

Original article

The influence of non-opiate analogue of leu-enkephalin to the cardiac consequences of intrauterine hypoxia of albino rats

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Abstract: *Objective* — Our study aimed to evaluate the possibility of correcting cardiac consequences of intrauterine hypoxia (IUH) by injecting leu-enkephalin analog, lacking affinity for opiate receptors, in the early postnatal period.

Material and Methods — To model IUH, we placed pregnant Wistar rats in a hypobaric chamber with an oxygen partial pressure of 52 mmHg. The procedure was repeated for 4 h daily over the 15th-19th days of gestation.

From the 2nd through the 6th days of their lives, the offspring were injected intraperitoneally with non-opiate leu-enkephalin analog at a dose of 100 µg/kg (NALE: Phe-D-Ala-Gly-Phe-Leu-Arg). This analog did not have affinity for opiate receptors. The 7- and 60-day old offspring of female rats subjected to IUH were investigated. The control group included the descendants of intact animals. We investigated gravimetric indicators, DNA-synthetic activity of cardiomyocytes (CMC) by tritium-labeled thymidine autoradiography method, the size of the CMC nuclei, as well as size and amount of nucleoli in the CMC nuclei. The activity of free radical oxidation was evaluated in cardiac homogenates by chemiluminescence.

Results — In 7-day old rats subjected to IUH vs. control animals, we observed decreases in body mass by 32.6%, in heart mass by 27.3%; in the proportion of ³H-thymidine labeled CMC nuclei by 32.7% in the left ventricle and by 30.4% in the right ventricle; in the number of nucleoli in the CMC nuclei (in the left ventricle: control – 2.384±0.027, IUH – 2.282±0.027*, p<0.05; in the right ventricle: control – 2.409±0.038; IUH – 2.240±0.012*, p<0, 05). Increase in CML indices of cardiac homogenates was revealed, indicating the activation of free radical oxidation. In 7-day old rats subjected to IUH and administration of the NALE peptide from the 2nd through the 6th days of their lives, the proportion of ³H-thymidine labeled nuclei in the CMC did not differ from the control (in the left ventricle: control – 12.79±0.89%, IUH + NALE – 10.98±0.95%, p>0.05; in the right ventricle: control – 11.61±0.78%; IUH + NALE – 11.26±0.58%, p>0.05). The number of nucleoli in the CMC nuclei of the left and right ventricles in the heart of 7-day old animals in the IUH + NALE group did not differ from the control too. The CML indices of heart homogenates in the IUH + NALE group were significantly lower than those in the IUH group.

In 60-day old male rats exposed to IUH, there was a decrease in heart mass by 18.5%, sizes of CMC nuclei by 7.5% and 16.1% in the left and right ventricles, respectively, and in the total nucleoli area in the CMC nuclei of the left ventricle (control – 3.953±0.085; IUH – 3.372±0.078*; p<0.05). In 60-day old male rats subjected to IUH and injections of the NALE peptide from the 2nd to the 6th days of their lives, heart mass (control – 692.73±26.81 mg; IUH + NALE – 631.0±29.79 mg; p>0.05) and the size of the CMC nuclei of the right ventricle (control – 54.25±0.84; IUH + NALE – 55.24±0.94; p>0.05) did not differ significantly from the control. The size of the nuclei, the number and size of the nucleoli in the CMC of the left ventricle, as well as the area of the nucleoli in the CMC of the right ventricle in 60-day old male rats of the IUH + NALE group significantly exceeded control group values.

Conclusion — Administration of the NALE peptide to albino rats subjected to IUH normalized DNA-synthetic activity and the number of nucleoli in the nuclei of CMC in 7-day old animals, and also reduced the severity of oxidative stress in the heart tissue. In 60-day old albino male rats exposed to IUH, injecting NALE from the 2nd to the 6th days of their lives eliminated declines in heart mass and sizes of the CMC nuclei and nucleoli, and also led to an increase in the values of the nucleus-and-nucleolus complex indices compared with the control.

Keywords: intrauterine hypoxia, cardiomyocytes, biologically active peptides.

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Introduction

Intrauterine hypoxia (IUH) induces pronounced changes in the myocardium of mammals. Resulting decrease in the proliferative and anabolic activities of cardiomyocytes (CMC) was noted by J.L.

Morrison et al. (2007) [1]; M. Ream et al. (2008) [2]; K.J. Botting et al. (2012) [3]. Myocardial damage in IUH conditions has significant clinical consequences [4], which can occur in the long run rather than at early stages of postnatal ontogenesis alone. D. Barker

(2003) described the phenomenon of the so-called “fetal programming” in individuals born with an IUH-caused low body mass (suggesting an early gestational age) in the form of arterial hypertension and coronary heart disease in adulthood [5]. Hence, there is a vital need to search for the factors that can restore myocardium condition after an unfavorable intrauterine period.

Opioid peptides are crucial among endogenous factors with a cardioprotective effect. Enkephalins, endogenous ligands of δ -opioid receptors, are considered the triggers of ischemic preconditioning of the myocardium [6].

The objective of our study was to evaluate the possibility of correcting early and long-term cardiac consequences of IUH by injecting a peptide leu-enkephalin analog in the neonatal period of ontogenesis.

Material and Methods

Study object

The study was performed on albino Wistar rats. The animals were kept in vivarium conditions at a temperature of 22 °C and *ad libitum* access to a standard granular food for laboratory rodents and water.

Tested compound

We used intraperitoneal administration of the Phe-D-Ala-Gly-Phe-Leu-Arg peptide (Almabion, LLC, Russian Federation). The amino acid sequence in this peptide was similar to the structure of leu-enkephalin synthetic analog Dalargin (Tyr--D-Ala-Gly-Phe-Leu-Arg), a non-selective agonist of μ/δ -opiate receptors. However, the peptide in our study lacked affinity for opiate receptors due to the absence of tyrosine at the N-terminal position [7]. Hence, we named the Phe-D-Ala-Gly-Phe-Leu-Arg peptide “non-opiate analog of leu-enkephalin”, or NALE peptide.

Study design and experimental groups

In order to conduct the experiment, 3-4 month old female rats were placed with sexually mature male rats at a 4:1 ratio. Pregnancy was established by the presence of sperm in vaginal smears. Pregnant female rats were subjected to 4 h hypoxic exposure daily over the 15th-19th days of gestation. A hypobaric hypoxia model was used: the animals were placed in an experimental hypobaric chamber, where atmospheric pressure was reduced to 250 mmHg, which corresponded to a partial oxygen pressure of 52 mmHg. Decreases and increases in pressure were conducted gradually, over 1 h, to exclude barotrauma, and the duration of stationary hypoxia was 2 h. Examination of the offspring of hypoxic female rats was conducted on the 7th and the 60th days of their ages. The control group included the progenies of intact animals.

Three experimental groups were formed:

1) The control group including the offspring of intact (not exposed to hypoxia) female rats subjected to daily intraperitoneal injections of 0.1 ml isotonic sodium chloride solution from the 2nd through the 6th days of their lives.

2) IUH treatment group encompassing the offspring of hypoxic female rats subjected to daily intraperitoneal injections of 0.1 ml isotonic sodium chloride solution from the 2nd through the 6th days of their lives.

3) IUH + NALE treatment group comprising of the offspring of hypoxic female rats subjected to daily intraperitoneal injections of NALE peptide (Almabion, LLC, Russian Federation) at a dose of 100 $\mu\text{g}/\text{kg}$ in 0.1 ml of solvent (isotonic sodium chloride solution) from the 2nd through the 6th days of their lives.

The animals were removed from the experiment by rapid decapitation under anesthesia with chloroform vapor. Body mass, absolute heart mass and heart mass index (heart mass to body mass ratio expressed in percent) of each animal were measured.

Conducting histological and morphometric study

Rat hearts underwent standard histological processing with paraffin tissue section preparation. To identify nucleolar organizer regions, heart sections (after dewaxing) were stained with silver nitrate according to the AgNOR method [8]. Morphometry of the CMC nucleus-and-nucleolus complex was executed on a MEKOS-C computer image analyzer: the size (area) of the CMC nucleus, the number and total area of the nucleoli in the CMC nucleus were evaluated. The CMC of subendocardial zones of the left and right heart ventricles were subjected to morphometry. The choice of myocardial zones was justified by the smallest representation of non-muscle heart cells in them [9] and their highest sensitivity to hypoxic effects [10].

Conducting autoradiographic study

DNA-synthetic activity of CMC in seven day old animals was evaluated using tritium-labeled thymidine autoradiography. One hour before euthanasia, animals were injected intraperitoneally with ³H-thymidine at a dose of 1 μCi per 1 g of a body mass. After a standard histological procedure, heart sections were dewaxed, covered with ILFORD nuclear emulsion (UK) and exposed in a lightproof container for 21 d. After exposure, autoradiographs were treated with an X-ray developer, fixed in a 33% sodium hyposulfite solution and stained with hematoxylin and eosin. The labeling index (LI, %) was determined by viewing 1000 CMC nuclei in the subendocardial layer of the myocardium of the left and right heart ventricles.

Conducting chemiluminescence study

To analyze the activity of free radical oxidation in the heart homogenates of 7-day old animals, the chemiluminescence (CML) method was used. CML was recorded on an LS 50B PerkinElmer Inc. luminescent spectrometer. The signal was standardized using the FinLab firmware. The following indices were determined: S_{sp} – total intensity of emitted light over 1 min of spontaneous CML; H1 – maximum amplitude of a fast flash of Fe^{2+} -induced luminescence; S_{ind1} – total intensity of emitted light over 2 min of Fe^{2+} -induced CML; H2 is the maximum amplitude of the H_2O_2 -induced luminol-dependent CML; S_{ind2} is the total intensity of emitted light over 2 min of H_2O_2 -induced luminol-dependent CML. CML intensity was expressed in relative units.

Statistical analysis

Statistical processing of experimental data was conducted using Statistica 6.0 software. After confirming the normality of the data distributions, the sample means and standard errors were determined – $M \pm \text{SEM}$. Comparison of the groups was performed using the *t*-test. Differences were considered significant at $p < 0.05$.

Table 1. Indicators of 7-day old albino rats subjected to IUH and neonatal administration of the non-opiate leu-enkephalin analog

	Control	IUH	IUH + NALE
Body mass, g	16.07±0.61	10.83±0.91, p<0.001	12.10±0.82, p<0.001
Heart mass, mg	116.12±5.58	84.38±8.07 p=0.010	89.29±9.86 p=0.040
Heart mass index, %	0.72±0.03	0.78±0.03 p=0.120	0.73±0.05 p=0.720
CMC labeling index of the LV, %	12.79±0.89	8.61±0.34 p<0.001	10.98±0.95 p=0.37
CMC labeling index of the RV, %	11.61±0.78	8.08±1.04 p=0.010	11.26±0.58 p=0.832
Nucleoli number in CMC nuclei of the LV	2.384±0.027	2.282±0.027 p=0.025	2.399±0.040 p=0.540
Nucleoli number in CMC nuclei of the RV	2.409±0.038	2.240±0.012 p<0.001	2.363±0.034 p=0.461

CMC, cardiomyocytes; LV, left ventricle; RV, right ventricle; IUH, intrauterine hypoxia; NALE, Phe-D-Ala-Gly-Phe-Leu-Arg peptide; P-level is presented for differences in relation to the control group.

Table 2. Chemiluminescence indices of heart homogenates in 7-day old albino rats subjected to IUH and neonatal administration of the non-opiate leu-enkephalin analog

	Control	IUH	IUH + NALE
S _{sp}	1.434±0.088	3.171±0.177 *p<0.001	2.271±0.133 *p=0.020, #p=0.001
S _{ind1}	3.802±0.173	7.186±0.368 *p<0.001	5.205±0.314 *P=0.002, #p=0.001
H1	1.251±0.095	3.908±0.198 *p<0.001	2.122±0.204 *p=0.002, #p<0.001
S _{ind2}	8.968±0.395	18.077±0.712 *p<0.001	10.996±0.432 *P=0.004, #p<0.001
H2	4.774±0.285	15.088±0.477 *p<0.001	6.181±0.415 *P=0.001, #p<0.001

IUH, intrauterine hypoxia; NALE, Phe-D-Ala-Gly-Phe-Leu-Arg peptide; * P-level is presented for differences in relation to the control group; # P-level is presented for differences in relation to the IUH group.

Table 3. Indicators of 60-day old albino male rats subjected to IUH and neonatal administration of the non-opiate leu-enkephalin analog

	Control	IUH	IUH + NALE
Body mass, g	144.86±5.75	130.75±4.33 p=0.151	139.50±6.08 p=0.542
Heart mass, mg	692.73±26.81	564.50±17.49 p<0.001	631.0±29.79 p=0.112
Heart mass index, %	0.48±0.02	0.44±0.01 p=0.091	0.46±0.02 p=0.453
Nucleoli number in CMC nuclei of the LV	2.542±0.064	2.660±0.073 p=0.180	2.860±0.068 p=0.007
Nucleoli number in CMC nuclei of the RV	2.448±0.058	2.570±0.068 p=0.271	2.495±0.066 p=0.560
Total area of the nucleoli in the CMC of the LV	3.953±0.085	3.372±0.078 p<0.001	4.725±0.115 p<0.001
Total area of the nucleoli in the CMC of the RV	3.383±0.074	3.325±0.091 p=0.432	3.665±0.089 p=0.016
Area of the nuclei in the CMC of the LV	51.84±0.94	47.97±0.98 p=0.005	61.54±0.91 p<0.001
Area of the nuclei in the CMC of the RV	54.25±0.84	45.52±0.92 p<0.001	55.24±0.94 p=0.741

CMC, cardiomyocytes; LV, left ventricle; RV, right ventricle; IUH, intrauterine hypoxia; NALE, Phe-D-Ala-Gly-Phe-Leu-Arg peptide; P-level is presented for differences in relation to the control group.

Results

Seven-day old animals exposed to IUH were characterized by a significant (32.6%) reduction in body weights. A decrease in heart mass index (by 27.3%) was also noted. Moreover, the heart mass index of 7-day old rats in a treatment group did not differ from the control (Table 1).

IUH caused a drop in the DNA-synthetic activity of myocardial cells in 7-day old animals: LI in the CMC of the left ventricle was reduced by 32.7% and of the right ventricle by 30.4% (Table 1). We also recorded a decrease in the nucleoli number of the CMC nuclei: by 4.3% in the left ventricle and by 7.0% in the right ventricle (Table 1).

In 7-day old animals subjected to IUH and five-time administration of the NALE peptide from the 2nd through the 6th days of their lives, a significant decrease in body weight (by 24.7%) and heart weight (by 23.1%) was registered, compared with the control group (Table 1). At the same time, injecting NALE peptide from the 2nd through the 6th days of life leveled out posthypoxic changes in DNA-synthetic processes and the nucleoli number in the CMC of experimental animals (Table 1). LI and nucleoli number in the CMC of the subendocardial layer of the left and right ventricles in 7-day old animals of the IUH + NALE group did not differ from the control values.

In the heart homogenates of 7-day old rats subjected to IUH, we recorded increases in the intensity of generation of reactive oxygen species (ROS) (S_{sp}) by 121.1%, in the content of peroxide radicals (H1) in the tissue by 212.4%, and in the rate of formation of peroxide radicals (S_{ind1}) by 89.0% (Table 2). We also discovered a decline in peroxide resistance of the substrate (increase in H2) by 216% and the activity of the antioxidant defense system (increase in S_{ind2}) by 101.6%. In the heart homogenates of 7-day old rats subjected to IUH and five-time administration of the NALE peptide from the 2nd to the 6th days of their lives, a significant increase in CML indices was also revealed with respect to the control. However, the difference from the control values was significantly less pronounced than in the group of animals that did not receive peptide correction. Percent changes from the S_{sp} indicator, H1, S_{ind1}, H2 and S_{ind2} were 58.4, 69.6, 36.9, 29.4 and 22.6, correspondingly. CML indices for the IUH + NALE group were significantly lower than for IUH group (Table 2).

In 60-day old male rats exposed to IUH, a decrease in the absolute heart mass by 18.5% was observed, compared with the parameter value in control group. An analysis of the morphometric parameter values of the CMC nucleus-and-nucleolus complex revealed a decrease in the size of the CMC nuclei of subendocardial parts of the left and right ventricles (by 7.5% and 16.1%, respectively), and in the average total area of the nucleoli in the CMC nuclei of the subendocardial layer of the left ventricle by 14.7% (Table 3).

60-day old male rats subjected to IUH and five-time administration of the NALE peptide from the 2nd through the 6th days of their lives had a body mass that did not differ from the control group value (Table 3). In the animals of this group, in contrast to the IUH group, we did not register any decrease in morphometric parameters of the CMC nucleus-and-nucleolus complex. Moreover, in male rats of the IUH + NALE group, compared with control animals, we detected an increase in the size of the nuclei by 18.7%, along with increases in the number of nucleoli by 12.5% in the CMC of the left ventricle and in the total

area of the nucleoli in the nuclei of the left and right ventricles' CMC by 19.5% and 8.3%, respectively.

Discussion

IUH induces a decrease in the DNA-synthetic activity of CMC in the developing heart. We have previously shown that albino rats exposed to IUH had a reduction in the DNA-synthetic activity of CMC at the age of 1-day old [11]. In the current study, a similar effect was shown for 7-day old animals. L.M. Reyes et al. (2018) reported a decrease in the proliferative activity of heart cells in mammals after prenatal hypoxia [12]. CMC proliferation occurs in mammals, mainly in the antenatal and early postnatal periods of ontogenesis [13]. S. Bae et al. (2003) stated a significant decrease in the CMC numbers and their hypertrophic changes in the heart of newborn rats that have undergone IUH [14]. The number of CMC and their ploidy determine the structural reserve of the heart over the subsequent ontogenetic periods [13].

In addition to impaired DNA synthesis in experimental newborn animals exposed to IUH, we detected a decline of the nucleoli number in the CMC nuclei at the ages of 1-day old [11] and 7-day old. A decrease in the nucleoli number and disruption in the structure of the CMC nucleolar complex reflects the condition of the cellular stress, and correlates with the induction of apoptosis and with increased content of p53 protein, which has both antiproliferative and proapoptotic effects on cells [15].

CMC structural changes were accompanied by significant oxidative stress at a tissue level. In heart tissue homogenates of experimental 7-day old animals, we recorded stimulation of free radical oxidation and decrease in antioxidant activity. Hypoxia is a classic inducer of oxidative stress in cells. Under IUH, oxidative stress is exacerbated by the low activity of antioxidant systems in fetal cells and cells of newborn mammals. An excess of reactive oxygen species (ROS) during oxidative stress causes epigenetic changes. It is shown that IUH, through an increase in the concentration of ROS in tissues, induces a decrease in the amount of protein kinase C ϵ in the hearts of rat fetuses, which is associated with DNA hypermethylation in the promoter of the C ϵ protein kinase gene [16]. Protein kinase C ϵ plays a significant role in the processes of CMC growth and adaptation of heart cells to damaging factors [17].

Deviations in the values of the indices reflecting the state of the heart in the animals subjected to IUH were maintained at least until the period of maturity. In 60-day old male rats subjected to IUH, we found a decrease in heart mass, a decrease in the size of the CMC nuclei, as well as a smaller total area of the nucleoli in the CMC nuclei of the left ventricle. In our previous study conducted on the same experimental model, we detected a decrease in the nucleoli number in the CMC nuclei [11]. The revealed changes may indicate the persistence of structural deficit in the myocardium of adult animals exposed to IUH, and a decrease in the functional reserves of the hearts in experimental animals [18]. This corresponds to the "fetal programming" concept [5], which describes the relationship between the fetal growth retardation syndrome, caused by unfavorable conditions for intrauterine ontogenesis, and the formation of cardiovascular pathology at later ontogenetic stages [19].

We assumed the possibility of correcting posthypoxic changes in the heart and eliminating unfavorable cardiac manifestations of fetal programming *via* using neonatal administration of the NALE peptide. The choice of the exposure period was justified by the fact that DNA methyltransferase activity, which determines

epigenetic changes, is closely related to the DNA-synthetic activity of cells [20]. The latter reaches the maximum for CMC in rats during the neonatal period. Administering opioid receptor ligands at early ontogenetic stages can cause hormonal imprinting of the endogenous opioid system and irreversible changes in its functioning [21]. This fact defined our interest in a biologically active peptide, lacking affinity for opioid receptors.

Administration of NALE peptide from the 2nd through the 6th days of life did not normalize gravimetric indices of experimental 7-day old animals. A decrease in gravimetric indices' values under the influence of IUH is formed in the antenatal period and is detected immediately after birth in 1-day old experimental rat pups [11]. In the current study, the introduction of the peptide in the neonatal period could not lead to the rapid restoration of body mass and heart mass of experimental animals. However, the effects of NALE have corrected the posthypoxic abnormalities of the DNA-synthetic activity of CMC and changes in the nucleolar complex of myocardial cells in 7-day old animals subjected to IUH. The corrective effect of the neonatal administration of the NALE peptide on the cardiac consequences of IUH may be due to its ability to normalize the processes of free radical oxidation at the tissue level under conditions of oxidative stress. CML-indicators of free radical oxidation in the heart homogenates of 7-day old rats subjected to IUH and neonatal administration of the NALE peptide had significantly lower values than those in animals undergoing IUH without peptide correction. The revealed effect implied the presence of pronounced antioxidant properties in the NALE peptide. Earlier, we discovered the antioxidant and cytoprotective effect of the NALE peptide *in vitro* [22], which indicated a direct action mechanism of the peptide without mediation by the regulatory systems of the body.

In 60-day old animals subjected to IUH, posthypoxic changes in the heart condition (decreased heart mass, decreased size of the nuclei and nucleoli in CMC) were completely offset by the introduction of the NALE peptide during the neonatal period. Moreover, in animals treated with the peptide from the 2nd through the 6th days of life, we detected an increase in the CMC nucleus-and-nucleolus complex indices *versus* control values. The discovered changes could be accounted for by increased anabolic activity of CMC in adult animals treated with the NALE peptide over the neonatal period.

The mechanisms of the "reprogramming" effect of the NALE peptide on CMC require further investigation. Currently, the research is underway to assess the involvement of the nitric oxide system and the nociceptin peptide system in the implementation of NALE effects.

Conclusion

Non-opiate leu-enkephalin analog injections in the neonatal period of ontogenesis eliminated a number of early and delayed pathological changes in the heart of experimental animals subjected to IUH. Thus, this particular Phe-D-Ala-Gly-Phe-Leu-Arg peptide can be considered a promising basic tool for the development of a pharmaceutical drug intended for the correction of IUH cardiac consequences.

Limitations

Each experimental group included 12-14 animals. While greater sample sizes could have increased the reliability of our

results, high complexity of experimental design and morphometric measurements have limited the numbers of animals involved in the study. Besides, we followed the ethical principles set forth in the “European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes” (Strasbourg, 1986) on using the minimum sufficient animal numbers to obtain statistically significant results.

The resulting morphometric indices may have had inaccuracies associated with morphometry conducted on tissue sections rather than isolated cardiomyocytes.

Ethical approval

The study took into account all international, national and institutional requirements for animal welfare and experimental procedures. The experiments were carried out in accordance with the guidelines of the “European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes” (Strasbourg, 1986). The study received permission from the Ethics Committee of the Federal State Budgetary Institution of Higher Education, The Far Eastern State Medical University of the Russian Federation Ministry of Healthcare, the protocol No. 2 of February 5, 2019). In total, 112 animals were included in our experiments.

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Conflict of interest: none declared.

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