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Morphofunctional features in mice treated by low and high Hsp70 doses

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Abstract

Aim. We sought to assess the effects of exogenous Hsp70 (single subcutaneous low- and high-dose injections) on organ structure and functions in adult mice.

Materials and methods. We randomized CD1 90-day-old male mice (n = 30) to three groups (10 mice per group). We injected the animals with single subcutaneous saline solution for Group 1 (control), low dose (500 μg/kg) of recombinant human Hsp70 (HspA1A) for Group 2, and high dose (5000 μg/kg) of the Hsp70 for Group 3. We examined the behavior of the mice on Day 3 after the injections (distance traveled, velocity, and bowel movement number). We lethalized the mice on Day 5 with further histological study and morphometrics of cerebral cortex, thymus, spleen, and liver. The statistics included one-factor ANOVA test with *post hoc* Tukey test.

Results. All study groups exhibited no significant difference of behavioral parameters. Some liver sinusoids were wider in control group and Hsp70 500 μg/kg group comparing to Hsp70 5000 μg/kg group. We obtained also data for morphometrics: no difference was found for the number of neurons in ganglionic cerebral cortex, the lymphocytic cellularity difference between thymic cortex and medulla, the number of lymphocytes in white splenic pulp, and the number of hepatocyte nuclei in the liver. Red splenic pulp exhibited 1774,5 ± 24,8, 1623,0 ± 26,7, 1553,6 ± 47,0 macrophages for control, low-dose and high-dose groups, respectively (p < 0,0001). Tukey test showed a significant difference between control group and each of Hsp70 groups 500 μg/kg (p = 0,012) and 5000 μg/kg (p < 0,0001).

Conclusion. Our study revealed no negative impact of subcutaneous Hsp70 administration at low and high doses on organ structure and functions in mice.

Keywords: heat-shock proteins; recombinant human Hsp70; chaperones; morpho-functional characteristics; exogenous Hsp70 in mice; HspA1A

MeSH terms:
STRESS, PHYSIOLOGICAL
HSP70 HEAT-SHOCK PROTEINS – ADMINISTRATION & DOSAGE
HSP70 HEAT-SHOCK PROTEINS – PHARMACOLOGY

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Ethics statements. The study complies with the standards of the EU Directive for the Protection of the Vertebrate Animals used for Experimental and other Scientific Purposes. All manipulations with animals were approved by the Local Bioethics Committee of the Sechenov First Moscow State Medical University (Sechenov University), No. 04-23 of 02.03.2023.
Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request. Data and statistical methods used in the article were examined by a professional biostatistician on the Sechenov Medical Journal editorial staff.

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Морфофункциональные изменения у мышей после однократного введения высоких и низких доз Hsp70

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Аннотация

Цель. Оценить влияние экзогенного Hsp70 (heat shock protein, белок теплового шока массой 70 кДа) на морфофункциональное состояние взрослых мышей при его однократном подкожном введении в высоких и низких дозах.

Материалы и методы. Изучались самцы мышей линии CD1 (n = 30) возрастом 90 ± 3 суток, рандомизированные на три группы по 10 животных в каждой. Вводили однократно подкожно: 1-й группе (контроль) – физиологический раствор, 2-й группе – низкую дозу Hsp70 (500 мкг/кг) и 3-й группе – высокую дозу Hsp70 (5000 мкг/кг) рекомбинантного человеческого HspA1A. На 3-й день регистрировали поведенческую активность: скорость перемещения, пройденный путь и количество дефекаций. На 5-й день мышей выводили из эксперимента с последующим гистологическим исследованием и морфометрией срезов коры головного мозга, тимуса, селезенки и печени. Статистическая обработка данных осуществлялась при помощи однофакторного дисперсионного анализа и апостериорного теста Тьюки.

Результаты. В изученных группах не обнаружено статистически значимой разницы поведенческих показателей. Синусоидные капилляры печени в группе контроля и группе Hsp70 500 мкг/кг оказались немного шире и полнокровнее по сравнению с Hsp70 5000 мкг/кг. При морфометрии клеток получены следующие результаты: число нейронов в ганглионарном слое коры больших полушарий головного мозга, разница лимфоцитарной клеточности между корковым и мозговым веществами тимуса, число лимфоцитов в белой пульпе селезенки, количество ядер гепатоцитов в печени в группах не различались. В красной пульпе селезенки количество макрофагов составило 1774,5 ± 24,8, 1623,0 ± 26,7, 1553,6 ± 47,0 в группах контроля, низкой и высокой доз Hsp70 соответственно (p < 0,0001). В тесте Тьюки статистически значимые различия получены между группой контроля и группами Hsp70 500 мкг/кг (p = 0,012) и 5000 мкг/кг (p < 0,0001).

Заключение. Исследование не выявило негативного влияния подкожного введения низких и высоких доз Hsp70 на морфофункциональные показатели у мышей.

Ключевые слова: белки теплового шока; рекомбинантный человеческий Hsp70; шапероны; морфофункциональная характеристика; экзогенный Hsp70 у мышей; HspA1A
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Соответствие принципам этики. Исследование проведено с соблюдением положений Европейской конвенции о защите позвоночных животных, которые используются для экспериментальных и других научных целей. Все манипуляции с животными проведены в соответствии с разрешением Локального этического комитета ФГБОУ ВО «Первый МГМУ им. И.М. Сеченова» Минздрава России (№ 04-23-02.03.2023).
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Hsp70 – 70 kilodalton heat shock proteins

The molecular origins of numerous pathological processes include a disrupted integrity of cellular proteins (proteome). Therefore, such disorders require therapeutic approaches to protect protein structure and/or restore their properties. Among the protectors, there is a diverse group of compounds called molecular chaperones. These macromolecules maintain proteostasis via a continuous support of a balanced proteome. Actually, molecular chaperones restore the proper native structure (tertiary or quaternary) of proteins, including through the formation of intermediate protein complexes with them and their subsequent dissociation. Their key regulation is due to a group of heat shock proteins weighing 70 kilodaltons (70 kilodalton heat shock proteins, Hsp70) [1].

Various members of the Hsp 70 family ensure homeodynamics in most of cells of the body by proteome quality control (PQC), thereby preventing the aggregation of misfolded proteins or their components. The mechanisms of PQC include both protection from errors in post-translational modifications (folding and refolding) and activation of proteasomes and autophagy. Moreover, Hsp70 either prevents apoptosis or directs the formation of apoptosomes [2].

It is obvious that Hsp70-induced biochemical reactions concern any cell of the body. However, the molecular chaperones are crucial for long-living cell populations, such as those of nervous tissue. For instance, Hsp70 contributes to the resistance of neurons to premature degradation [3, 4], mitigating glial reactions to proinflammatory cytokines [5, 6]. Recently, we have seen a surge of interest in pharmacological options to induce or modify Hsp70 pathways in nervous tissue [7]. This tendency directed many research teams to examine the role of Hsp70 in neuronal protection from neurodegeneration, such as Alzheimer's disease or cerebral ischemia [8]. However, most authors focus on easy-to-obtain therapeutic options only. As a result, no comprehensive understanding is present for the fundamental machinery of Hsp70 activation in neurons and glia *in vivo* [9]. In addition, Hsp70 affects different intracellular pathways, so its hyperexpression or activity may lead to some adverse effects. For example, it is possible in stress that increases the production of molecular chaperones. Perhaps, a targeted delivery of Hsp70 would be a safer but a more expensive approach. Its feasibility depends on the possible benefit of exogenous Hsp70 in neurons and glia compared to the Hsp70 of nervous tissue.

The study aims to assess morphofunctional changes in the organs of the nervous and immune systems, as

[2] https://rscf.ru/project/23-25-00448/ (accessed: 12.06.2023).

well as in the liver, upon intake of excessive amounts of exogenous Hsp70.

MATERIALS AND METHODS

Animal manipulation

All experiments with animals were carried out in accordance with domestic and international guidelines[3,4,5]. The study included 30 male CD1 mice. The number of animals corresponded to the standard rules for experimental design in rodents [10]. We received the animals from the Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia. The mice stayed in a vivarium at a constant room temperature of 20–22 °C and humidity of 50–60% with free access to clean water and granulated food *ad libitum*.

On Day 0 of the study, the animals aged 90 ± 3 days with body weight of 33.2 ± 2.3 g. The age satisfied a need to assess changes in the organs of the nervous and immune systems. Exactly, the cerebral cortex should be mature and there should be no evidence of thymic involution. Since male and female mice got thymic involution at different age, the only sex is preferred [11, 12].

Hsp70 family's member 1A (HspA1A) is the most stress-related molecular chaperone [13]. It served to affect nervous system with the following assessment by behavior study and histological study with morphometrics. We also studied the systemic adverse effects upon administering Hsp70 by a histological examination of the liver (as it metabolizes exogenous Hsp70) as well as of the thymus and spleen. The experimental design is presented in Fig. 1.

After a two-week quarantine, we randomized the mice using the random number method into 3 groups of 10 rodents. Animals of each group stayed separately, 5 animals per cage, in a separate room of the vivarium. All individuals received single subcutaneous injections on Day 0: Group 1 – saline (control), Group 2 – low dose of Hsp70 (recombinant human HspA 1A, RAS Institute of Biomedicine, Russia, 500 μg/kg), Group 3 – high dose of Hsp70 (same medication and manufacturer, 5000 μg/kg).

On Day 3, all mice underwent a physiological "open field" test for two minutes at the time range 8:00 am – 8:30 am at 40 lx ("Open field for mice", NPK Open Science LLC, Russia). An hour before the test, the mice stayed calm. We recorded parameters of behavioral

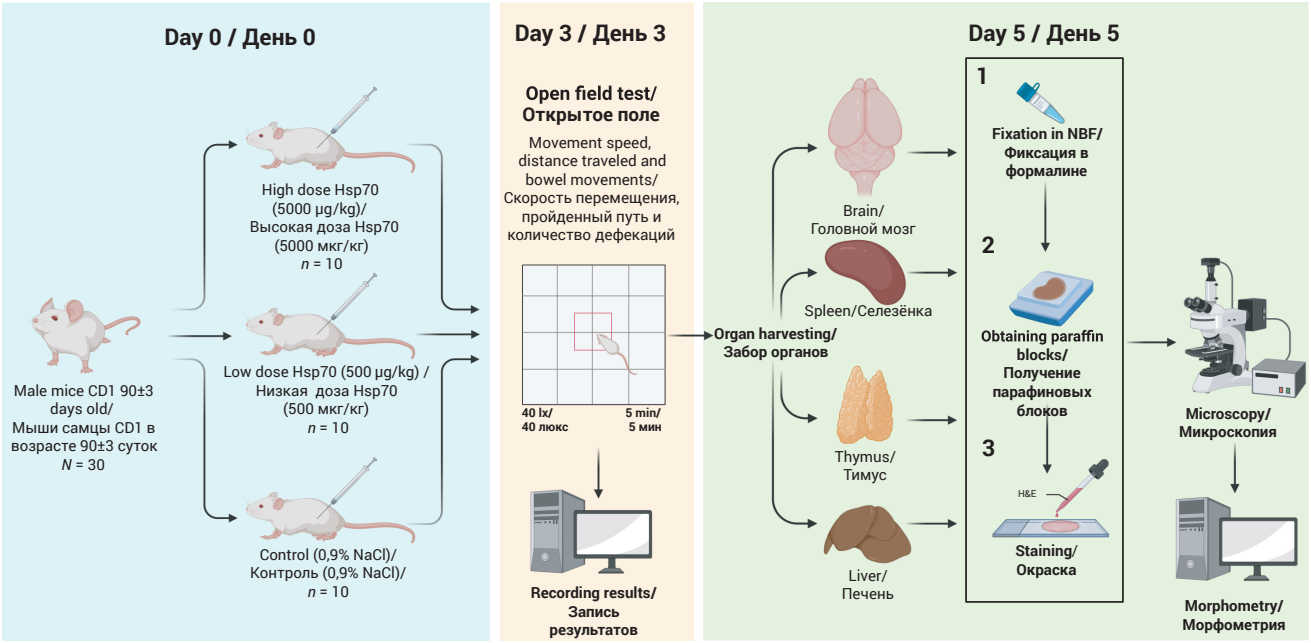

FIG. 1. Experimental design.
РИС. 1. Схема эксперимента.

Note: Hsp70 (heat shock 70 kDA) / HspA1A (heat shock 70 kDa protein 1A), recombinant; NBF – Neutral Buffered Formalin.
Примечание: Hsp70 (heat shock 70 kDA, белок теплового шока массой 70 кДа) / рекомбинантный человеческий HspA1A.

[3] https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf (accessed: 12.06.2023).
[4] https://www.internet-law.ru/gosts/gost/61242/ (accessed: 12.06.2023).
[5] https://www.internet-law.ru/gosts/gost/62388/ (accessed: 12.06.2023).

activity: movement speed, distance traveled and bowel movements.

On Day 5, the animals used in the experiment were killed by increasing the concentration of carbon dioxide in inhaled air [14].

Morphological study

The brain, thymus, spleen, and liver were collected. After fixation in 10% buffered formalin (ErgoProduction LLC, Russia) and standard processing through isopropyl alcohol of increasing concentrations, we prepared histological 5 μm slides with following hematoxylin and eosin staining [15]. We analyzed six fields of view on each slide using an AxioImager.A1 microscope with an Axiocam 305 color camera and Zen 3.3 software (all manufactured by Zeiss, Germany).

Morphometrics

For cell and nuclei identification, we used an open-sourced software QuPath 0.3.2 (Queen's University Belfast, UK), which was trained as part of ongoing research to recognize various structures in organs and tissues of interest [16].

In the brain, we counted the number of neurons in the ganglionic layer of cerebral hemispheres.

In the thymus, we assessed the quality of T-cell selection. For this, we separately counted the number of lymphocytes in the cortex and medulla to calculate the difference further with neither endothelial or reticuloepithelial cells counted.

In the spleen, we recorded the number of lymphocytes in the periarteriolar lymphatic sheaths for the white pulp and the number of macrophages in the red pulp.

In the liver, we assessed the number of hepatocyte nuclei in the parenchyma of the hepatic lobules without central veins and triads. For the liver morphometrics, we only considered rounded contrasted nuclei. Since individual hepatocytes are polyploid, directly determining cell count was not feasible.

Statistical analysis

We tested the normality of distribution with Kolmogorov–Smirnov test. All obtained data are given as the median and interquartile range (25^{th}; 75^{th} percentiles), as well as the mean with its standard error. We employed one-way analysis of variance (ANOVA) to compare groups. In case of significant differences, we provided pairwise comparisons with *post hoc* Tukey's test, $p < 0.05$. We used Microsoft Excel (Microsoft, USA) and OriginPro (OriginLab, USA) software packages.

RESULTS

Open field test

Analysis of motor activity showed that the rodents moved with an average speed of 8.9 ± 1.0 cm/s in control group, 8.4 ± 0.6 cm/s – in 500 μg/kg Hsp70 group, and 8.4 ± 0.7 cm/s in 5000 μg/kg Hsp70 group (Fig. 2A). For the distance traveled, mice did 2671.7 ± 292.1 cm in control group, in 500 μg/kg Hsp70 group – 2507.8 ± 167.0 cm, in 5000 μg/kg Hsp70 group – 2524.7 ± 222.7 cm (Fig. 2B). In control group, mice had 1.7 ± 0.5 bowel movements during the test time, in 500 μg/kg Hsp70 group – 2.5 ± 0.5, and in 5000 μg/kg Hsp70 group – 2.2 ± 0.7 bowel movements (Fig. 2C). The number of boluses per bowel movement varied within 1–3 pieces in all groups. No statistically significant differences for all studied parameters were found between the groups in the open field test.

Histological study of the brain revealed intact cyto- and myeloarchitectonic features. Neuronal and glial distribution stayed similar in mice of all groups (Fig. 3). The histological imaging of all groups remained normal.

The visualization of cortical and medullar structure was appropriate with no signs of structural changes and no visual differences between the groups (Fig. 3).

Slides of the white pulp of spleen also reveal no structural signs of damage. No accumulation of specific pigments occurred in the red pulp with some macrophages clearly visible, and their number lower in 5000 μg/kg Hsp70 group (Fig. 3).

In the liver, the normal structure stayed intact for all three groups. Sinusoidal capillaries slightly widened with a stronger blood flow in control group and 500 μg/kg Hsp70 group compared to 5000 μg/kg Hsp70 group (Fig. 3).

In the mice that comprised the control group, morphometrics (Fig. 4A) of ganglionic layer in the cerebral cortex detected 34.6 ± 1.6 neurons per one field of view, in 500 μg/kg Hsp70 group – 25.2 ± 1.4 cells, in 5000 μg/kg Hsp70 group – 27.2 ± 1.7 neurons. On sections of the thymus (Fig. 4B), the difference of lymphocyte number between the cortex and medulla for control group was 398.7 ± 30.4 cells, for 500 μg/kg Hsp70 group is 274.9 ± 36.1 cells, for 5000 μg/kg Hsp70 group – 407.6 ± 42.8 cells. In periarteriolar lymphatic sheaths (Fig. 4C), control group had 2091.6 ± 33.4 lymphocytes, 500 μg/kg Hsp70 group – 2040.0 ± 15.7 cells, 5000 μg/kg Hsp70 group – 1987.4 ± 20.9 cells. Despite individual peaks, the distribution was normal, and all three samples overlapped. There was no significant difference between the groups in the number

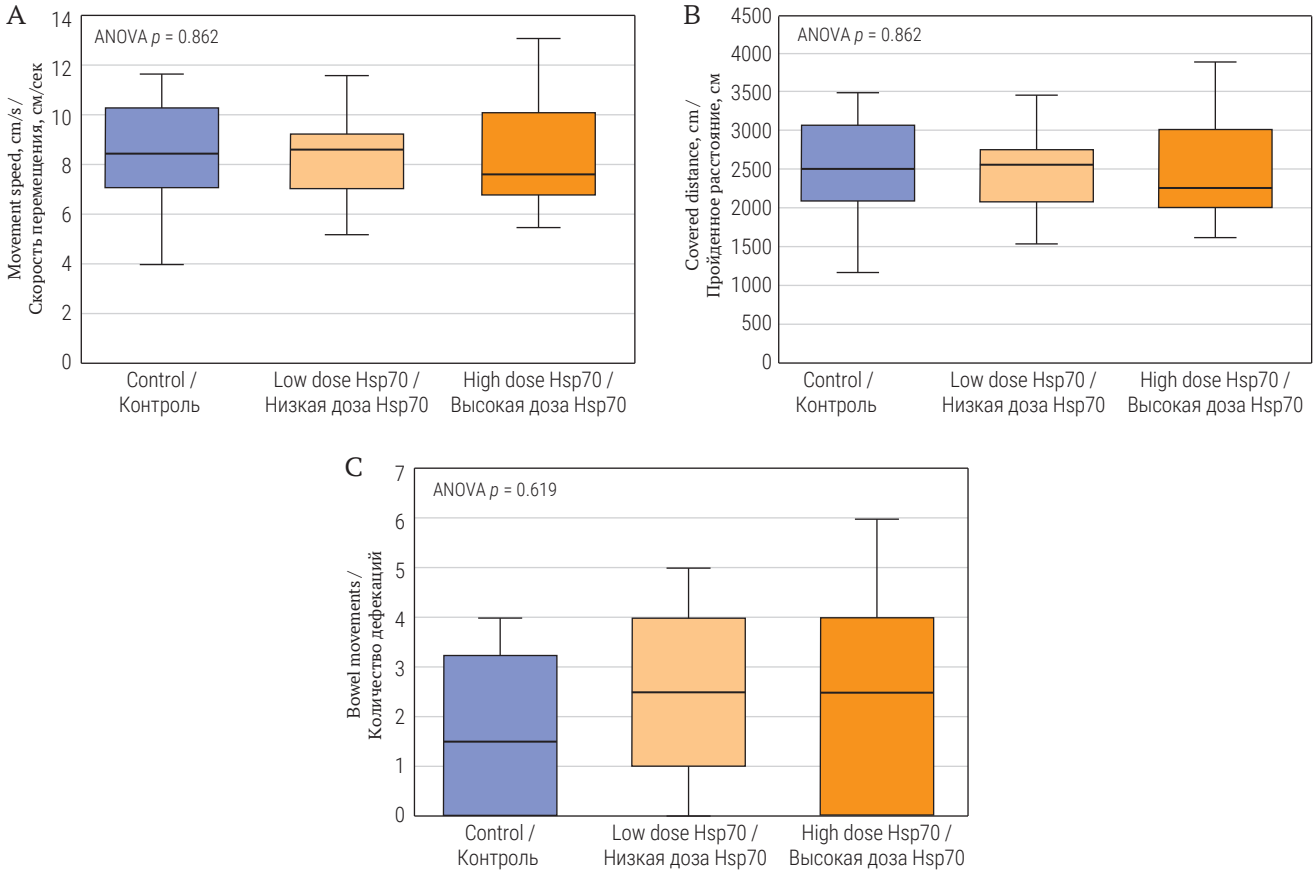

FIG. 2. Behavior of adult mice in "open field" test on Day 3 after single injections of Hsp70 at low or high dose.
РИС. 2. Поведенческие показатели взрослых мышей в тесте «открытое поле» на третьи сутки после однократного введения низкой или высокой дозы Hsp70.

of neurons, difference in lymphocyte number between the cortex and medulla, and number of lymphocytes in the periarteriolar lymphatic sheaths.

In the red pulp (Fig. 4D), morphometrics revealed differences between the groups. Thus, control group had 1774.5 ± 24.8 macrophages. In Hsp70 groups, the number of macrophages decreased in a dose-dependent manner: 500 μg/kg – 1623.0 ± 26.7 cells, 5000 μg/kg – 1553.6 ± 47.0 cells. The differences between the groups were statistically significant ($p < 0.0001$). In *post hoc* Tukey's test, the differences were shown between the control group with both Hsp70 groups, 500 μg/kg ($p < 0.05$) and 5000 μg/kg ($p < 0.0001$).

Liver morphometrics (Fig. 4E) detected 122.5 ± 4.2 hepatocytes in control group, 129.5 ± 3.6 cells in 500 μg/kg Hsp70 group, 115.7 ± 3.6 cells in 5000 μg/kg Hsp70 group. The obtained p value crossed the accepted level ($p = 0.053$). Tukey's test revealed the difference between 500 μg/kg Hsp70 and 5000 μg/kg Hsp70 groups to be significant.

DISCUSSION

Our results showed that subcutaneous administration of Hsp70 (recombinant human HspA1A) did not affect the functional reactions in mice, as they remained within the values defined for the control group at both low (500 μg/kg) and high doses (5000 μg/kg) of the drug. Tolerance of neurons of the Ganglionic (Layer V) of the cerebral cortex to single high doses of subcutaneously administered Hsp 70 has also been noted.

Thymus resistance to the possible damaging effects of exogenous Hsp70 was found, despite the age of rodents close to the beginning of involution. Indifferent and mediator cellular elements of the immune system in periarteriolar lymphatic sheaths were observed upon the administration of Hsp70. Using the liver tissue, it was also demonstrated that recombinant human Hsp70 administration had no negative effects in metabolically active organs. Interestingly, the administration of high doses of Hsp70 led to a statistically significant reduction in the number of hepatocytes, while both samples did

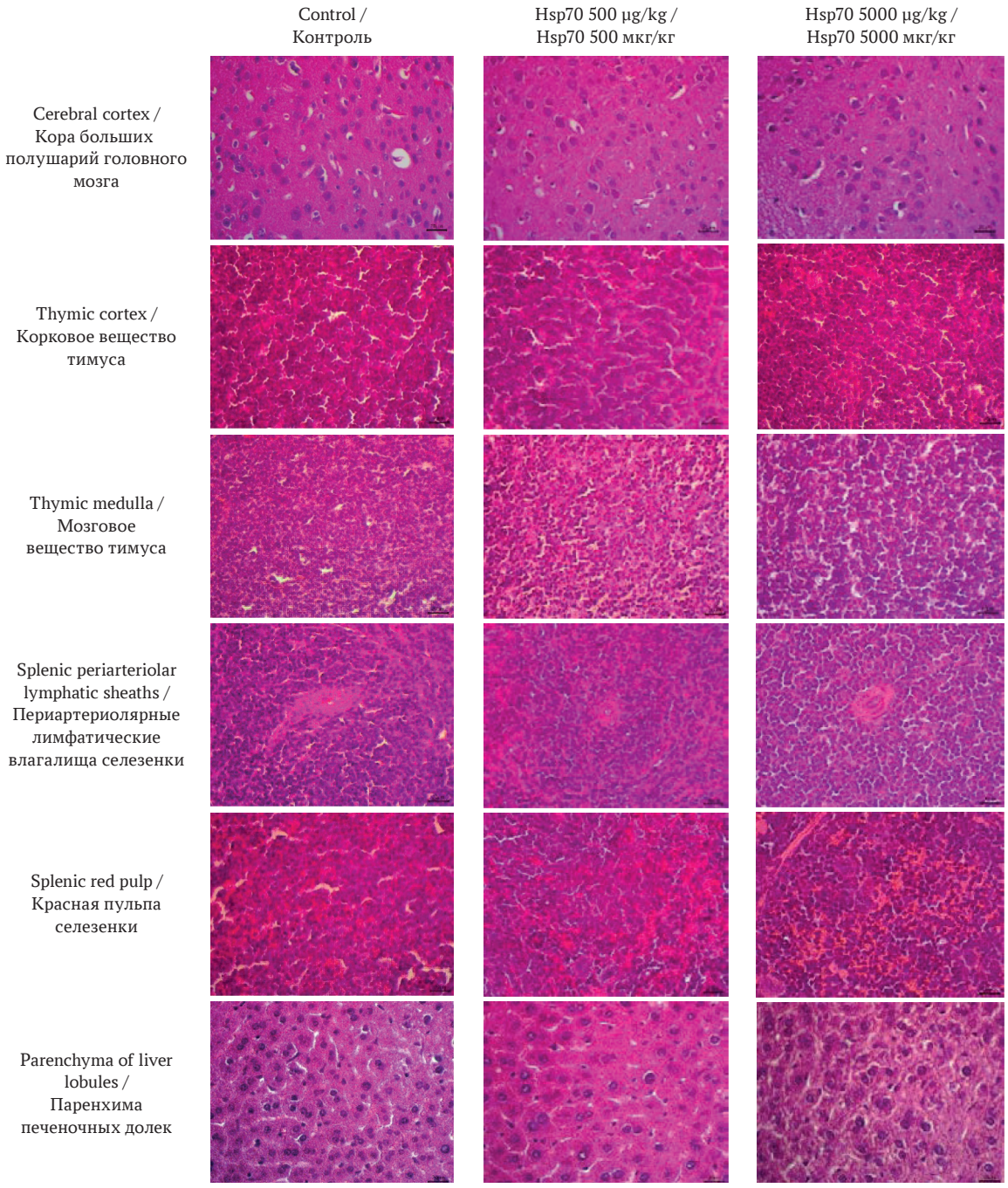

FIG. 3. Histological slides of murine organs on Day 5 after single injections of Hsp70 at low or high dose. Hematoxylin and eosin staining, ob.×40, oc.×20.
РИС. 3. Гистологические препараты органов мышей на пятые сутки после однократного введения низкой или высокой дозы Hsp70. Окраска гематоксилином и эозином, об.×40, ок.×20.

not differ from those for the control group. Probably, high doses of Hsp70 have a minor effect on the hepatic parenchyma, nevertheless, it remains within the normal variability range.

At the same time, there was a tendency towards a less prominent blood filling of sinusoidal capillaries with increasing the dose of Hsp70. This effect may be due to the interaction of Hsp70 with phagocytic cells in the red pulp of the spleen. The decrease in the number of macrophages we observed could contribute to changes in the metabolism of heme components, which, in theory, could affect the utilization of heme breakdown products. This phenomenon requires careful detailed study in the future.

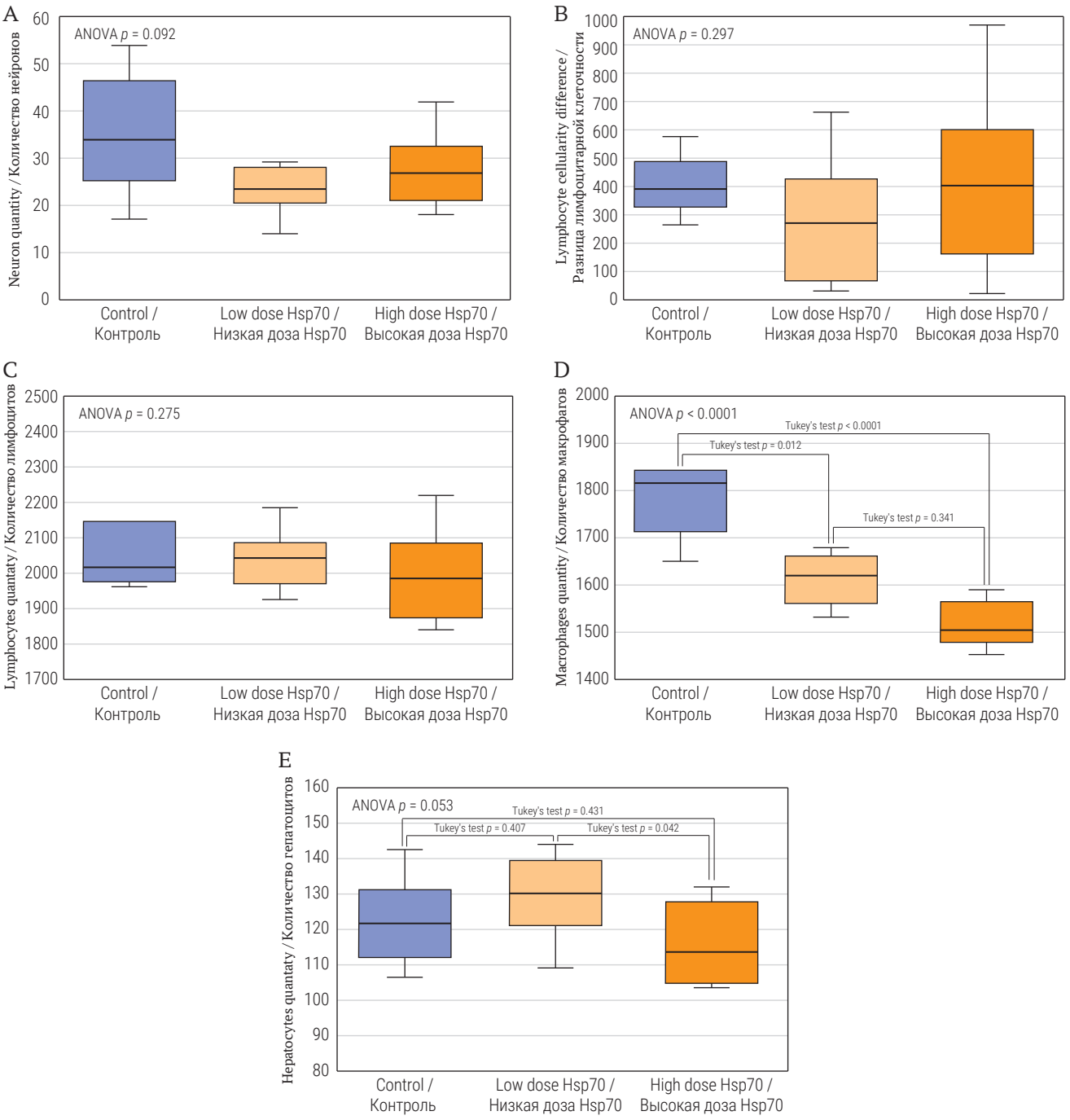

FIG. 4. Morphometrics in mice on Day 5 after single injections of Hsp70 at low or high dose, 6 fields of view. Ob.×40, oc.×20.
РИС. 4. Морфометрические показатели у мышей на пятые сутки после однократного введения низкой или высокой дозы Hsp70 при подсчете в 6 полях зрения. Об.×40, ок.×20.

We suggest that the changes we observed in the red pulp of the spleen tending to reduce the number of macrophages with increasing doses of Hsp70 (despite the fact that *post hoc* analysis did not obtain data on the dose-dependent nature of such changes) may serve as an adaptive reaction of antigen-presenting cells to the effect of the marker of the injected exogenous Hsp70 as a stress protein. At the same time, the differences in the monocyte-macrophage link between the study groups are consistent with reported data [17]. We noted that there are data in science literature that have demonstrated the opposite effect, especially when using long-term exposure to Hsp70 and with the simultaneous course of tumors [18, 19].

The use of Hsp70 for this study required careful selection of the relevant dosing regimen. The randomization design for administration of 500 or 5000 μg/kg of recombinant human HspA1A once subcutaneously as low- and high-dose regimens, respectively, was developed after reviewing alternatives described in the literature. Thus, Hsp70 administered intraperitoneally at 200 μg/kg for 21 days [17] or subcutaneously at 50 μg/kg for 14 days [20] did not cause tissue damage. Non-injectable use without pathologic reactions has been described for intranasal administration, for example, 2 μg/kg for 9 months [21]. There are developments on targeted delivery of recombinant human Hsp70 by encapsulation, which demonstrated systemic pro-inflammatory properties of the protein in the form of an effective reduction in the production of reactive oxygen species and tumor necrosis factor [22].

We focused on the subcutaneous route of administration as the least studied, including the penetration of exogenous Hsp70 across the blood-brain barrier. Considering the range of Hsp70 doses according to the sources, we chose the highest possible dose of 5000 μg/kg of recombinant human HspA1A, which did not lead to fatal consequences. The same principle explains the choice of a single exposure, which can compensate for the excess of administered Hsp70.

Limitations of the study

The range of values for the data obtained is large because the sample was small. This is a necessary limitation of the pilot experiment due to ethical considerations.

Directions for further research

Based on the results obtained, it is not entirely clear whether the blood-brain barrier allows exogenous Hsp70 to enter the nervous tissue when administered subcutaneously. This prompts a comparison of different methods of used to assess the state of nervous tissue when a given protein is administered.

CONCLUSIONS

Subcutaneous administration of high and low doses of exogenous Hsp70 to three-month-old mice showed no statistically significant effect on organs of the nervous (cerebral cortex) and immune systems (thymus, spleen), as well as the liver. The obtained functional and histological results demonstrate the absence of adverse effects at a single administration of even high (5000 μg/kg) doses of recombinant human HspA1A. This provides an opportunity for further developments in the application of Hsp70 in neurodegenerative diseases, considering minimal undesirable morphofunctional manifestations. In addition, the results of the study facilitate a further dosage of recombinant human Hsp70 when administered subcutaneously.

AUTHOR CONTRIBUTIONS

Gennadii A. Piavchenko, and Artem A. Venediktov contributed equally to this work and should be considered as co-first authors. Gennadii A. Piavchenko, Artem A. Venediktov, and Sergey L. Kuznetsov designed the study. Gennadii A. Piavchenko, Artem A. Venediktov, and Egor A. Kuzmin provided manipulations with animals and physiological testing. Egor A. Kuzmin made histological slides. Gennadii A. Piavchenko, and Artem A. Venediktov carried out morphometrics and statistical calculations for the results, and wrote the text of the article. All the authors read and approved the final version of this manuscript.

ВКЛАД АВТОРОВ

Г.А. Пьявченко и А.А. Венедиктов в равной степени внесли вклад в работу и должны считаться первыми соавторами. Г.А. Пьявченко, А.А. Венедиктов и С.Л. Кузнецов разработали дизайн исследования. Г.А. Пьявченко, А.А. Венедиктов и Е.А. Кузьмин осуществляли подготовку животных и проводили физиологические тесты. Е.А. Кузьмин изготавливал гистологические препараты. Г.А. Пьявченко, А.А. Венедиктов выполняли морфометрию, статистическую обработку результатов и написали текст работы. Все авторы утвердили окончательную версию статьи.

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