

## The Effects of Severe Hypoxia on Nitric Oxide Parameters in Hypoxia-tolerant Rodent: *Nannospalax nehringi*

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### Abstract

Blind mole rats (BMRs) are solitary rodents which are tolerant to severe hypoxia. The aim of this study is to reveal the changes in nitric oxide (NO) and nitric oxide enzymes (NOS), which are involved in many physiological and pathological processes related with hypoxia, in BMRs under severe hypoxia. For this purpose, 12 subadult (11-15 months) male *Nannospalax nehringi* were captured in Kars location. Captured BMRs were divided into two groups as Normoxic (NG) and Hypoxic (HG) randomly ( $n=6$ ). NG were kept in completely dark, normoxic conditions for 52 h. HG were kept inside completely dark glovebox chamber with 7% oxygen flow for 52 h. After experimental protocol, NG were sacrificed under normoxic conditions and HG were sacrificed inside glovebox chamber with 7% oxygen. NO, iNOS, eNOS, nNOS and MDA levels of plasma and homogenized tissue samples were detected spectrophotometrically. All parameters of each sample were found to be high in HG compared to NG. But especially, NO was high in the lung tissues of HG. Additionally eNOS level of the kidney, liver and lung, iNOS levels of the liver and eNOS, and nNOS levels of the brain were found to be markedly high. Consequently, our data on NO and NOS enzyme production in *Nannospalax nehringi* tissues under hypoxia are compatible with the data obtained from other animals, but it contains differences in some points. We believe that these differences are different evolutionary adaptations of BMRs to hypoxia..

**Keywords:** Hypoxia, NO, iNOS, eNOS, nNOS, MDA, *Nannospalax nehringi*

## Hipoksi-toleranslı Rodentte Şiddetli Hipoksinin Nitrik Oksit Parametreleri Üzerine Etkileri: *Nannospalax nehringi*

### Öz

Kör fareler (BMR) ölümcül hipoksiye oldukça toleranslı soliter rodentlerdir. Bu çalışmanın amacı, hipoksi ile ilişkili birçok fizyolojik ve patolojik süreçte rol alan nitrik oksit (NO) ve nitrik oksit enzimlerinin (NOS), şiddetli hipoksi altındaki BMR'lerdeki değişimlerini ortaya çıkarmaktır. Buna yönelik olarak Kars arazisinden 12 subadult (11-15 ay) erkek *Nannospalax nehringi* yakalandı. Yakalanan BMR'ler Normoksi (NG) ve Hipoksi grubu (HG) olmak üzere rastgele iki gruba ayrıldı ( $n=6$ ). NG, 52 saat boyunca tamamen karanlık, normoksik koşullarda tutuldu. HG, 52 saat boyunca tamamen karanlık, içerisinde %7'lik oksijen akıtılan glovebox kabin içerisinde tutuldu. Deney sonunda NG, normoksi altında ve HG %7 oksijen içeren glovebox kabin içerisinde öldürüldü. Plazma ve homojenize edilen doku örneklerinden NO, iNOS, eNOS, nNOS ve MDA seviyeleri spektrofotometrik olarak belirlendi. Tüm örneklerde ait her parametrenin, NG'ye kıyasla HG'de yüksek olduğu belirlendi. Fakat özellikle, NO'nun, HG akciğer dokularında oldukça yüksek olduğu tespit edildi. Ayrıca böbrek, karaciğer ve akciğer eNOS seviyesinin, karaciğer iNOS seviyesinin ve de beyin eNOS ve nNOS seviyelerinin oldukça yüksek olduğu görüldü. Sonuç olarak, hipoksi altındaki *Nannospalax nehringi*'de NO ve NOS enzim üretimi hakkında elde ettiğimiz veriler, diğer hayvanlardan elde edilen verilerle uyumlu olsa da bazı noktalarda farklılıklar içermektedir. Bu farklılıkların, BMR'lerin hipoksiye karşı sahip olduğu farklı evrimsel adaptasyonlar olduğunu düşünmektedir.

**Anahtar sözcükler:** Hipoksi, NO, iNOS, eNOS, nNOS, MDA, *Nannospalax nehringi*



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## INTRODUCTION

Hypoxia is deficiency of normal oxygen tension in tissues and it is a deadly condition for the most animal species. Prolonged exposure to hypoxia results with necrosis, apoptosis and autophagy in cellular level, and so ischemic damages [1]. In cellular level hypoxia also displays various effects on mitochondria, because of mitochondria are major consumers of oxygen [2]. Electron Transport Chain (ETC) components can produce reactive oxygen species (ROS) during ATP production and release them into different compartment of the mitochondria [3]. But under hypoxic situations ETC components are reorganized for adaptation to hypoxic environment and inducing of reductive carboxylation increases ROS production [2]. In multicellular animals, ROS-mediated cellular dysfunctions can be fatal. Therefore, for avoiding from the overproduction of ROS, organisms evolved some protective mechanisms. Upregulation of HIF-1 (Hypoxia Inducible Factor 1) and Nrf2 (Nuclear Factor (Erythroid-derived 2)-Like 2) are examples of cellular hypoxia-protective mechanisms [4,5] and enhanced hematocrit levels or inducing angiogenesis are examples of systemic mechanisms [6,7]. These adaptive mechanisms regulate oxygen economy and so, protect organism against deadly effects of the hypoxia by decreasing ROS production. However, some local mediators such as Nitric Oxide (NO) also play a role in protection from the negative effects of hypoxia.

Nitric Oxide shows local effects and it has an important role in preserving of homeostatic functions under hypoxia, because rapidly inactivated after diffusing into the bloodstream [8]. Releasing of NO as response to low oxygen tension is a compensator mechanism that mediate to enhancing of blood volume in tissues by relaxing of smooth muscles around the vessels. Nevertheless, effects of NO on pulmonary system is different. Pulmonary vascular resistance increases while systemic vascular resistance decreases under hypoxia via NO-mediated and each of these responses is known as protective mechanisms [9]. NO also takes part in different pathological and physiological process like apoptotic balance, proliferation and regulation of the adhesion molecules [10].

Nitric Oxide can be produced enzymatically via Nitric Oxide Synthase (NOS) isoforms or other metabolic process such as cytocrom-c-mediated production [11]. Most known NOS isoforms are Endothelial Nitric Oxide Synthase (eNOS), Inducible Nitric Oxide Synthase (iNOS) and Neuronal Nitric Oxide Synthase (nNOS). Evolutionally, NOS enzyme isoforms found in mammals are thought to have formed after a second duplication following the early tetrapod evolution [12]. Although all isoforms are expressed in various tissues, nNOS is commonly found in neural tissues, eNOS found in vascular endothelial tissues and the third isoform, iNOS, found in a larger tissue spectrum [12,13].

The clades of evolution tree have different looking organisms

that adapted to countless lifestyles. In hypoxic habitats, it is possible to find species, which have evolutionary advantages against the deadly effects of hypoxia. There are many hypoxia-tolerant species in Pisces, Amphibia, Reptilia and Aves and also in Mammalia [14,15]. *Nannospalax nehringi* also known as blind mole rats (BMRs) are one of the hypoxia-tolerant animals which belong to Spalacidae family in rodentia [16,17]. BMRs live in highly hypoxic underground tunnels and they differ from other rodents in terms of managing of oxygen economy. They have evolved many physiological, morphological and anti-cancer strategies in millions year [18-20]. Theirs total lung capacity, alveolar surface area and capillary volume is higher than white rats, therefore, theirs pulmonary diffusion capacities are higher by 43% [21]. BMRs have also high mitochondria, myoglobin, neuroglobin and cytoglobin density [22]. Because of these unique features, BMRs are one of the non-model organisms that are frequently studied.

In the light of this information, BMRs are unique animals for understanding the effects of hypoxia. For this purpose, for the first time, we reported levels of NO, eNOS, iNOS and nNOS under normoxia and severe hypoxia levels, and MDA (Malondialdehyde) levels as a stress indicator in *Nannospalax nehringi*.

## MATERIAL and METHODS

### Experimental Design

This study was carried out twelve subadult (11-15 months) male *Nannospalax nehringi* (2n=50) which collected from Kars, Turkey. Age determination of BMRs were performed with molar tooth crests methods [23]. The research ethics committee approval for the capture of the BMRs was provided with proper injunction of Kafkas University Local Ethics Committee for Animal Experiments (KAÜ-HADYEK 2018/070). All animals were housed at the Kafkas University, Veterinary Medicine Faculty, Physiology Research Laboratory in individual cages under constant darkness conditions (24D:0L). Animals were fed with fresh vegetable and fruit as *ad libitum*. For the laboratory orientation, animals were housed at the same conditions for three months. After orientation, animals were divided two groups as Normoxic and Hypoxic Group, randomly. Animals in the Normoxic Group (n=6) were housed at normoxic conditions and the constant darkness (24D:0L) for 52 h. Animals in the Hypoxic Group (n=6) were housed in hypoxic glovebox maintained with 7% O<sub>2</sub> + 93% N<sub>2</sub> mixture gas (20 liter/min) for 52 h. Animals in the Normoxic Group were sacrificed with cervical dislocation method under sevoflurane anesthetized after the experiment protocol. Animals in the Hypoxic Group were killed with cervical dislocation method under sevoflurane anesthetized in hypoxic glovebox after the experiment protocol. Tissue samples were homogenized in phosphate buffer (pH: 7.4) and stored at -80°C immediately. Plasma samples were obtained by centrifuged blood (4000 rpm at +4°C) and stored at -80°C.

### Biochemical Analysis

Nitric Oxide levels were determined according to the method described by Miranda et al.<sup>[24]</sup> in that nitrate is reduced to nitrite by  $\text{VaCl}_3$ , and then in acidic environment nitrite was reacted with sulphanilamide to produce colored diazonium compound, which was read at 540 nm. eNOS and iNOS activities are determined by commercial ELISA kit (LSBio, USA). nNOS activity is also determined with commercial ELISA kit (Biocompare USA).

Malondialdehyde as an end product of lipid peroxidation concentrations were measured by the method of Yoshioka et al.<sup>[25]</sup> based on the reaction between thiobarbituric acid and MDA. The end products were read at 535 nm.

Protein content of each sample were measured using bovine serum albumin as standard according to the method of Lowry et al.<sup>[26]</sup>. Results were calculated as  $\mu\text{mol/L g protein}$  in NO and MDA, IU/L g protein in iNOS, eNOS and nNOS.

### Statistical Analysis

SPSS 20.0.0 software was used for statistical evaluation of data, which were expressed as median  $\pm$  standard deviation. Importance level of difference among the groups was determined by variance analysis test (ANOVA) and Tukey multiple comparison test.  $P<0.05$  was considered as significant.

## RESULTS

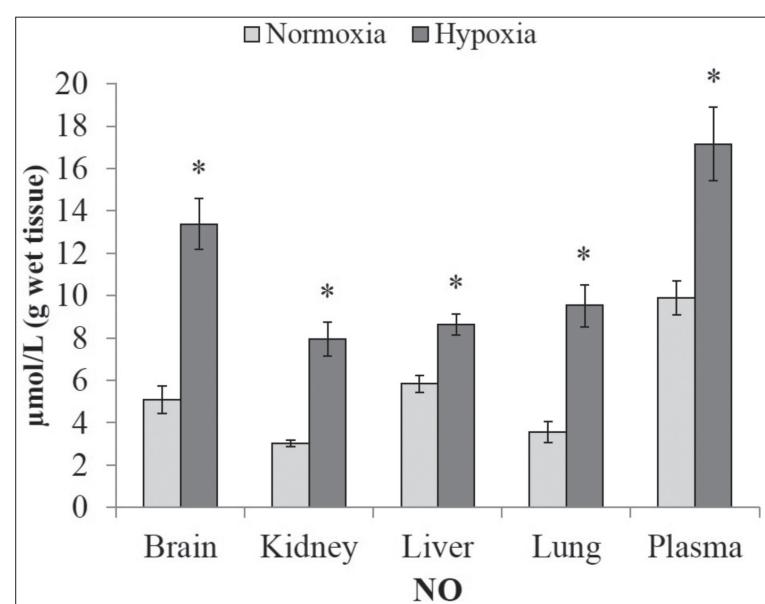
The NO levels of the brain, kidney, liver, lung and plasma are shown in Fig. 1. It was founded that, NO levels of all tissues in Hypoxic Group was increased when compared with Normoxic Group ( $P<0.001$ ). Also, in the brain, kidney and lung tissues, this increase was found to be two times more than the Normoxic Group.

The results of the brain, kidney, liver, lung and plasma eNOS and iNOS levels, and nNOS levels of brain tissues are summarized in Fig. 2, 3 and 4 respectively. As a result of statistical analyzes, significant differences was found between all tissues of animals in Hypoxic and Normoxic Group ( $P<0.001$ ). Liver, lung, and plasma eNOS levels were found to increase approximately four-fold under hypoxia, while brain and liver levels increased ten-fold. Additionally, a three-fold increase was observed in liver iNOS levels, while the levels of iNOS in all tissues increased significantly under hypoxia. Also it was determined that, hypoxia causes approximately four-fold increase in nNOS levels of brain tissues.

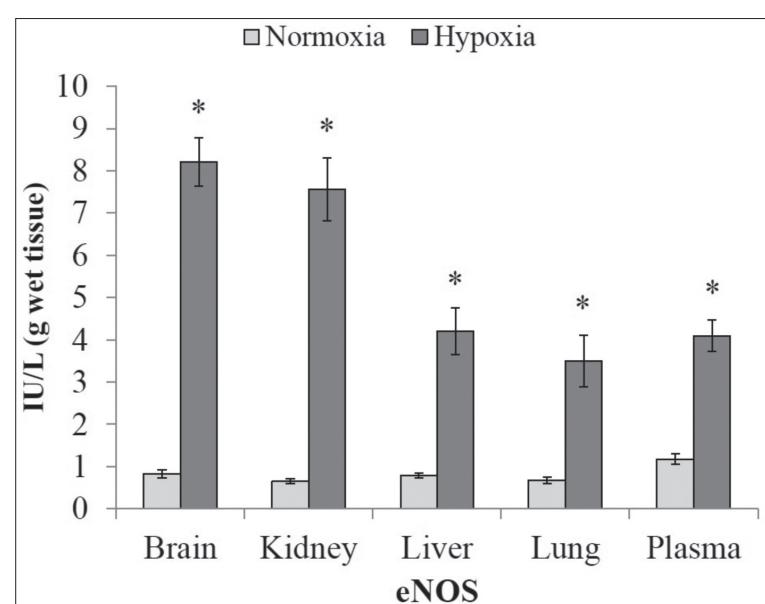
The MDA levels of the brain, kidney, liver and plasma are shown in Fig. 5. Statistically significant increase was determined in MDA levels of all tissues under hypoxia when compared with Normoxic Group ( $P<0.001$ ).

## DISCUSSION

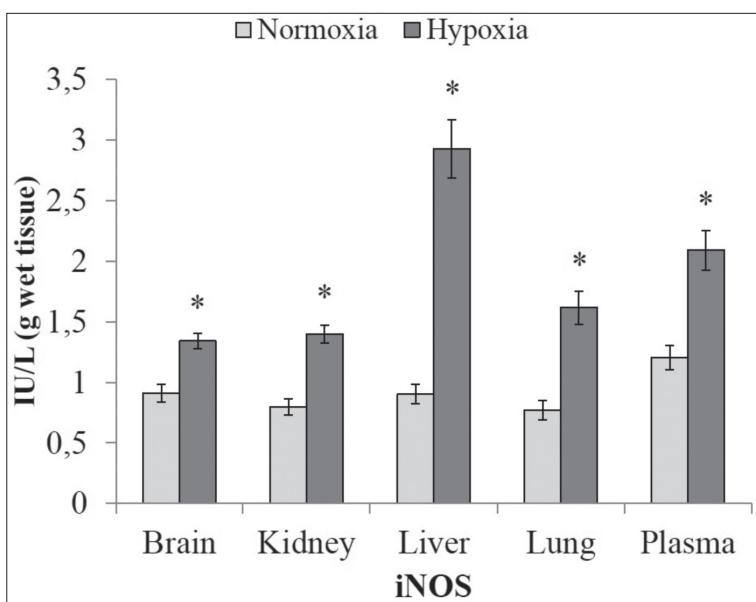
As a result of the mutual harmony among the organisms, our world has an average oxygen concentration between 20-21% at sea level<sup>[27]</sup>. Therefore, the oxygen pressure that many organisms can tolerate is in quite limited



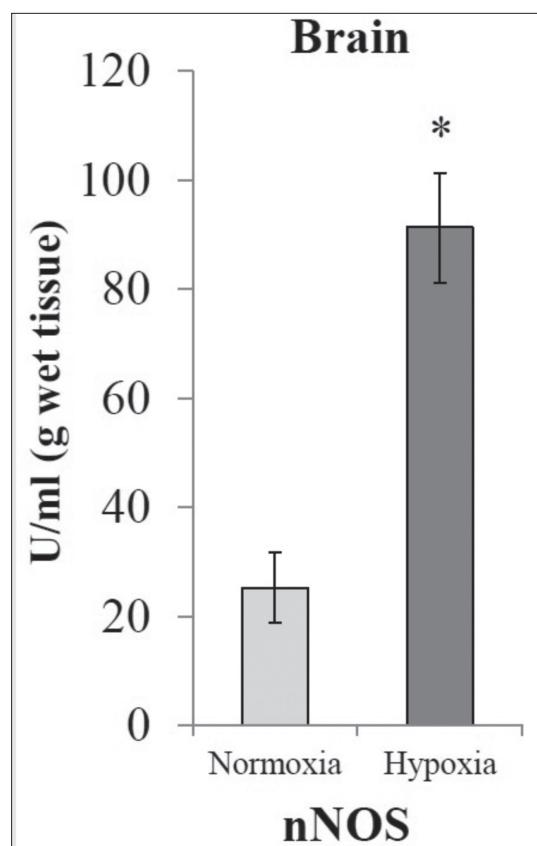
**Fig 1.** NO levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (\*  $P<0.001$ )



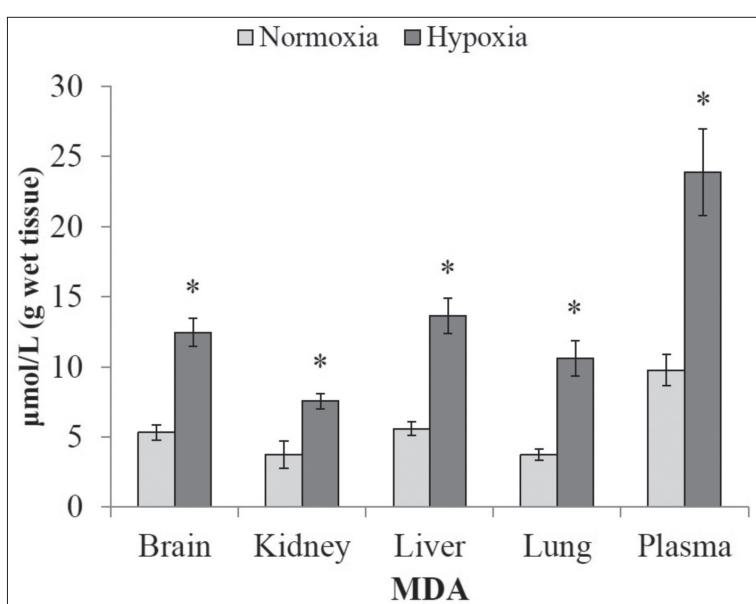
**Fig 2.** eNOS levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (\*  $P<0.001$ )



**Fig 3.** iNOS levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (\*  $P < 0.001$ )



**Fig 4.** Brain nNOS levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (\*  $P < 0.001$ )



**Fig 5.** MDA levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (\*  $P < 0.001$ )

range. Organisms try to maintain their homeostasis by processing the molecules that they can get energy, with various chemical reactions. Many organisms need to oxygen for these processes and they can not survive without oxygen. Habitats of BMRs are highly hypoxic and their characteristics are change with soil structure and seasonally [20]. In present study, no losses of reflexes or behavior changes were observed in the animals during the 52 h hypoxia administration. But hypoxia is a mortal condition for the most organisms. Long-term hypoxia causes various types of cell death [28,29] or, at best, cellular damages by producing to ROS [30]. The most

affected biomolecules from the free radicals are lipid-structured molecules. Therefore, evaluating of MDA which the end product of lipid peroxidation is one of the methods that using for following the ROS producing [31,32]. We observed that hypoxia causes an increase in the MDA levels of all BMRs tissues.

Nitric Oxide have an important role in adapting to hypoxia because they cause smooth muscles relaxing, thereby they mediate to enhancing of blood volume in the tissue via vasodilatation [33,34]. It is seen that there are different NO levels among different human populations, depending on their geographical characteristics. Erzurum et al. [35] showed that, Tibetan highlanders who live at the high altitudes and durable to the hypobaric hypoxia, that have higher NO levels than other people who live at the sea levels. Interestingly, NO also plays a central role in protective effect of intermittent-hypoxia against different conditions including cardiovascular diseases and ischemia [36]. Thus, it can be say that, NO may one of the key for adapting to hypoxia in the evolutionary process. In our study, we observed that, NO levels in brain, kidney, liver and plasma samples of BMRs increase up to 2.6, 1.47 and 1.7 times respectively. However, it is known that NO has different effects on pulmonary system [37].

Using of NOS antagonists increases pulmonary and systemic vascular resistance under normoxic conditions, and these data refer to protective role of NO against the hypoxia-mediated damages [9]. This phenomenon also have tested in different animal species. It was observed that hypoxic ventilation has no any effect on the NO levels of buffer-perfused rabbit's lung [38]. On the contrary, a decrease in both NO levels of isolated pig lungs [39] and, aortic and pulmonary arterial nitrite levels in cardiopulmonary bypassed pigs [40] exposed to hypoxia have been shown. In the present study, we detected approximately 2.6 fold increase in NO products levels of BMRs lung tissues. We think that this may be another adaptation that BMRs have. However, cause of these different results is may be originate from differentiation of experimental methods.

Nitric Oxide Synthases inhibition results with low blood flow and critical oxygen pressure levels in cortical and medullary area of kidney [41]. Also, eNOS specifically plays an important role in the controlling of vascular tension which effects glomerular filtration rate in the kidney [42]. Similarly, our data show that, kidney of BMRs have high NOSs expression under the hypoxia. Especially eNOS have 11.6 fold increase in kidney. It indicate that, eNOS is an important component for running of physiological activities under hypoxia in the kidney of BMRs. Lung is another critical organ for the animals. Balasubramaniam et al.[43] showed that hypoxia impairs alveolarization in the eNOS deficient mouse, and also decreased expression of the eNOS is related with impaired integrity of injured lung [44]. Also, it was showed that, levels of eNOS protein increase in lung of rats but not iNOS after hypoxic exposure [45]. Nevertheless, nNOS is not considered to have an important role in the lungs [45]. However, we observed a significantly increase both eNOS and iNOS levels in lung tissues of BMRs. Previous studies have demonstrated that pulmonary diffusion capacities of BMRs are higher than rats [21]. High eNOS and iNOS levels under hypoxia may be another component of these adaptations. Also, it is known that, iNOS production increases in ischemic human liver [46]. Similarly, we found hypoxia causes significant increase of iNOS and eNOS production in the liver tissue of BMRs. We believe that this is a compensative process which are protect the vital organ liver.

Nitric Oxide production is also closely associated with HIF-1 $\alpha$  and HIF-1 activity can be prevent or activate by the NO sources because of their compounding pharmacological actions [47]. HIF-1 $\alpha$  is stabilized under hypoxia and controls the iNOS and eNOS transcription, but in brain, nNOS activity is necessary for the HIF-1 $\alpha$  stability [33]. Brain is the major organ that sensitive to O<sub>2</sub> pressure. Therefore, effects of NOS enzymes on the brain are more complicate and nNOS plays a critical role in hypoxia protection in the central nervous system. It is thought that nNOS is a negative regulator for inhibiting of NO overexpression, because of nNOS is evaluated as NOS derivative which associated with

presence of O<sub>2</sub> [48]. Hypoxia-ischemia models in mice brain causes markedly increase in both protein and mRNA levels of nNOS [49]. van den Tweel et al.[50] observed that hypoxia-ischemia induced brain injury in neonatal rats and cause an increase in nNOS while eNOS decreases after hypoxia-ischemia but iNOS is not effect. Additionally, inhibition of nNOS and iNOS decreases adverse effects of hypoxia-mediated brain ischemia [51]. Therefore some researchers argue that nNOS inhibition afford neuroprotection in ischemia models [52,53]. In our study, we have found that hypoxia increases eNOS expression 9.8 fold, iNOS expression 1.47 fold and nNOS expression 3.6 fold in the brain tissues. Unlike the most animals, BMRs live in hypoxic underground tunnels and severe hypoxia is a part of BMRs life. For this reason, we believe that high increase of the brain nNOS and iNOS levels may be another protective element that *Nannospalax lehmingi* have against the hypoxia.

Consequently, our data indicate that, although production of NO and NOS enzymes in tissues of *Nannospalax lehmingi* are compatible with data obtained from other animals, it contains differences in some points. We think that these differences may be another characteristic features that BMRs have. Therefore, further research should aim to test our data with different animal research methods for reveal the properties of NO in BMRs.

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