of exposure to NO donor (Kobayashi et al., 2008).

The direction of the change in force in response to NO donors depends on the interval between contractions, i.e. more frequent stimulations lead to more increase in the force of contraction in muscle in response to NO. NO plays a role in exercise-induced maintenance and adaptation of muscular contraction by regulating its glucose metabolism, force of contraction, fatigue and antioxidant systems activities (Lambertucci et al., 2012).

L-arginine has been given to the training athletes not only as an amino acid but as a donor of NO which enhances the muscular performance through increasing blood flow, glucose uptake and oxygen consumption (Sureda and Pons, 2012). NO has multiple roles in the circulatory and metabolic responses through modulating glucose uptake, metabolism, and mitochondrial bioenergetics during acute bout of exercise (Henstridge et al., 2009).

The present study was designed to examine the role of nitric oxide on diaphragmatic muscle contraction under different frequencies of stimulation and modulation of this effect by hemoglobin.

MATERIALS AND METHODS

The experimental protocol and animal handling were approved and performed according to the guidelines of animal use of the Ethical committee of Faculty of Medicine - Al-Azhar University. The following experimental procedures were done during December 2013 till January 2014 in the laboratory room of physiology department – Al-Azhar Faculty of Medicine. Thirty adult male albino rats of local strain weighing 140 - 155 g were chosen to be the model of the present study. They were left for two weeks in the laboratory room before any experimental interference for acclimatization with free access to water and rat chow pellets. Rats were kept in suitable cages (40 x 30 x 30 per 5 rats) at room temperature with the natural light/dark cycle. Rats were divided into two equal groups:

Group I: Diaphragms were electrically stimulated by supramaximal stimuli of 0.5 msec duration and low frequency of 0.5 Hz, directly and indirectly through their nerve supply to induce simple muscle twitch.

Group II: Diaphragms were electrically stimulated by supramaximal stimuli of 0.5 msec duration and high frequency of 100 Hz, directly and indirectly through their nerve supply to induce tetanic contraction.
Diaphragmatic muscle preparation: Thoracic and abdominal cavities were opened immediately after sacrificing the animals, the phrenic nerve, the diaphragm with its origin on the ribs and central tendon were excised, transferred to a disecting dish containing oxygenated Krebs’ solution aerated with carbogen. Diaphragmatic preparations were mounted in bath of 50 ml of Krebs’ solution at 37 °C and aerated with carbogen, stimulated directly and indirectly.

Effect of L-arginine (known as NO donor): L-arginine was added in a dose of 4.7 mM to the bath to investigate the effect of NO application on rat’s diaphragm exposed to either direct or indirect electrical stimulation .(Queiroz and Alves Do-Prado, 2001).

Effect of bovine hemoglobin (NO scavenger): In order to prove that the observed responses were NO mediated, bovine hemoglobin (NO scavenger) was added in a dose of 50 mM to the bath containing L-arginine. A contact time of 4 minutes was allowed before repeating either direct or indirect stimulation (Ambiel and Alves Do-Prado, 1997).

Contractile measurements: Maximal twitch force (Δ Y) from initial twitch to peak, contraction time (Δ X) from initial to peak tension and half relaxation time (½ Rt) from the middle of the crown of the twitch tension until tension had fallen half way to the initial tension prior to the contraction were measured for group I. In tetanic contractions (group II), only its maximum contraction force was measured.

Statistical analysis: Data input and analysis were done using SPSS computer program. All results were expressed as mean ± standard error. Mean values of the different groups were compared using a one-way analysis of variance (ANOVA). Least significant difference (LSD) post hoc analysis was used to identify significantly different mean values. P value ≤ 0.05 was accepted to denote a significant difference.

RESULTS

Indirect supramaximal stimulus of 0.5 msec duration and 0.5 Hz frequency showed that the maximal twitch force (Δ Y) in presence of Krebs’ solution ranged from 3.4 g/cm² to 8.5 g/cm² with a mean value of 5.89 ± 1.738 g/cm². Addition of L-arginine led to significant improvement of Δ Y ranged from 5.5 g/cm² to 11.4 g/cm² with a mean value 8.079 ± 2.011 g/cm² (+37.16 %). Addition of bovine hemoglobin to L-arginine bath led to returning of Δ Y back nearly to its original value with a mean value of 5.894 ± 1.753 g/cm² with a little change of - 0.06 % as compared to its value in Krebs’ solution.

The contraction time (Δ X) in presence of Krebs’ solution ranged from 0.0312 msec to 0.0925 msec with a mean value of 0.0496 ± 0.016 msec. Addition of L-arginine led to significant increase of Δ x ranging from 0.0426 msec to 0.126 msec with a mean value of 0.0699 ± 0.021 msec (+ 40.92 %). Addition of bovine hemoglobin to L-arginine bath led to returning of Δ x back nearly to its original value with a mean value of 0.0450 ± 0.015 msec with a little change of - 9.27 % as compared to its value in Krebs’ solution.

The half relaxation time (½ Rt) in presence of Krebs’ solution ranged from 0.0127 msec to 0.178 msec with a mean value of 0.075 ± 0.0454 msec. Addition of L-arginine led to significant decrease of ½ Rt by -36 % as compared to its value in Krebs’ solution. Its value ranged from 0.0129 msec to 0.0797 msec with a mean value 0.048 ± 0.022 msec. Addition of bovine hemoglobin to L-arginine bath led to returning of ½ Rt back nearly to its original value with a mean value of 0.069 ± 0.018 msec with a little change of - 8 % as compared to its value in Krebs’s solution only (Table 1).

Direct supramaximal stimulus of 0.5 msec duration and 0.5 Hz frequency showed that Δ Y in presence of Krebs’ solution ranged from 6.4 g/cm² to 12.2 g/cm² with a mean value of 10.283 ± 1.796 g/cm². Addition of L-arginine led to significant decrease of Δ Y from 5.6 g/cm² to 7.735 g/cm² with a mean value 7.585 ± 1.535 g/cm² (by -14.53 %). Addition of bovine hemoglobin to L-arginine bath led to returning of Δ Y back nearly to its original value with a mean value of 10.05 ± 1.841 g/cm² with a little change of -2.26 % as compared to its value in Krebs’ solution.

The contraction time (Δ X) in presence of Kreb’s solution only was ranged from 0.0268 msec to 0.06874 msec with a mean value of 0.049 ± 0.012 msec. Addition of L-arginine led to significant decrease of Δ x ranging from 0.0215 msec to 0.0576 msec with a mean value 0.037 ± 0.014 msec (-24.48 %). Addition of bovine hemoglobin to L-arginine bath led to returning of Δ X back nearly to its original value with a mean value of 0.048 ± 0.013 msec with a little change of -2 % as compared to its value in Krebs’ solution.
The half relaxation time (½ Rt) in presence of Krebs’ solution ranged from 0.0159 msec to 0.0632 msec with a mean value of 0.033 ± 0.014 msec. Addition of L-arginine led to significant increase of ½ Rt by +81.8% as compared to its value in Krebs’ solution, its value ranged from 0.0159 msec to 0.0792 msec with a mean value 0.060 ± 0.018 msec. Addition of bovine hemoglobin to L-arginine bath led to returning of ½ Rt back nearly to its original value with a mean value of 0.039 ± 0.012 msec with +18% change of as compared to its value in Krebs’ solution (Table 2).

Indirect supramaximal high frequency stimulation (100 Hz) showed that Δ Y in presence of Krebs’ solution ranged from 11.1 g/cm² to 20 g/cm² with a mean value of 14.654 ± 2.467 g/cm². Addition of L-arginine led to significant increase of Δ Y from 15 g/cm² to 24 g/cm² with a mean value 17.793 ± 2.463 g/cm² (+21.42%) as compared to its value in Krebs’ solution. Addition of bovine hemoglobin to L-arginine bath led to returning of Δ Y back nearly to its original value with a mean value of 14.532 ± 2.491 g/cm² with a little change of -0.8% as compared to its value in Krebs’ solution (Table 3).

Direct supramaximal high frequency stimulation (100 Hz) showed that Δ Y in presence of Krebs’ solution ranged from 24 g/cm² to 28.5 g/cm² with a mean value of 25.753 ± 1.746 g/cm². Addition of L-arginine led to significant decrease of Δ Y from 19.8 g/cm² to 24.4 g/cm² with a mean value 22.773 ± 2.244 g/cm² (-11.1%) as compared to its value in Krebs’ solution. Addition of bovine hemoglobin to L-arginine bath led to returning of Δ Y back nearly to its original value with a mean value of 25.720 ± 1.776 g/cm² with a little change of -0.128% as compared to its value in Krebs’ solution (Table 4).

### Table (1): Changes Δ Y, Δ X and ½ RT with low frequency of 0.5 Hz in group I with indirect electrical stimulation (n = 15).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experiments</th>
<th>Experiment 1: Krebs’ solution</th>
<th>Experiment 2: Krebs’ solution + L-arginine</th>
<th>Experiment 3: Krebs’ solution + L-arginine + Bovine Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ Y (g/cm²) mean ±SD</td>
<td>5.89 ± 1.738</td>
<td>8.079 ± 2.011</td>
<td>5.894 ± 1.753</td>
<td></td>
</tr>
<tr>
<td>% differences</td>
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<td>P value</td>
<td>&lt; 0.005</td>
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<tr>
<td>Δ x (msec) mean ±SD</td>
<td>0.046 ± 0.016</td>
<td>0.0699 ± 0.021</td>
<td>0.1545 ± 0.015</td>
<td>0.046 ± 0.018</td>
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<tr>
<td>% differences</td>
<td>+40.92%</td>
<td>-9.27%</td>
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<tr>
<td>P value</td>
<td>&lt; 0.001</td>
<td>&gt; 0.05</td>
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<tr>
<td>½ Rt (msec) mean ±SD</td>
<td>0.075 ± 0.045</td>
<td>0.048 ± 0.022</td>
<td>0.069 ± 0.018</td>
<td>0.046 ± 0.018</td>
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<tr>
<td>% differences</td>
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<td>-8%</td>
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<tr>
<td>P value</td>
<td>&lt; 0.009</td>
<td>&gt; 0.05</td>
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</tr>
</tbody>
</table>

Δ Y: Maximal twitch force.
Δ x: Contraction time.
½ Rt: Half relaxation time.

n = No of rats in the group.

### Table (2): Changes Δ Y, Δ X and ½ Rt with low frequency of 0.5 Hz in group I with direct electrical stimulation (n = 15).

<table>
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<th>Parameters</th>
<th>Experiments</th>
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<th>Experiment 2: Krebs’ solution + L-arginine</th>
<th>Experiment 3: Krebs’ solution + L-arginine + Bovine Hb</th>
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</thead>
<tbody>
<tr>
<td>Δ Y (g/cm²) mean ±SD</td>
<td>10.283 ± 1.796</td>
<td>7.585 ± 1.535</td>
<td>10.05 ± 1.841</td>
<td>10.05 ± 1.841</td>
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<tr>
<td>% differences</td>
<td>-14.53 %</td>
<td>-2.26 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt; 0.0001</td>
<td>&gt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ x (msec) mean ±SD</td>
<td>0.049 ± 0.012</td>
<td>0.037 ± 0.014</td>
<td>0.048 ± 0.013</td>
<td>0.048 ± 0.013</td>
</tr>
<tr>
<td>% differences</td>
<td>-24.48%</td>
<td>-2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt; 0.0001</td>
<td>&gt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>½ Rt (msec) mean ±SD</td>
<td>0.033 ± 0.014</td>
<td>0.060 ± 0.018</td>
<td>0.039 ± 0.012</td>
<td>0.060 ± 0.018</td>
</tr>
<tr>
<td>% differences</td>
<td>+81.8%</td>
<td>-18%</td>
<td></td>
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<tr>
<td>P value</td>
<td>&lt; 0.0001</td>
<td>&gt; 0.05</td>
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</tr>
</tbody>
</table>

Δ Y: Maximal twitch force.
Δ x: Contraction time.
½ Rt: Half relaxation time.

n = No of rats in the group.

### Table (3): Changes Δ Y with high frequency of 100 Hz in group II with indirect electrical stimulation (n = 15).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experiments</th>
<th>Experiment 1: Krebs’ solution</th>
<th>Experiment 2: Krebs’ solution + L-arginine</th>
<th>Experiment 3: Krebs’ solution + L-arginine + Bovine Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ Y (g/cm²) mean ±SD</td>
<td>14.654 ± 2.467</td>
<td>17.793 ± 2.463</td>
<td>14.532 ± 2.491</td>
<td>14.532 ± 2.491</td>
</tr>
<tr>
<td>% differences</td>
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<td>-0.8%</td>
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<tr>
<td>P value</td>
<td>&lt; 0.0001</td>
<td>&gt; 0.05</td>
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</tr>
</tbody>
</table>

Δ Y: Maximal twitch force.

n = No of rats in the group.
DISCUSSION

Nitric oxide (NO) has earned the reputation of being a signaling mediator with many diverse and often opposing biological activities. The diversity in response to this simple diatomic molecule comes from the enormous variety of chemical reactions and biological properties associated with it (Douglas et al., 2008).

The present study was designed to examine the role of nitric oxide in diaphragmatic muscle contraction under different frequencies of direct and indirect stimulation and modulation of this effect by hemoglobin.

Results of the present work showed that addition of L-arginine to the rat muscle preparation led to significant increase in the force of muscle contraction and contraction time associated with decreased half relaxation time in indirectly stimulated muscle at 0.5 Hz.

These results were in agreement with Deshmukh et al. (2010) who reported that treatment of skeletal muscle with spermine (nitric oxide donor) causes increased intracellular cyclic GMP (cGMP) levels and promote glucose transport in the skeletal muscle fibers resulting in increased force of muscle contraction.

It has been reported that ATP-sensitive potassium channels and nitric oxide (NO) have been suggested to contribute in mediating arteriolar dilatation and active hyperemia in diaphragm leading to enhanced blood supply and contraction force (Danialou et al., 2008). Also, it has been reported that, in restraint position, the peak twitch of diaphragm significantly decreased and force-generating capacity reduced at low frequency stimulation which increased after L-arginine pre-incubation. In addition, serum NO level increased significantly in the restraint position and the diaphragmatic nNOS mRNA expression upregulated significantly in the restraint position (Xiang et al., 2012).

Ferguson et al. (2014) has reported that exercise intolerance is a characteristic feature of diseases, such as chronic heart failure and diabetes, is associated with reduced nitric oxide bioavailability from nitric oxide synthase, resulting in an impaired microvascular O2 driving pressure and metabolic control. Infusions of the potent NO donor sodium nitroprusside augment NO bioavailability and decrease the mean arterial pressure thereby reducing its potential efficacy for patient populations. These indicate that supplementation delivered to the muscle directly through superfusion enhances the blood-myocyte oxygen driving pressure following the onset of muscle contraction.

It has been reported that nitric oxide signalling plays a key role in exercise/contraction. exercise/contraction-induced metabolic responses in skeletal muscle via an AMPK-dependent mechanism. During exercise/contraction, increased nitric oxide levels are associated with induction of glucose uptake in skeletal muscle.

NO precursor L-arginine acting at the presynaptic level produced a dose-dependent increase in the amplitude of muscle contraction in rat neuromuscular preparation when stimulated indirectly at 0.2 Hz (Deshmukh et al., 2010).

The mechanism by which NO precursor enhances muscle contraction could be attributed to linked nitric oxide signalling to AMPK activation and glucose uptake. It has been reported that sodium nitroprusside increases AMPK-α1-, but not AMPK-α2-associated activity. In rodent skeletal muscle, sodium nitroprusside-induced increases in AMPK-isoform-specific activity occurred independently of changes in ATP, creatinine phosphate or glycogen levels (Lira et al., 2007).

It has also been reported that arginine improves glucose and lipid metabolism in skeletal muscle, in parallel with increased phosphorylation of Akt and AMPK-α. These effects are mediated by the NO/cGMP pathway. Thus, arginine treatment enhances signal transduction and has a beneficial effect of metabolism in skeletal muscle through direct activation of Akt and AMPK pathways (Barbosa et al., 2013).

<table>
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<th>Experiment 3: Krebs’ solution + L-arginine + Bovine Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ Y (g/cm²) mean ±SD</td>
<td>25.753 ± 1.746</td>
<td>22.773 ± 2.244</td>
<td>25.720 ± 1.776</td>
<td></td>
</tr>
<tr>
<td>% differences</td>
<td>-11.1 %</td>
<td>-0.128 %</td>
<td></td>
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<tr>
<td>P value</td>
<td>&lt; 0.0001</td>
<td>&gt; 0.05</td>
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</table>

Δ Y: Maximal twitch force.
In an attempt to clarify whether the improved muscle contraction that followed the addition of L-arginine when stimulated indirectly was due to its effect at a pre or post-synaptic level, its effect on directly stimulated muscle was studied. Results obtained showed that in vitro addition of L-arginine to the rat diaphragm preparation directly stimulated at low frequency of 0.5 Hz. The amplitude of maximum contraction and the contraction time of the simple muscle twitch significantly reduced and associated with increased half relaxation time.

These results were in accordance with Vitecek et al. (2012) who reported that L-arginine (NO precursor) reduced the amplitude of maximum contraction of directly stimulated skeletal muscle using a frequency of 0.2 Hz and that endogenous NO synthesis inhibits muscle contractility especially at low frequency stimulation and reported that NO might acts directly on modulating regulatory proteins via its redox effect or thiol groups of the Ca\(^{2+}\) release channels of the sarcoplasmic reticulum which are likely responsible for this interaction. Reactive thiols present on the myosin head are another potential target. This modulation would reduce maximal force generation. Thus, the effect of NO appeared to be mediated through cGMP-dependant and cGMP-independent mechanisms involved in excitation contraction coupling and sarcoplasmic reticulum Ca\(^{2+}\) flux.

In the present work, following indirect electrical stimulation of rat diaphragm at 100 Hz, tetanic contractions of the muscle developed and recorded, the force of contraction increased by 21.42% after the addition of L-arginine to the rat diaphragm preparation. These results were in agreement with Shuman and Madison (2000) who suggested that during high frequency stimulation, the endogenous and exogenous NO increases basal acetyl choline release from central and peripheral cholinergic neurons. During high frequency stimulation, the presynaptic and postsynaptic components are activated through a variety of signal transduction cascades. Also, Lawler and Hu (2000) documented that high frequency stimulation induces changes in muscle contraction partially modulated by NO via cGMP-independent mechanisms, and that the tonic production of NO would originate from the muscle fibers to serve as a feedback signal for synaptic maintenance. They also reported that NO affects sarcoplasmatic calcium release in skeletal myotubes and hypothesized that NO reduces Ca\(^{2+}\) level in the activated skeletal myotubes through oxidation of thiols associated with the sarcoplasmic reticulum Ca\(^{2+}\) release channel. So, NO leads to decreased force of contraction in directly stimulated rat skeletal muscle when NO donor is added to it.

In the present work and in order to prove that the obtained results were NO mediated, bovine hemoglobin (NO scavenger) was added to the bath containing L-arginine, when the rat diaphragm directly stimulated at low and high frequencies. The maximum contraction force increased and returned back to its original value before adding L-arginine. Also, when indirect stimulation was performed, the maximum contraction force decreased and returned back to its original value before adding L-arginine either with low or high frequency. Therefore, it could be concluded that the observed results were due to NO enriched medium.

REFERENCES


الملخص العربي

دور أكسيذ إلىيحريك على ضغط الحساب الحادژ تحت التنبية بترددات مختلفة

حامد محمد عثمان - سعد كمال طه - محمد السيد عبد الفتاح - محمد محمود خميس

قسم السيولجيا الطبية - كلية الطب - جامعة الأزهر - وجامعة الفيوم

خلفية البحث:

يؤد أكسيذ الأطرشيل أدءة ممازح في تأثير صفات الجسم حيث يشارك في التحكم في نشاط الأوعية الدموية كمضاد للجهاز العصبي السبئاوي. ويوجد أكسيذ النبتيز في الغلاف الخارجي للنوع الثاني من العضلات الآرادية. وقد تبين أن انقباضات الحساب الحاجز تتأثر بال تعرض لأكسيذ النبتيز.

الهدف من البحث:

تقييم تأثير أكسيذ النبتيز على انقباض عضللة الحساب الحاجز تحت تأثير ترددات كهربائية مختلفة بانتظاب المباشر وغير المباشر.

مواد وطرق البحث:

استخدَم في هذا العمل ثلاثون ذكرًا من السلالات المحلية من البزضاء البالغة وقد تقسيمهم إلى مجموعتين متساويتين: المجموعة الأولى (تم تأريض الحساب الحاجز لتآثر كهربائي بتهد منخفض 5 هرتز)، والمجموعة الثانية (تم تأريض الحساب الحاجز لتآثر كهربائي بتهد مرتفع 100 هرتز).

النتائج:

وجد أن أكسيذ النبتيز يقلل من قوة انقباض عضللة الحساب الحاجز بالنتيجة الكهربائي المباشر عند مختلف الترددات الكهربائية المنخفضة (5 هرتز) والمرتفعة (100 هرتز) بينما يزيد من قوة انقباض عضللة الحساب الحاجز بالنتيجة الكهربائي الغير مباشر عند مختلف الترددات الكهربائية المنخفضة (5 هرتز) والمرتفعة (100 هرتز). وتم التوصل إلى هذه النتائج في أن أضافة الهموجلوبين الحيوي إلى الحساب الحاجز المزدح بشكل يمكن في وجود الأرقام (كمملي و مخفي و معنى) لأكسيذ النبتيز في حالتي النفاخ الكهربائي المباشر وغير المباشر و عند مختلف الترددات المنخفضة و غير المباشر و عند مختلف الترددات المنخفضة والمرتفعة.

الاستنتاج:

أكسيذ النبتيز له تأثير ثانوي يقلل و يخفف الألفاظ العضلي عند التأثير الكهربائي المباشر و غير المباشر على التوالي و التكلفة الطبيعي يقلل كناسك و لافتات لأكسيذ النبتيز. يوجب تأثيره على الألفاظ العضلي عند التأثير الكهربائي المباشر و غير المباشر عند مختلف الترددات المنخفضة والمرتفعة.