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The nitric oxide production in tissues of 7- and 16-week-old rats under mobility restriction

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The electron paramagnetic resonance method was used to study the intensity of nitric oxide production by analyzing the amount of NO-containing paramagnetic complexes in the heart, liver, and spinal cord tissues of 7- and 16-week-old rats growing under restricted mobility conditions. Nitric oxide production was assessed by the intensity of the EPR signal belonging to the $(DETC)_2$-Fe^{2+}NO complex. The results obtained show that growth under conditions of restricted motility leads to an increase in NO production in all organs under study, with the greatest increase observed in 7-week-old rats.

Keywords: nitric oxide, heart, liver, spinal cord, electron paramagnetic resonance.

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Introduction

Mobility — is the basic property of animals and humans, inherent part of life and growth of each organism. Normal functioning of the body has been developed together with the extensive mobility. Life in modern manufacturing and urban environment offers an absolutely unusual form of activity for human body and systems which is far from the necessary requirements inherent to evolution in terms of mobility and safety. Labour and every day life automatization and computerization, passive leisure, transport development and other conditions determine modern inactive lifestyle. In the course of life, often under the influence of some environmental requirements, mobility level changes upwards and downwards. When lifestyle is changed in such way so that mobility becomes low, then the body shall adapt to this new state. In this conditions, specific adaptation develops limited to structural and metabolic malfunctions of many organs and body systems. Growing restriction of mobility becomes a serious threat to health, in particular increases the risk of coronary vascular diseases.

A wide factual evidence has been acquired regarding the influence of limited mobility on human and animals, however, large part of data was obtained during solution of certain space medicine problems. Limited mobility causes morphofunctional shifts in main life-supporting systems: nervous, cardiovascular, muscular, endocrine up to pathological states depending on duration and degree of limited mobility [1–6]. The main factor causing this process is the reduction of sensory input resulting in reduced central nervous system tone, change in synapse structure and function, muscle trophicity [6].

Participation of a free-radical compound — nitrogen oxide (NO) - in the development mechanisms of various pathological states of body is of great interest. NO is known as one of the most important signal molecules which control physiological functions of body and cell metabolism, it is widely spread in nervous and cardiovascular systems [7–14]. Excessive generation of NO may notably reduce smooth muscle cell tone, degrade endothelium function and directly suppress myocardial contractile function, which is observed in toxic and hemorrhagic shock, acute myocardial infarction [14–16]. On the one hand, NO has toxic action associated with mitochondrial oxidative phosphorylation, formation of free-radical peroxynitrite-anion compound which blocks a set of neural receptors, inactivates superoxide dismutase (SOD) enzyme and causes free-radical oxidation extension resulting in cell death [16–19]. In addition to vasodilatory, neurotransmitter and stress-limiting properties, participation of NO in oxidative stress reactions, glutamate-calcium pathway and inflammation is evident [13]. NO performs its physiological functions by bonding with ferrous ions (Fe) as part of haem or through S-nitrosylation of proteins and is involved in a range of biochemical reactions[13,20–23]. NO toxicity mechanism includes covalent modification of proteins in interaction with their thiol groups, and direct DNA damage. At the same time, there is opposite opinion that excessive NO serves as a compensatory factor. NO activates soluble heme-containing guanylate cyclase, increases cyclic guanosine monophosphate (cGMP) synthesis, and can protect neurons in toxic action of glutamate. Thus, dual nature of NO inherent in many natural modulators is manifested; protective and damaging properties of NO are probably defined by intracellular concentration [13,21–26]. NO

system is known to be one of the multifunctional factors of stress and adaptive response control. It is known that NO system plays an important role in body adaptation to various changes in environment and external conditions causing stress (Manukhina, Malyshev, 2000; Manukhina, 2006; Sitdikov, Zaripova, Gainutdinov, 2017). Currently, it has been proved that without normal cell metabolism of NO, optimum health condition maintenance and body adaptation to various environment factors, including to physical loads, are impossible [25–28].

Thus, NO system serves as one of the promising targets for therapeutic interventions in limited mobility conditions. The purpose of the research is comparative analysis of NO content in spinal marrow, heart and liver tissues in rats growing in unlimited and limited mobility conditions. Limited mobility was achieved by placement of the animals in special pencase-like cages beginning from the age of 3 weeks.

1. Research methods and management

Various partial and full animal immobilization methods are reported. Simulation of limited mobility of animals kept in small-size cages is widely used. Taking into consideration the characteristic of daily mobility restriction, simulation of growing mobility restriction with gradual increase in rat immobilization time in pencase-like cages meets the main requirements. By moving the partition, the volume of the pencase may be changed in accordance with size of the animal. During the first two days, mobility restriction time is 1 h, and is further increased by 2 h in each 2 days. The obtained experimental model allowed to create the same degree of „rigidity“ of mobility restriction for all animals which is a prerequisite for obtaining comparable results. This model is characterized by the absence of additional damaging factors and ease of implementation and gives opportunity to assess the observed changes as the result of body adaptation to mobility reduction. It should be noted that the used mobility restriction is not rigid and obviously reduces the stress reaction. Animals were let outside every day and they were able to compensate the forced hypokinesia.

The study was carried out on laboratory scrub rats which were subdivided into 2 groups: I — reference group kept in standard vivarium conditions; II — test group kept in restricted mobility conditions. The animals were subdivided into 2 groups by age: rats of age 7 weeks and weeks. Baby rats were growing in restricted mobility conditions until they reached the age of 3 weeks, therefore the mobility restriction duration was 30 days in the first group and 90 days in the second group. In each age group $n = 15$. One of the most efficient and direct NO detection and quantitative determination methods for biological samples is EPR spectroscopy with spin trap. The spin trap method is based on NO radical reaction with spin trap. A complex of Fe2+ with diethyldithiocarbamate (DETC) was used for NO capture and formation of stable ternary complex $(DETC)_2$-Fe^{2+}-NO [29,30]. To produce this complex in the body, DETC-Na aqueous solution at a dose of 500 mg/kg per 2.5 ml of water was introduced abdominally and iron citrate solution [iron (II) sulfate ($FeSO_4 \cdot 7H_2O$, Sigma, USA) at a dose of 37.5 mg/kg + sodium citrate, 187.5 mg/kg intramuscularly (the method was detailed before) [31,32]. Nitrogen oxide trap was introduced 30 min before dissection. The DETC-Fe (II) complex interacts with NO, and a stable radical $(DETC)_2$-Fe^{2+}-NO is formed. This complex is paramagnetic ($SFe = 1/2$, and $IN = 3/2$) and can be recorded by EPR method [30]. The complexes are characterized by an easily recognized EPR spectrum with g-factor $g = 2.035$ and triplet extra-thin structure. The amount of NO was assessed by the intensity of typical EPR signal belonging to $(DETC)_2$-Fe^{2+}-NO complex. The signals were compared by integral intensity, since the integral intensity of the EPR signal is directly proportional to paramagnetic complex concentration [30]. 30 min after introduction, the rat anesthetized with urethan was secured on the surgical table, dissected, the removed organs were quickly dried and frozen in liquid nitrogen in capillaries for measurements. For the study, heart, liver and spinal marrow tissues samples were taken. EPR spectra of the prepared samples were recorded using „Bruker“ EMX/plus ER-200E-SRC X range EPR spectrometer with ER 4112HV temperature accessory at 77 K. In all experiments, the following parameters were kept constant: microwave power — 30 mW, modulation — 5 G, amplification — 4104, time constant — 100 ms, spectrum recording time — 50 s and number of accumulations — 8. For accumulations and spectra recording, „Bruker“„Aspect 3000“ spectrometer computer was used. Directly before the measurement, the prepared sample cut to the shape of the measurement cell was weighed. Sample weight shall be about 100 mg. EPR spectra amplitude is always rated to the sample weight and EPR signal amplitude of the reference sample (EPR signal measurement techniques were detailed before in [31]).

Average measured value and standard error of mean M±SEM were calculated during statistical processing. Using Student-t criterium and Mann−Whitney U-criterium, valid differences were checked between average NO levels in tissues of rats of different ages. The differences were considered relevant at $p < 0.05$.

2. Results and discussion

EPR method was used to study heart, marrow and liver tissue of rats of age 7 and 16 weeks growing in restricted mobility conditions and reference rats of the corresponding age. In all measured EPR spectra, typical triplet signal from the spin trap complex $(DETC)_2$-Fe^{2+}-NO was recorded, its integral intensity was directly proportional to NO content in the sample.

For comparison of EPR spectra of heart tissues of 7- and 16-week rats growing in restricted mobility conditions,

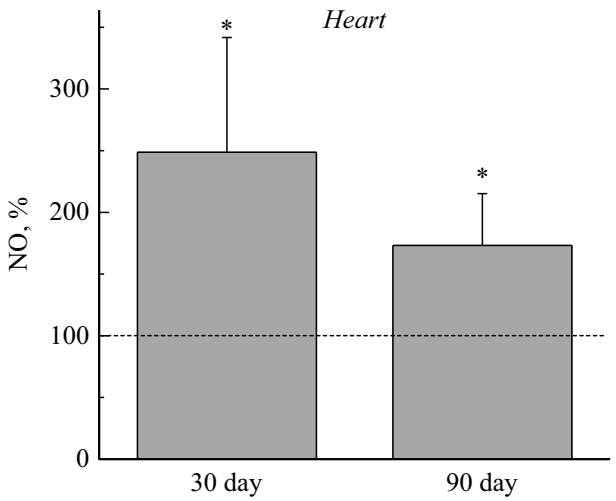

Figure 1. The change in NO production in rat heart tissues after 30- and 90-day mobility restriction compared with the reference group. Y-axis — change in integral intensity of signal from $(DETC)_2$-Fe^{2+}-NO complex in percentage compared with the reference group. Confidence compared with the reference group is: $^*p < 0.05$.

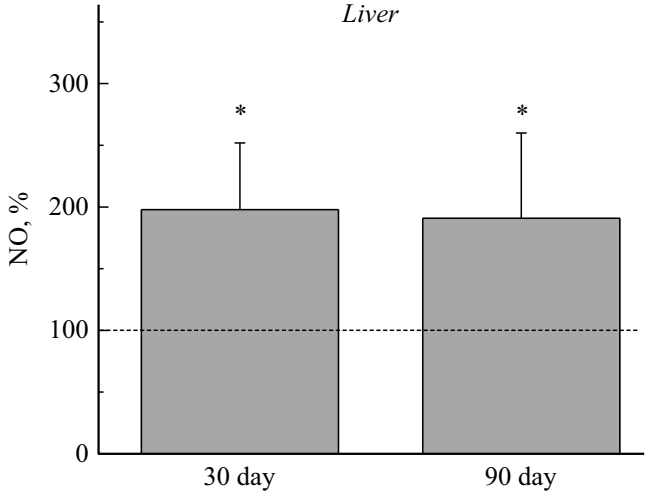

Figure 2. The change in NO production in rat liver tissues after 30- and 90-day mobility restriction compared with the reference group. Y-axis — change in integral intensity of signal from $(DETC)_2$-Fe^{2+}-NO complex in percentage compared with the reference group. Confidence compared with the reference group is: $^*p < 0.05$.

increase in NO content in heart tissues was detected (Figure 1): after staying in 30-day hypokinesia conditions by 148.7%, after 90-day hypokinesia — by 73.2% compared with reference group rats ($p < 0.05$). Growth in 30- and 90-day restricted mobility conditions caused increase in NO content in rat liver tissue by 98% and 91%, respectively, compared with the reference group ($p < 0.05$), Figure 2. In marrow tissues of baby rats growing in restricted mobility conditions during 30 days, increase in NO production intensity by 125% was detected relative to its level in reference rats of the corresponding age ($p < 0.05$). And for mobility restriction during 90 days, NO production in marrow tissues did not differ from that in reference group rats (Figure 3).

Thus, growth in restricted mobility conditions causes increased NO production in all test tissue samples, except the marrow tissues in rats growing with 90-day mobility restriction. Rat body reaction to hypokinesia depends on the mobility restriction time. The most pronounced increase in NO content in rat tissues was detected in 30-day mobility restriction conditions. Most probably, this is caused by the age: 7-week age — beginning of adolescence. Also, during 1 month of restricted mobility local stress-limiting systems are activated at organ level,and nitrogen oxide generation system is one of them. Probably, growth in 30-day mobility restriction conditions causes mobilization of all stand-by adaptation mechanisms. For mobility restriction during 90 days, increase in nitrogen oxide production was also detected, however, it was not so pronounced as for 30-day hypokinesia. This is probably associated with the development of adaptive effect of the adequate stress reaction by this time. NO system activation is one of those mechanisms due to which the body prevents stress damages.

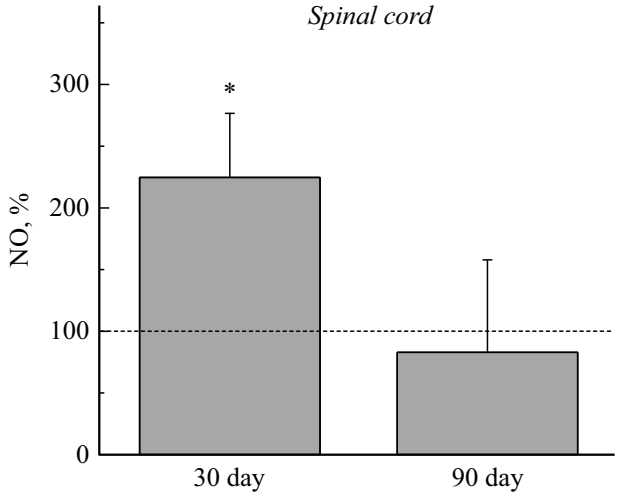

Figure 3. The change in NO production in rat marrow tissues after 30- and 90-day mobility restriction compared with the reference group. Y-axis — change in integral intensity of signal from $(DETC)_2$-Fe^{2+}-NO complex in percentage compared with the reference group. Confidence compared with the reference group is: $^*p < 0.05$.

Conclusion

The increase in NO production intensity detected by us during mobility restriction suggests the presence of tight links between NO level in the body and mobility conditions, in particular in a growing body. Since our model is composed of two components: directly hypokinesia and stress from the applied procedures. Thus' there are NO-dependent mechanisms of body response to immobilization stress. This is important, because any experimental mobility

restriction technique includes stress component which cannot be extracted as it is. It is interesting that the increase in NO level in cells regardless of its source efficiently prevents significant growth of its amount in stress and related tissue damage by means of enzyme inducible form inhibiting (iNOS) or through formation of protective antioxidant or other proteins [33], i.e. there is a complex feedback mechanism. The obtained data expand the understanding of the role of nitrogen oxide and NO-synthases in the activity of internal organs of rats growing in stress conditions in early postnatal ontogenesis. In mobility restriction conditions, NO system may stabilize and prevent destruction of skeletal muscles and other tissues and launch molecular adaptation mechanisms Since it is known that hypokinesia causes significant changes in cardiovascular system, internal organs, blood flow system and oxygen supply, then it can be suggested that a part of these changes was caused by stationary increase in nitrogen oxide production in key tissues of the body.

Compliance with ethical standards

The research was carried out in accordance with the Basel Declaration and recommendation of the local bioethical committee of Kazan Federal University.

Conflict of interest

The authors declare that they have no conflict of interest.

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