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НАУЧНО-ПРАКТИЧЕСКИЙ МЕДИЦИНСКИЙ ЖУРНАЛ

ORIGINAL STUDY:
FUS/HSP70 transgenic mice in neurodegeneration

ORIGINAL STUDY:
VEGF-A and monocyte subpopulations in IHD

ORIGINAL STUDY:
Bone turnover markers in children with CKD

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Overexpression of HSP70 in mice with mutant FUS protein is accompanied by a mitigated neurodegeneration in limbic system

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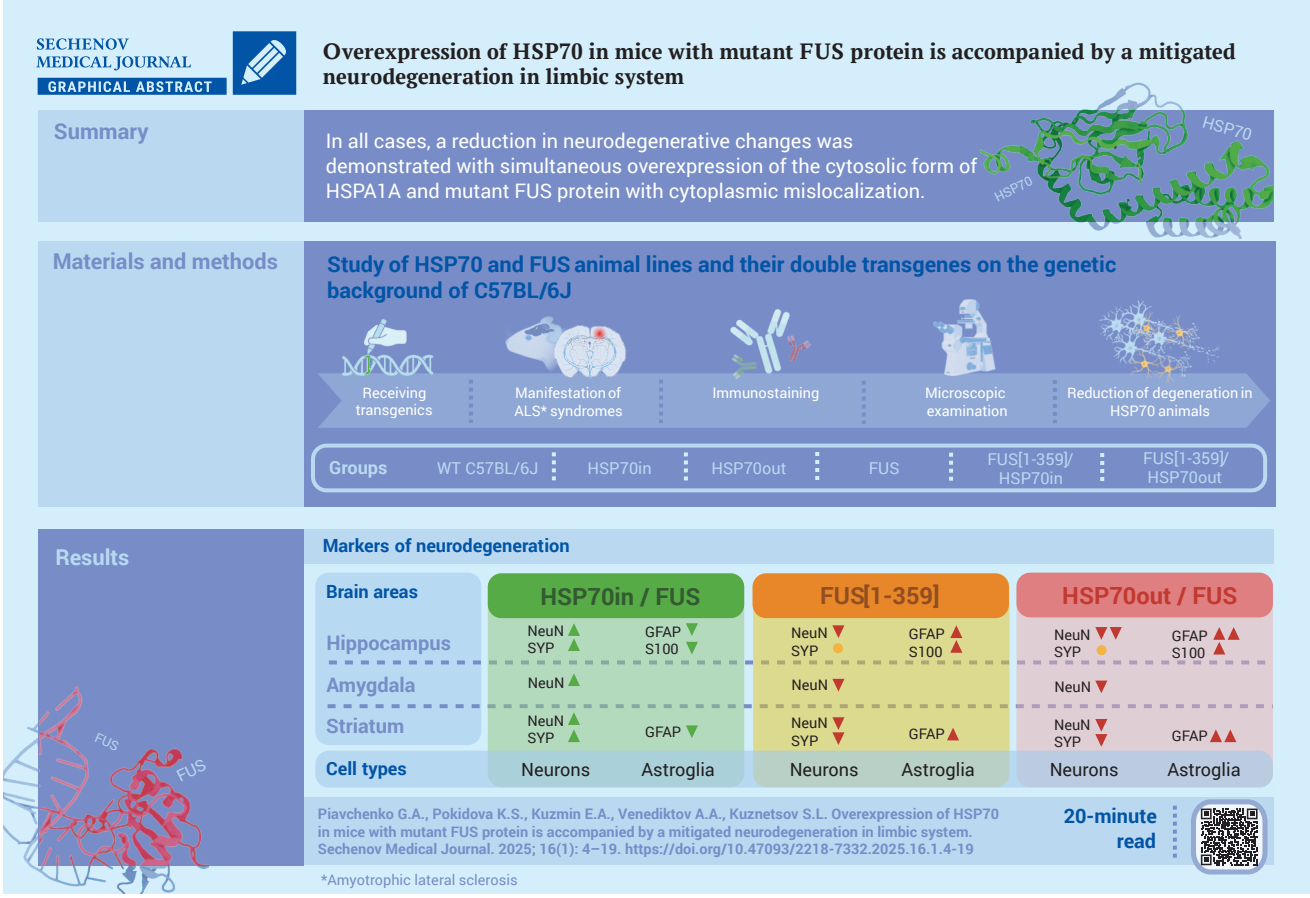

Abstract

Aim. To study morphological and developmental changes in the structure of mice's limbic system, which overexpress 70 kDa heat shock proteins (HSP70) and co-express mutant fused-in-sarcoma (FUS) protein with amyotrophic lateral sclerosis (ALS).

Materials and methods. The study was based on mice (n = 36; six for each group) of six lines either without FUS mislocalization: C57Bl/6 (wild-type); extracellular (*HSP70out*) or intracellular (*HSP70in*) overexpression of HSP70 family 1A protein; or with FUS mislocalization: transgenic mice with ALS-FUS (*FUS[1-359]*); and double transgenic animals (*FUS[1-359]/HSP70out* and *FUS[1-359]/HSP70in*). All FUS expressing mice were symptomatic with myasthenia up to limb paralysis in some animals. When the mice were 20 weeks old, they were killed by staining histological slides of their brain using hematoxylin and eosin, toluidine blue by Nissl, immunofluorescent antibodies for the neuronal nuclear marker (NeuN) of the caudoputamen, septal nuclei, and hippocampus, as well as glial fibrillar

acid protein (GFAP), S100β protein, and synaptophysin for the hippocampus. The number of neurons and the number of cells with a positive reaction to antibodies were counted. The statistical processing included ANOVA and were compared with the results from the Tukey test for.

Results. Statistically significant differences were observed for the FUS-expressing groups compared to FUS-negative groups: (1) a reduction in the number of neurons and $NeuN^+$-cells in the caudoputamen and amygdala, especially for *FUS[1-359]/HSP70out* group; (2) an increase in the number of hyperchromic neurons in the subiculum, cornu Ammonis (CA1), and dentate gyrus, with a significantly greater increase for *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to *FUS[1-359]/HSP70in* group; (3) an increase in the number of $GFAP^+$- and $S100β^+$-cells in the hippocampus, with the most pronounced change for the *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to c *FUS[1-359]/HSP70in* group.

Conclusion. An overexpression of HSP70 family 1A protein and co-expression of mutant FUS protein in the cytoplasm is accompanied by mitigated neurodegeneration in the structure of the limbic system compared with the expression of mutant FUS protein alone.

Keywords: molecular chaperones; heat shock proteins; amyotrophic lateral sclerosis; amygdala; caudoputamen; hippocampus; septal nuclei.

MeSH terms:
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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 from 02.03.2023.
Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request.
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Гиперэкспрессия белков теплового шока HSP70 у мышей с мутантным белком FUS сопровождается меньшими нейродегенеративными изменениями в структурах лимбической системы

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Аннотация

Цель. Изучить морфологические изменения в структурах лимбической системы у мышей с гиперэкспрессией белков теплового шока молекулярной массой 70 кДа (70 kDa heat shock proteins, HSP70) и экспрессией мутантного белка fused-in-sarcoma («слитый при саркоме»; FUS) с развитием бокового амиотрофического склероза.

Материалы и методы. Объектом исследования служили мыши (*n* = 36) линии C57Bl/6 (wild-type) и трансгенных линий, разделенные на 6 групп по 6 мышей в каждой. Три группы были FUS-отрицательные: контрольная, с вне и внутриклеточной гиперэкспрессией белка 1A семейства HSP70: *HSP70out* и *HSP70in*; три группы – FUS-положительные: *FUS[1-359]*, *FUS[1-359]/HSP70out* и *FUS[1-359]/HSP70in*. У всех FUS положительных мышей развивалась мышечная слабость вплоть до паралича. На 20-й неделе жизни мышей выводили из эксперимента, гистологические препараты головного мозга окрашивали гематоксилином и эозином, толуидиновым синим по Нисслю или иммунофлуоресцентными антителами к нейрональному ядерному маркеру (NeuN) для препаратов каудопутамена, септальных ядер и гиппокампа, а также к глиальному фибриллярному кислому белку (GFAP), белку S100β и синаптофизину для препаратов гиппокампа; подсчитывали количество клеток. Сравнение средних значений между группами проводили при помощи однофакторного дисперсионного анализа и теста Тьюки.

Результаты. В группах с экспрессией FUS наблюдались статистически значимые различия по сравнению с FUS отрицательными группами: (1) снижение количества нейронов и $NeuN^+$-клеток в каудопутамене и миндалевидном теле, наиболее выраженное изменение отмечено в группе *FUS[1-359]/HSP70out;* (2) увеличение количества гиперхромных нейронов в основании гиппокампа, зоне Аммонова рога (CA1) и зубчатой извилине, прирост был значимо больше в группах *FUS[1-359]* и *FUS[1-359]/HSP70out* по сравнению с *FUS[1-359]/HSP70in;* (3) рост количества $GFAP^+$- и $S100β^+$-клеток в гиппокампе, увеличение было значимо больше в группах *FUS[1-359]* и *FUS[1-359]/HSP70out* по сравнению с *FUS[1-359]/HSP70in*.

Заключение. Одновременная гиперэкспрессия белка 1A семейства HSP70 и экспрессия мутантного белка FUS в цитоплазме клеток сопровождается меньшей выраженностью нейродегенеративных изменений в структурах лимбической системы по сравнению с экспрессией только мутантного белка FUS.

Ключевые слова: молекулярные шапероны; белки теплового шока; боковой амиотрофический склероз; миндалевидное тело; каудопутамен; гиппокамп; септальные ядра

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ЛИМБИЧЕСКАЯ СИСТЕМА – ПАТОЛОГИЯ
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Abbreviations:

ALS – amyotrophic lateral sclerosis
BLAa/BLAp – basolateral amygdalar nucleus, anterior and posterior parts
BLAv – basolateral amygdalar nucleus, ventral part
CA – cornu Ammonis
CeL – central amygdalar nucleus, lateral part
CP – caudoputamen
DG – dentate gyrus
FUS – fused-in-sarcoma protein
GFAP – glial fibrillary acidic protein
HSP70 – 70 kDa heat shock proteins
HSPA1A – heat shock proteins member 1A
IF – immunofluorescent study
LA – lateral amygdalar nucleus
LSc – lateral septal nucleus, caudal part
NeuN – neuronal nuclear marker
SUBd – subiculum, dorsal part
SYP – synaptophysin
WT – wild-type animals

HIGHLIGHTS

An excess of molecular chaperones HSP70 in the cytosol may have a beneficial effect on the course of the amyotrophic lateral sclerosis variant associated with the accumulation of FUS protein in the cytoplasm rather than in the nucleus.

Simultaneous expression of both intracellular human HSP70 (HSPA1A exactly) and mutant FUS in mice leads to greater neuronal survival in certain areas of the striatum, amygdala, and hippocampus compared to FUS expression without HSPA1A.

Activation of neuroglia in the structures of murine limbic system in simultaneous mutant FUS and HSPA1A expression is less pronounced than in mice with FUS expression only.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder primarily affecting the motor regions of the nervous system [1]. The disorder is characterized by high rates of disability and mortality, with no effective treatment methods available [2]. The development of ALS treatment principles is challenging due to the fact that ALS can arise from mutations in various genes [3] with different clinical phenotypes [4]. Given these differences, research that examines molecular mechanisms that counteract key pathological changes in the most common genetic variants of ALS is in high demand.

One of the genes that can mutate leading to the development of ALS encodes human RNA-binding protein “fused-in-sarcoma (FUS)” and is normally localized in the nucleus [5]. Aberrant FUS protein leaves the cell nucleus, and its pathological accumulation in the cytoplasm of neurons underlies the FUS variant of ALS, FUS-ALS, and associated cell death [6]. A promising direction in studying this problem is the search for experimental conditions to reduce the severity of neurodegenerative changes related to the cytoplasmic accumulation of FUS.

Thus, pathological protein accumulation in the cytoplasm can be controlled using molecular

[2] Карточка проекта фундаментальных и поисковых научных исследований, поддержанного Российским научным фондом. Изучение нейроиммунологических эффектов экстра- и внутриклеточного HSP70 при нейродегенеративном повреждении мозга у мышей. https://rscf.ru/project/23-25-00448/ (дата обращения: 01.12.2024).

chaperones, particularly 70 kDa heat shock proteins (HSP70) [7]. It has been also shown that HSP70 can affect the accumulation of mutant FUS protein in FUS-ALS [7, 8].

It should be emphasized that HSP70 member 1A (HSPA1A) is a constant, homeostatic component of the cytosol [9], which does not prevent the development of FUS-ALS. It is likely that the amount or activity of HSPA1A in patients with FUS-ALS is insufficient to prevent a pathological FUS accumulation. One may therefore assume that overexpression of HSPA1A favorably affects neuronal survival in ALS with cytoplasmic FUS accumulation [7].

In ALS, damage occurs in the motor areas of the cerebral cortex, the limbic system, as well as motor neurons in the spinal cord and motor plaques [1, 10]. However, changes in the structures of the limbic system have been the least studied, although the basal ganglia are involved in the pathogenesis of ALS [10]. For example, as of 01.12.2024, a search in the PubMed database using tags "FUS + ALS + brain", "FUS + ALS + spinal cord", "FUS + ALS + muscle" reveals more than 200 publications, while "FUS + ALS + hippocampus" reveals 21 publications, "FUS + ALS + striatum" yields 2 publications, and "FUS + ALS + amygdala" did not appear in any publications.

Aim of the study: to investigate morphological changes in the structures of the limbic system in mice overexpressing HSP70 and expressing mutant FUS protein with ALS development.

MATERIALS AND METHODS

Animal experiments

The study was conducted using 36 wild-type (WT) and transgenic mice weighing 32.0±2.5 g. The mice were divided into six groups (n = 6 per group). Only healthy animals (except ALS) were included. The mice were obtained from the vivarium at Sechenov University.

Group 1 included C57Bl/6 mice (WT), selected using the random number method. The randomization for Groups 2 to 6 was based on the genotype of the animals. Group 2 included transgenic animals with the human *FUS[1-359]* gene, expressing mutant FUS protein with the development of FUS-ALS. The FUS expression model described in the work of T.A. Shelkovnikova et al. [11] was implemented. Group 3 (*HSP70out*) included transgenic mice with extracellular expression of HSPA1A, and the fourth group (*HSP70in*) included mice with intracellular expression of HSPA1A. Groups 2 to 4 were formed with respect to polymerase chain reaction data, including only animals with hemizygous genes of *FUS[1-359]* and HSPA1A. Homozygous animals with the phenotype corresponding to Groups 2 to 4 served to breed double transgenic mice: Group 5 (*FUS[1-359]/HSP70out*) and Group 6 (*FUS[1-359]/HSP70in*).

The mice were kept in the vivarium at a temperature of 20–22 °C and with a humidity of 55–60% with free access to clean water and granulated food *ad libitum*. In groups expressing FUS, starting from 10–12 weeks of age, muscle weakness signs gradually increased, progressing up to paralysis in some animals. These symptoms primarily affected one of the hind limbs sometimes affecting both ones or manifesting in the forelimbs. At the same time, sexual activity decreased, but food intake did not. These changes were not observed in FUS-negative groups.

At Week 20, the animals were killed by decapitation (Fig. 1). Anesthesia was induced with 5 mg/kg of xylazine hydrochloride (Interchemie, Netherlands) and 40 mg/kg of tiletamine/zolazepam (Virbac, France).

Preparation of histological specimens

The brains were placed in 10% neutral buffered formalin (ErgoProduction LLC, Russian Federation). Twenty-four hours later, the organs were put into isopropyl alcohol of increasing concentrations (Biovitrum LLC, Russian Federation) and then finally placed into paraffin. Coronal sections 3 μm thick were made with HM325 rotary microtome (Thermo Fisher Scientific, USA), transferred onto silane-coated adhesive slides (Minimed LLC, Russian Federation), and dried at 37 °C.

Staining of specimens

For the purposes of a histological study, the specimens were stained with hematoxylin and eosin or toluidine blue by Nissl method. For immunofluorescent (IF) staining, after incubation in a dewaxing retrieval buffer solution (pH 9.0, 20×, lot XF05RT4N9592, Elabscience, China), the sections were washed with 10% PBS (Eco-Service LLC, Russian Federation), bovine serum albumin (lot RM-T1725/1000, Biosera, France) was added for 30 min, and then primary antibodies were added with accordance to the manufacturers' recommendations.

In order to identify neurons, monoclonal antibodies to neuronal nuclear marker (NeuN) (1:1000, clone SR45-07, item number ET1602-12, lot H661803001, Huabio, PRC) were used. And in order to detect activated astrocytes, we employed monoclonal antibodies to glial fibrillary acidic protein (GFAP) (1:500, clone SA03-04, item number ET1601-23, lot HO0913, Huabio, PRC) and S100β protein (1:1000, clone SC57-02, item number ET-1610-3, lot H661380007, Huabio, PRC) were used. To assess the level of synaptic contacts, polyclonal antibodies to synaptophysin (SYP) (1:200, item number O407-2, lot HG0614, Huabio, PRC) were used. After staining with primary antibodies, the samples were washed three times, and polyclonal

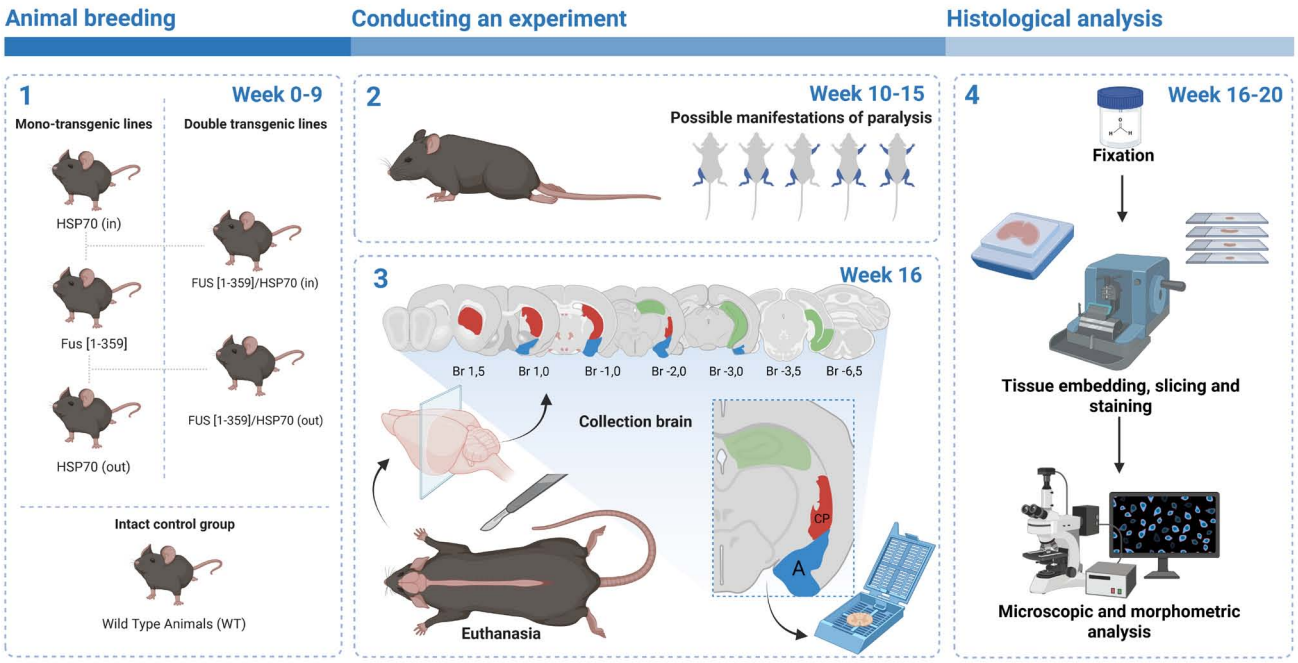

FIG. 1. Design of the experimental study.

Note. WT – wild type.

secondary antibodies (Anti-Rabbit-TRITC, 1:100, item number E-AB-1053, lot 22038, Elabscience, PRC) were applied, followed by DAPI staining (item number E-IR-R103, Elabscience, PRC). We washed the specimens with PBS and mounted them with coverslip.

Morphometric study

On the relevant sections, we identified the structure of rodent limbic system (6 fields of view per animal). According to the stereotaxic atlas [12], the following were identified: caudoputamen (CP); caudal part of the lateral septal nucleus (LSc); hippocampal formations, including dorsal part of the hippocampal subiculum (SUBd), regions of the hippocampus proper, or cornu Ammonis (CA1 and CA3), and dentate gyrus (DG); amygdala nuclei, namely lateral nucleus (LA), ventral part of the basolateral nucleus (BLAv), anterior and posterior parts of the basolateral nucleus (BLAa/BLAp), and lateral part of the central nucleus (CeL). Photographs were taken using the Axio Imager.A1 complex with ×40 and ×100 objective magnification, including the Axiocam 305 color camera and Zen 3.10 software (Zeiss, Germany).

The number of neurons per field of view was counted for CP, LSc, and amygdala structures. For hippocampal formations and LSc, the number of hyperchromic neurons was counted, and in CP, the number of neurons with eosinophilic cytoplasm, too. For antibody staining, microphotographs of hippocampal structures, CP, and LSc were taken at fluorescent wavelengths from 540 to 620 nm. The total number of NeuN-positive cells per field of view in CP and LSc zones and the ratio of $NeuN^+$ cells to DAPI-stained cells in hippocampal structures were counted.

The following changes were considered as neurodegenerative ones: a reduction in the total number of neurons per field of view, the appearance of hyperchromic neurons or neurons with eosinophilic cytoplasm, as well as a reduction in the number of NeuN-positive cells or the NeuN/DAPI ratio compared to the control group (WT). In the hippocampus and LSc zone, the proportion of GFAP-positive cells per field of view, given as percents, and the number of S100β-positive cells per field of view were also counted. An increase in GFAP and S100β expression was considered to be indicative of an astrocyte activation, and together with neurodegenerative changes, an indicator of neuroinflammation. For the hippocampus and CP zone, the level of SYP expression was calculated as the ratio of SYP expression area to the field-of-view area, given as percentages. The SYP expression area was assessed using open-source machine learning software QuPath 0.5.0 [13].

Statistical analysis

IF study data were assessed for normality using Shapiro–Wilk test. Mean values of the samples were compared using single-factor analysis of variance (ANOVA) with Tukey's post hoc test. Differences with a p value of less than 0.05 were considered statistically significant, with a sample size sufficient for 80% power and in accordance with the 3R principles. Statistical calculations were performed using OriginPro software (OriginLab, USA).

RESULTS

Histological study

Microphotographs and graphs of cell counts for brain sections in CP and LSc zones are shown in Fig. 2. In the CP zone, the total number of neurons decreased in all groups expressing mutant FUS compared to WT, *HSP70in*, and *HSP70out* groups (Fig. 2E). However, in the *FUS[1-359]/HSP70out* group, the number of neurons was lower than in *FUS[1-359]* and *FUS[1-359]/HSP70in* groups.

Neurons with eosinophilic cytoplasm were found only in the CP zone at the histological study (Fig. 2F). Their number increased significantly in all FUS-expressing groups, reaching maximum values in *FUS[1-359]/HSP70out* group. In *FUS[1-359]* and *FUS[1-359]/HSP70in* groups, the number of neurons with eosinophilic cytoplasm decreased but remained higher than in the groups without aberrant FUS.

In contrast to the CP zone, in the LSc zone, the number of neurons did not decrease in all FUS-expressing groups compared to WT group (Fig. 2G). Such a decrease in the number of neurons was characteristic only for *FUS[1-359]/HSP70out* group. At the same time, the number of hyperchromic neurons in the LSc zone (Fig. 2H) was higher in all FUS-expressing groups compared to WT group. The smallest number of hyperchromic neurons was a feature in *FUS[1-359]/HSP70in* group, compared to *FUS[1-359]* and *FUS[1-359]/HSP70out* groups.

Microphotographs and graphs of cell counts for brain sections in the hippocampal region are shown in Fig. 3. In the SUBd (Fig. 3G), CA1 (Fig. 3H), and DG (Fig. 3J) zones, the number of hyperchromic neurons increased in all FUS-expressing groups compared to the WT, *HSP70in*, and *HSP70out* groups. However, in *FUS[1-359]/HSP70in* group, the number of hyperchromic neurons was lower than in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups. In the CA3 zone, no statistically significant differences in hyperchromic neurons between groups were found (Fig. 3I).

Microphotographs and graphs of cell counts per field of view for brain sections in the amygdala region are shown in Fig. 4. In the BLAv (Fig. 4H), BLAa/BLAp (Fig. 4I), and CeL (Fig. 4J) zones, the total number of neurons decreased in the *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to WT, *HSP70in*, and *HSP70out* groups. However, in *FUS[1-359]/HSP70in* group, a decrease in the number of neurons relative to WT group was only revealed in the CeL zone. In the LA zone (Fig. 4G), the pattern of changes at the graph is similar to changes in other amygdala zones, but only a slight decrease in the number of neurons in *FUS[1-359]* group compared to the WT group where the changes were statistically significant.

Immunofluorescent study

The results of IF study for the CP and LSc zones are shown in Fig. 5. In both zones, a decrease in the number of $NeuN^+$ cells was observed in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups. The level of $NeuN^+$ cells in *FUS[1-359]/HSP70in* group was higher than in other FUS-expressing groups, while in the CP zone, it was comparable to WT group, and in the LSc zone, it was significantly lower than in WT group (Fig. 5C, 5D).

Fig. 6 shows the results of the IF study for hippocampal structures. In the DG and CA1 zones, the proportion of $GFAP^+$ and the number of $S100\beta^+$ cells increased significantly in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups, and to a lesser extent in *FUS[1-359]/HSP70in* group compared to WT group (Fig. 6H, 6I, 6K, 6L). Minor differences in SYP expression were observed only between FUS-expressing groups (Fig. 6M).

In the CA1 zone, a sharp decrease in the $NeuN^+$/$DAPI^+$ ratio was noted (Fig. 6J) in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups. In contrast, in *FUS[1-359]/HSP70in* group, this ratio was not only higher than in other FUS-expressing groups but also did not differ significantly from the WT group.

Fig. 7 shows the results of the IF study in the LA zone. In all FUS-expressing groups (*FUS[1-359]*, *FUS[1-359]/HSP70out*, and *FUS[1-359]/HSP70in*), the number of $NeuN^+$ cells was lower compared to WT group and groups with monoexpression of HSP70. At the same time, among the groups with mutant FUS, NeuN expression was higher in *FUS[1-359]/HSP70in* group and lower in *FUS[1-359]/HSP70out* group.

DISCUSSION

A key component of this work was the appropriate selection of transgenic mouse lines expressing HSP70 and FUS. The ALS model line was chosen based on the following criteria: 1) a model with the effectiveness in developing pathological and clinical manifestations of the FUS variant of ALS is well studied and aberrant FUS expression is proven; 2) there are pronounced neurodegenerative manifestations; 3) the mice survive up to an age where they can be crossed with rodents of other lines to breed double transgenic animals [6, 11]. The line expressing HSPA1A was chosen because this variant of HSP70 is capable of interacting with the FUS protein in its cytoplasmic mislocalization [14].

Our study revealed neurodegenerative changes in mutant FUS expression: a reduction in the number of neurons, an increase in the number of hyperchromic neurons, a decrease in NeuN expression and $NeuN^+$/$DAPI^+$ ratio, and co-expression of intracellular HSPA1A and mutant FUS in mice that is associated with lower levels of neurodegeneration. In the initial description of this ALS model, a neurodegenerative damage to

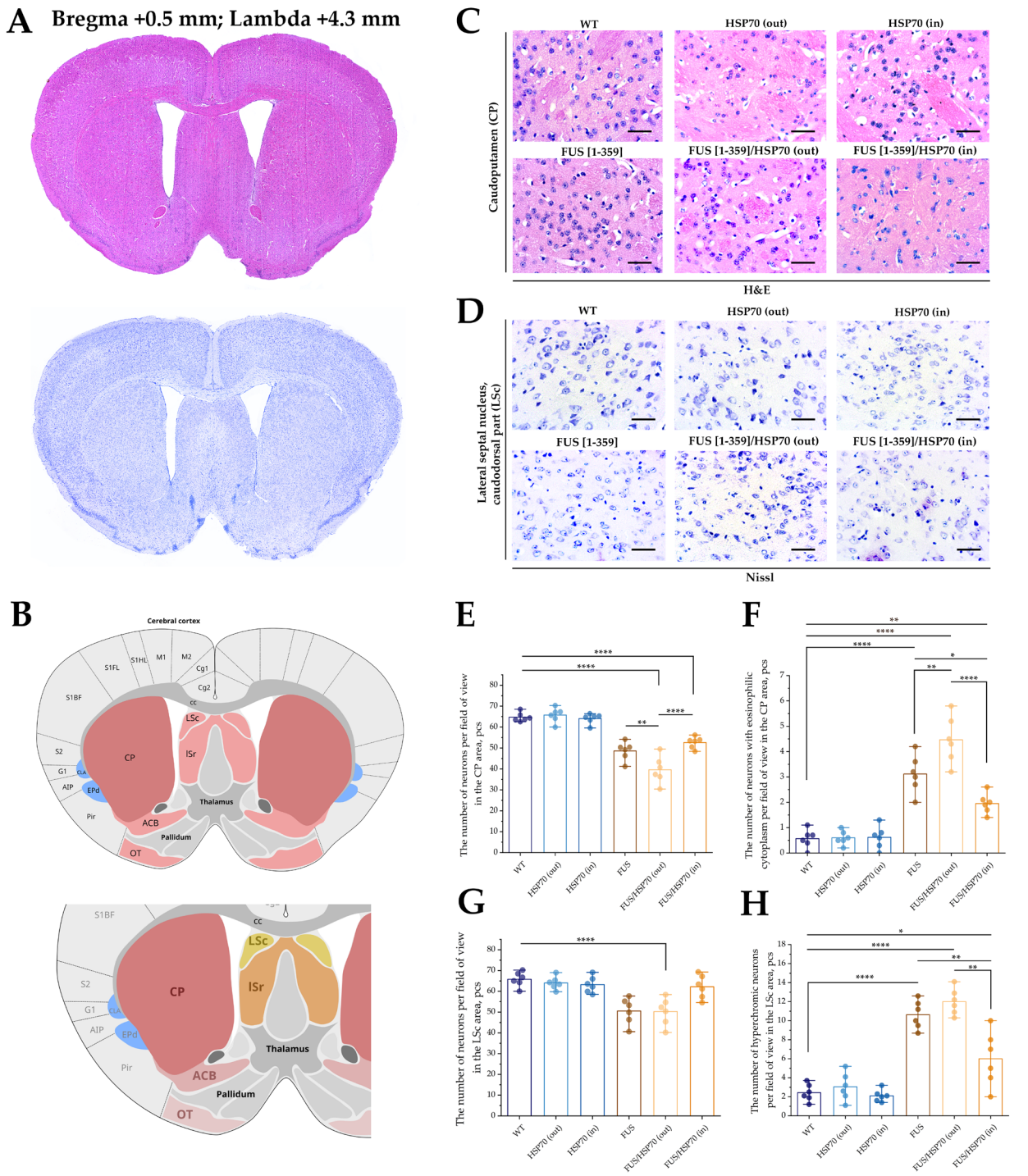

FIG. 2. Histological study of the caudoputamen and the caudal part of lateral septal nucleus in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; CP – caudoputamen; LSc – caudal part of the lateral septal nucleus.

A. Coronal brain sections stained with hematoxylin and eosin (above) and Nissl toluidine blue (below).
B. Stereotactic coronal section with localization of CP and LSc zones.
C. Histological slides of murine brains, CP, hematoxylin and eosin, ob. ×40. Scale bar: 20 μm.
D. Histological slides of murine brains, LSc, toluidine blue, ob. ×40. Scale bar: 20 μm.
E. The number of neurons per field of view in CP, hematoxylin and eosin.
F. The number of neurons with eosinophilic cytoplasm in CP, hematoxylin and eosin.
G. The number of neurons per field of view in LSc, Nissl toluidine blue.
H. The number of hyperchromic neurons in LSc, Nissl toluidine blue.

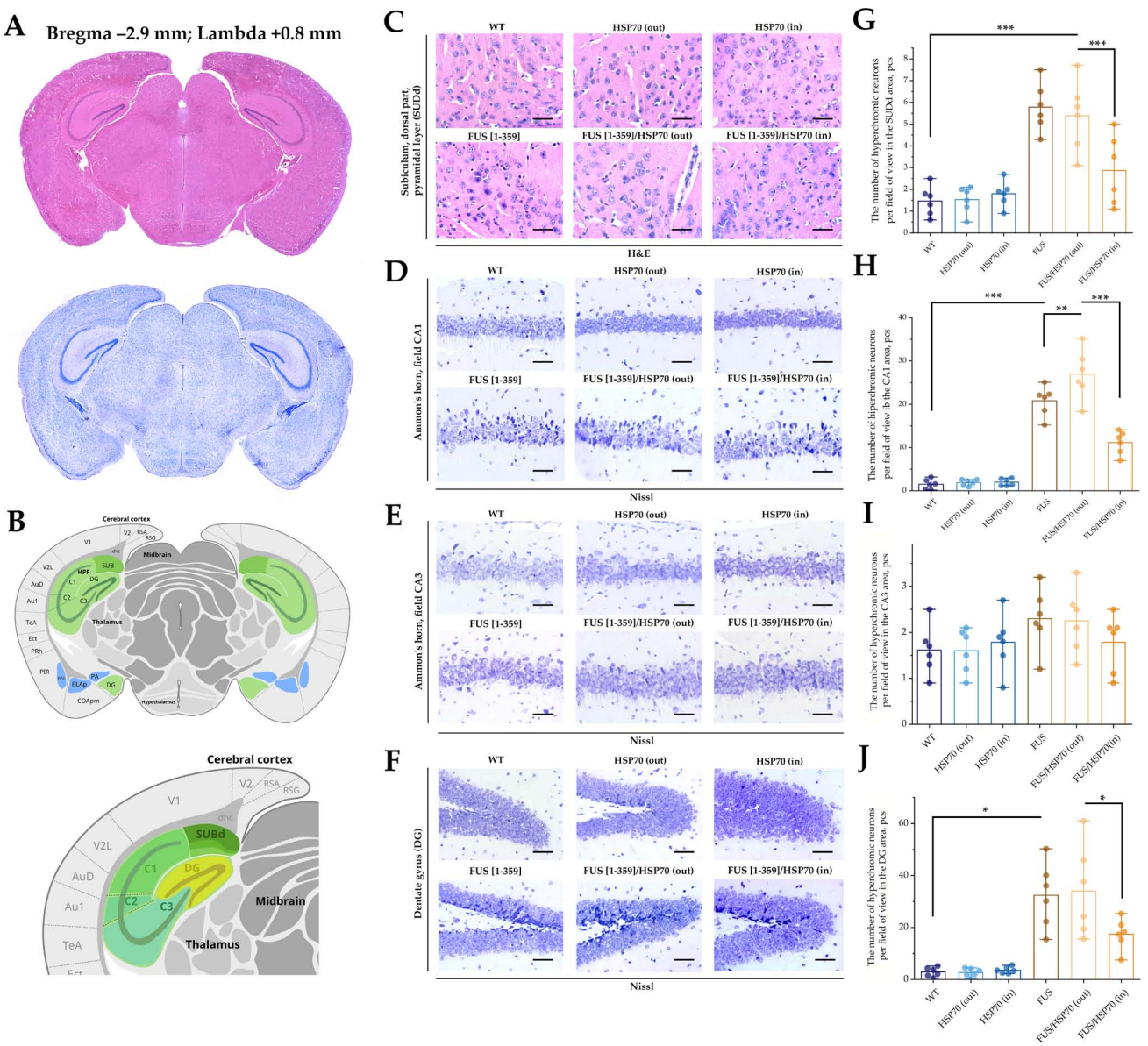

FIG. 3. Histological study of hippocampus in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; SUBd – subiculum; CA – cornu Ammonis; DG – dentate gyrus.

A. Coronal brain sections, stained by hematoxylin and eosin (above) and toluidine blue by Nissl (below).
B. Stereotactic coronal section with localization of hippocampal zones: SUBd, CA1 and CA3, DG.
C. Histological slides of murine brains, SUBd, hematoxylin and eosin, ob. ×40. Scale bar: 20 μm.
D. Histological slides of murine brains, CA1, toluidine blue, ob. ×40. Scale bar: 20 μm.
E. Histological slides of murine brains, CA3, toluidine blue, ob. ×40. Scale bar: 20 μm.
F. Histological slides of murine brains, DG, toluidine blue, ob. ×40. Scale bar: 20 μm.
G. The number of hyperchromic neurons in SUBd, hematoxylin and eosin.
H. The number of hyperchromic neurons in CA1, Nissl toluidine blue.
I. The number of hyperchromic neurons in CA3, Nissl toluidine blue.
J. The number of hyperchromic neurons in DG, Nissl toluidine blue.

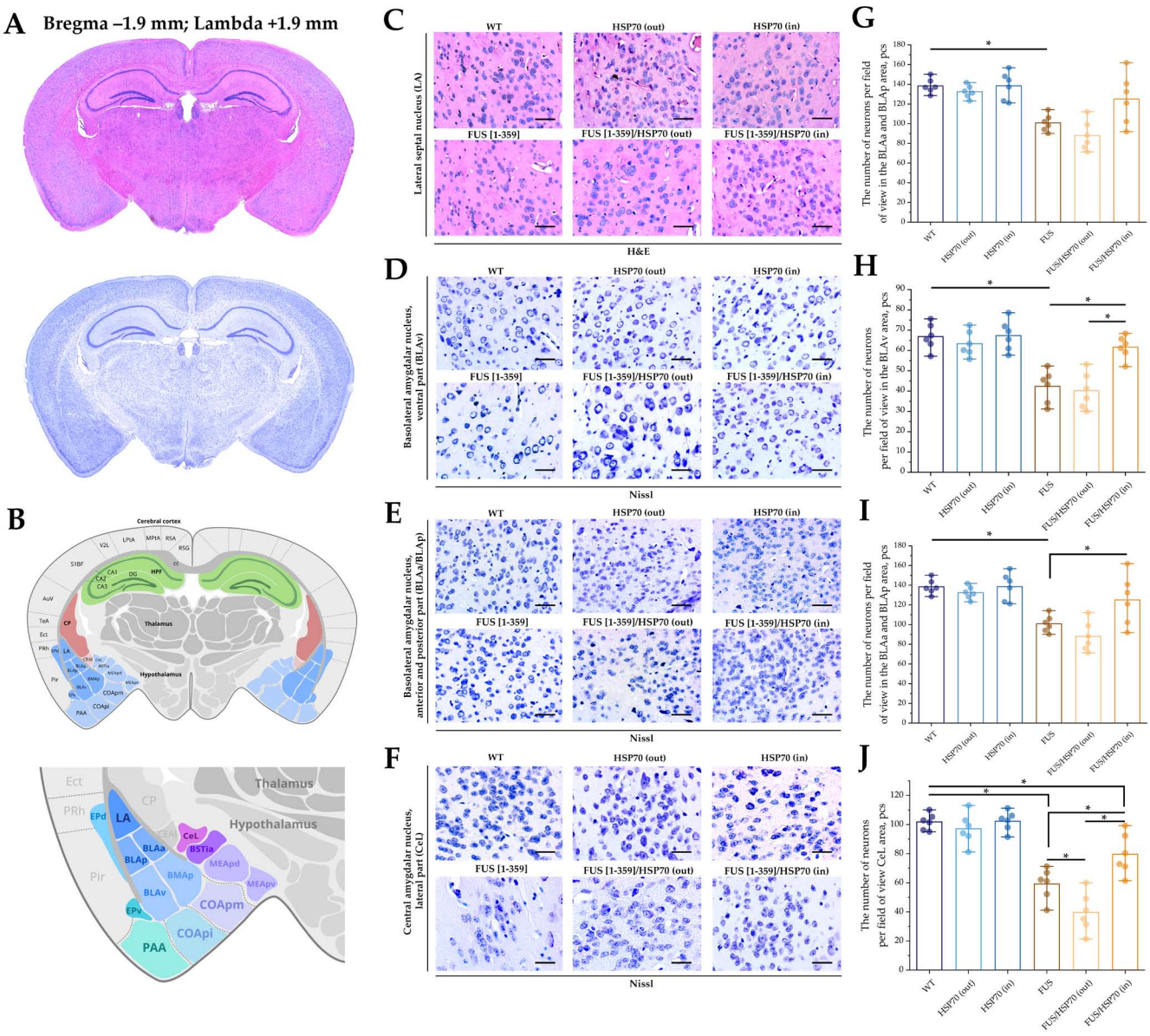

FIG. 4. Histological study of hippocampus in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; LA – lateral amygdalar nucleus; BLAv – basolateral amygdalar nucleus, ventral part; BLAa/BLAp – basolateral amygdalar nucleus, anterior and posterior parts; CeL – central amygdalar nucleus, lateral part.

A. Coronal brain sections, stained by hematoxylin and eosin (above) and toluidine blue by Nissl (below).
B. Stereotactic coronal section with localization of amygdalar zones: LA, BLAv, BLAa/BLAp and CeL.
C. Histological slides of murine brains, LA, hematoxylin and eosin, ob. ×40. Scale bar: 20 μm.
D. Histological slides of murine brains, BLAv, toluidine blue, ob. ×40. Scale bar: 20 μm.
E. Histological slides of murine brains, BLAa/BLAp, toluidine blue, ob. ×40. Scale bar: 20 μm.
F. Histological slides of murine brains, CeL, toluidine blue, ob. ×40. Scale bar: 20 μm.
G. The number of neurons per field of view, LA, hematoxylin and eosin.
H. The number of neurons per field of view, BLAv, toluidine blue.
I. The number of neurons per field of view, BLAa/BLAp, toluidine blue.
J. The number of neurons per field of view, CeL, toluidine blue.

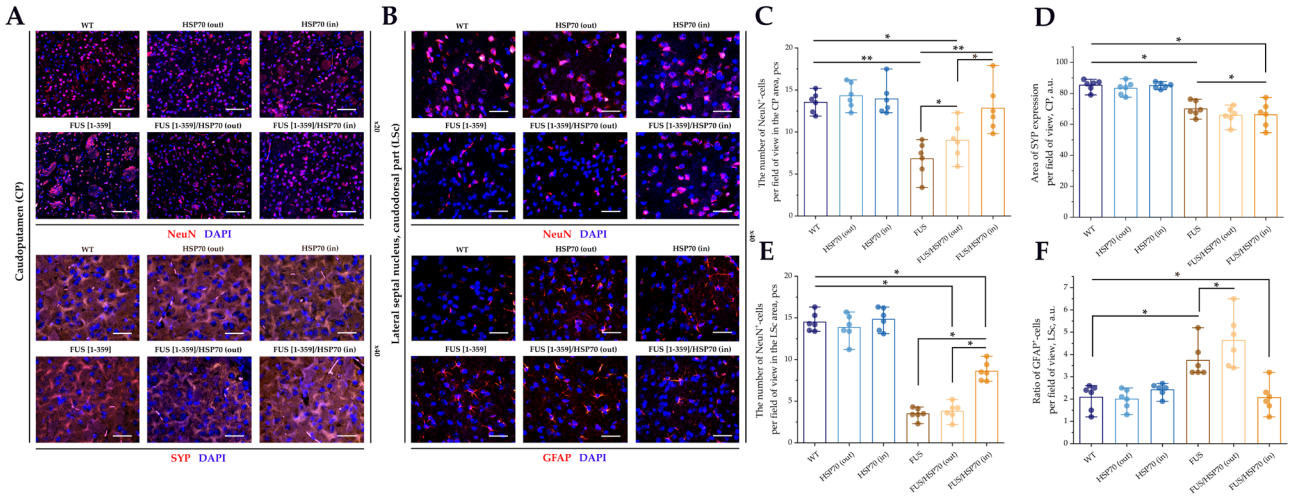

FIG. 5. Immunofluorescent study of caudoputamen and septal nuclei in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; ** $p < 0.01$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; CP – caudoputamen; LSc – caudal part of the lateral septal nucleus; GFAP – glial fibrillary acidic protein; NeuN – neuronal nuclear marker; SYP – synaptophysin.

A. Brain sections, CP, ob. ×100. Scale bar: 50 μm. Anti-NeuN and DAPI (above), Anti-SYP and DAPI (below).
B. Brain sections, LSc, ob. ×100. Scale bar: 50 μm. Anti-NeuN and DAPI (above), Anti-GFAP and DAPI (below).
C. The number of $NeuN^+$ cells per field of view, CP.
D. Area of SYP expression per field of view, CP.
E. The number of $NeuN^+$ cells per field of view, LSc.
F. Ratio of $GFAP^+$-cells per field of view, LSc.

brainstem structures was noted without specifying the nature of changes at the limbic system [11].

This study demonstrated a reduction in the number of neurons in the CP zone in all FUS-expressing animals. According to the literature, CP structures are susceptible to neurodegenerative changes in humans with FUS-associated ALS [15]. It has also been previously shown that electrical activity in the CP zone increases in mice expressing mutant FUS [16]. In this study, the animals with extracellular expression of HSPA1A (group *FUS[1-359]/HSP70out*) had neuronal survival in the CP even lower than in monotransgenic animals expressing mutant FUS (group *FUS[1-359]*), while in animals with intracellular expression (group *FUS[1-359]/HSP70in*), it was higher. Similar data were obtained for the number of cells with a positive reaction to NeuN, a known marker of mature neurons. Since the presence of excessive HSPA1A does not cause neurodegenerative changes in the CNS, as we had showed previously [9], it can be assumed that extracellular expression of HSPA1A exacerbates the pathological changes in the FUS variant of ALS. At the same time, co-expression of HSPA1A and FUS in the cytoplasm of CP neurons positively affects their survival.

In the CP zone, neurons with eosinophilic inclusions in the cytoplasm were also found, and their number increased in mutant FUS-expressing groups, especially in *FUS[1-359]/HSP70out* group. Similar "cherry spot" inclusions have previously been found in the nuclei of hippocampal neurons in the FUS variant of frontotemporal dementia [17]. A. Murakami et al. also reported the detection of FUS-positive inclusions in the striatum in autopsy material [18]. This finding may represent more evidence of FUS-associated neurodegenerative changes. However, there is insufficient experimental material for comparison to consider eosinophilic inclusions a specific marker.

For limbic system parts (septal nuclei, hippocampus) with a high concentration of neurons per unit area and, consequently, a higher probability of bias during comparison, the study considered not the total number of neurons, but the number of hyperchromic cells. This parameter is commonly used to assess neurodegenerative changes [19]. In the study of the LSc zone, the number of hyperchromic neurons, as well as cells expressing NeuN, increased in all FUS-expressing groups. These data are consistent with the general trend of changes in the CP zone, namely a more pronounced level of neurodegenerative changes in *FUS[1-359]* and *FUS[1-359]/HSP70out* groups compared to *FUS[1-359]/HSP70in* group. To date, no previous studies have been revealed in the accessible databases demonstrating changes in the number of neurons or hyperchromic neurons in the septal nuclei of animals expressing mutant FUS or in ALS-suffered humans.

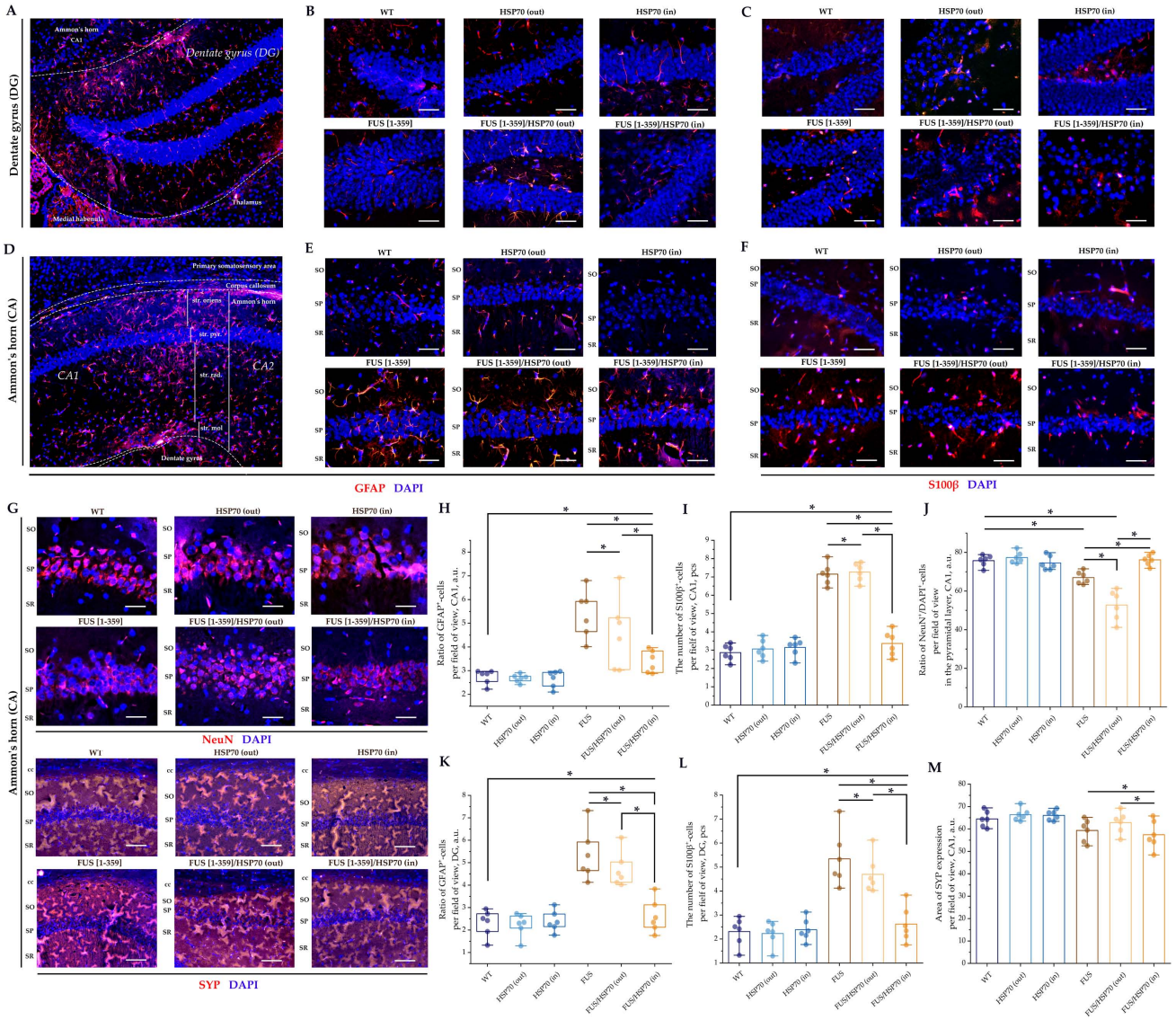

FIG. 6. Immunofluorescent study of hippocampal structure in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: * $p < 0.05$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; DG – dentate gyrus; CA – Cornu Ammonis; GFAP – glial fibrillary acidic protein; NeuN – neuronal nuclear marker; SYP – synaptophysin.

A. Brain sections, DG, ob. ×40. Scale bar: 20 μm. Anti-GFAP and DAPI.
B. Brain sections, DG, ob. ×100. Scale bar: 50 μm. Anti-GFAP and DAPI.
C. Brain sections, DG, ob. ×100. Scale bar: 50 μm. Anti-S100β and DAPI.
D. Brain sections, CA1, ob. ×40. Scale bar: 20 μm. Anti-GFAP and DAPI.
E. Brain sections, CA1, ob. ×100. Scale bar: 50 μm. Anti-GFAP and DAPI.
F. Brain sections, CA1, ob. ×100. Scale bar: 50 μm. Anti-S100β and DAPI.
G. Brain sections, CA1, ob. ×100. Scale bar: 50 μm. Anti-NeuN and DAPI (above), Anti-SYP and DAPI (below).
H. The ratio of GFAP$^+$ cells per field of view, %, CA1.
I. The number of S100β$^+$ cells per field of view, CA1.
J. NeuN+/DAPI+ ratio per field of view, %, CA1.
K. The ratio of GFAP$^+$ cells per field of view, %, DG.
L. The number of S100β$^+$ cells per field of view, DG.
M. SYP expression area per field of view, %, CA1.

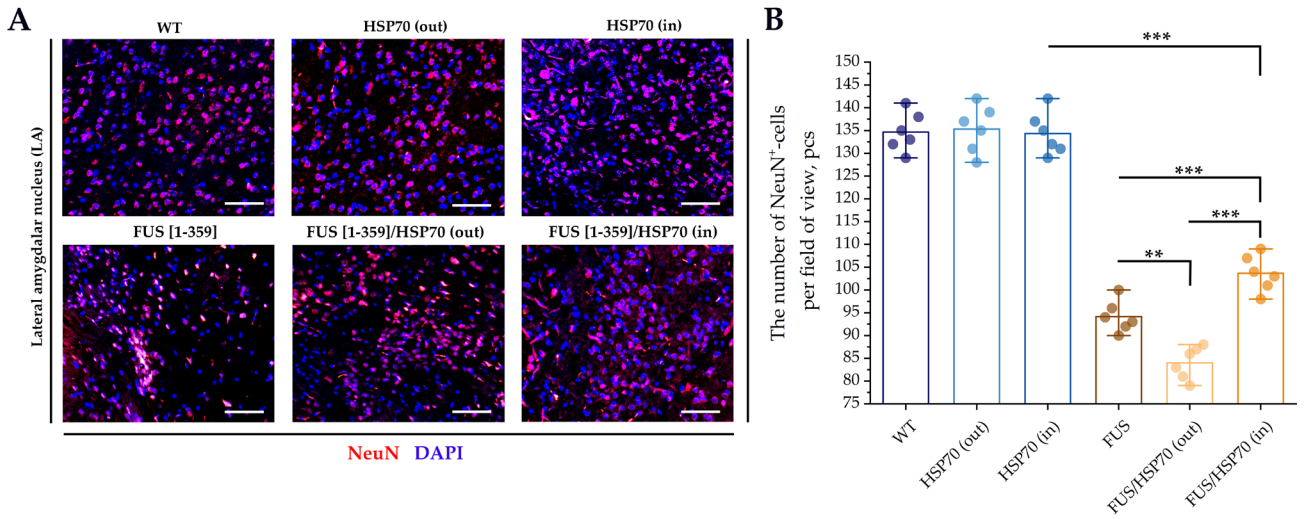

FIG. 7. Immunofluorescent study of amygdala in mice with FUS type of amyotrophic lateral sclerosis and overexpression of HSP70.

Note: ** $p < 0.01$; *** $p < 0.001$; HSP70 – 70 kDa heat shock proteins; FUS – fused in sarcoma protein; WT – wild type, control group; LA – lateral amygdalar nucleus; NeuN – neuronal nuclear marker.

A. Brain sections, LA, ob. ×100. Scale bar: 50 μm. Anti-NeuN and DAPI.
B. The number of $NeuN^+$ cells per field of view, LA.

In the hippocampal regions SUBd, CA1, and DG, this trend was maintained between the groups as described above for the CP and LSc zones. Thus, the number of hyperchromic neurons increased with mutant FUS expression, but in mice with simultaneous intracellular overexpression of HSPA1A (group *FUS[1-359]/HSP70in*) it was lower than in other mice expressing mutant FUS. These changes were accompanied by a decrease in NeuN expression in groups with mutant FUS, least pronounced in *FUS[1-359]/HSP70in* group. In the hippocampus, there was also an increase in the number of $GFAP^+$ and $S100\beta^+$ cells, and for these parameters, the differences between groups were consistent with the differences in the increase in the number of hyperchromic neurons. Since GFAP and S100β are markers of activated astrocytes, their increase may reflect the involvement of astroglia into FUS accumulation-driven reactions.

The hippocampus, due to its role in cognitive functions, is of particular interest in ALS. Its sensitivity to changes in FUS activity has been demonstrated in the study by Kino et al. [20]. In mice expressing mutant FUS, cognitive functions and spine formation in the hippocampus were reduced, although there was no cytoplasmic mislocalization of FUS in the hippocampus [21]. It has also been shown that spine density in the hippocampus and cognitive functions stay reduced in transgenic mice expressing mutant FUS and manifesting ALS [22]. However, in our study, no differences were found in the area of hippocampal tissue with a positive reaction to SYP. It is possible that synaptic function is less involved in FUS pathology in ALS than spine density.

An increase in FUS expression, neurodegenerative changes, and subsequent recovery of the number of neurons in a study investigating ischemic effects on the hippocampus were in some cases reported to be accompanied by a transient increase in HSP70 expression [23]. It can be assumed that HSP70 in the hippocampus helps to mitigate the negative effects of FUS accumulation (Fig. 8).

In the amygdala, in most nuclei except the lateral nucleus, the number of neurons decreased in all FUS-expressing groups, but in *FUS[1-359]/HSP70in* group it was minimal. This is consistent with the trend observed in other studied parts of the limbic system. There is little data in the literature about amygdala involvement in ALS, but it has been reported that mutant FUS expression is absent in the amygdala in the FUS variant of ALS [24].

In addition, we have previously examined changes in the primary motor cortex and spinal cord in the same models [25]. In all cases, a reduction in neurodegenerative changes was demonstrated with simultaneous overexpression of the cytosolic form of HSPA1A and mutant FUS protein with cytoplasmic mislocalization.

Limitations of the study

Extrapolation of animal experiments to humans is limited. This study does not cover all areas of the central nervous system. For example, the diversity of cortical areas in humans is inherently greater, as mice lack gyri; mice do not have speech regulation parts in the brain, etc.

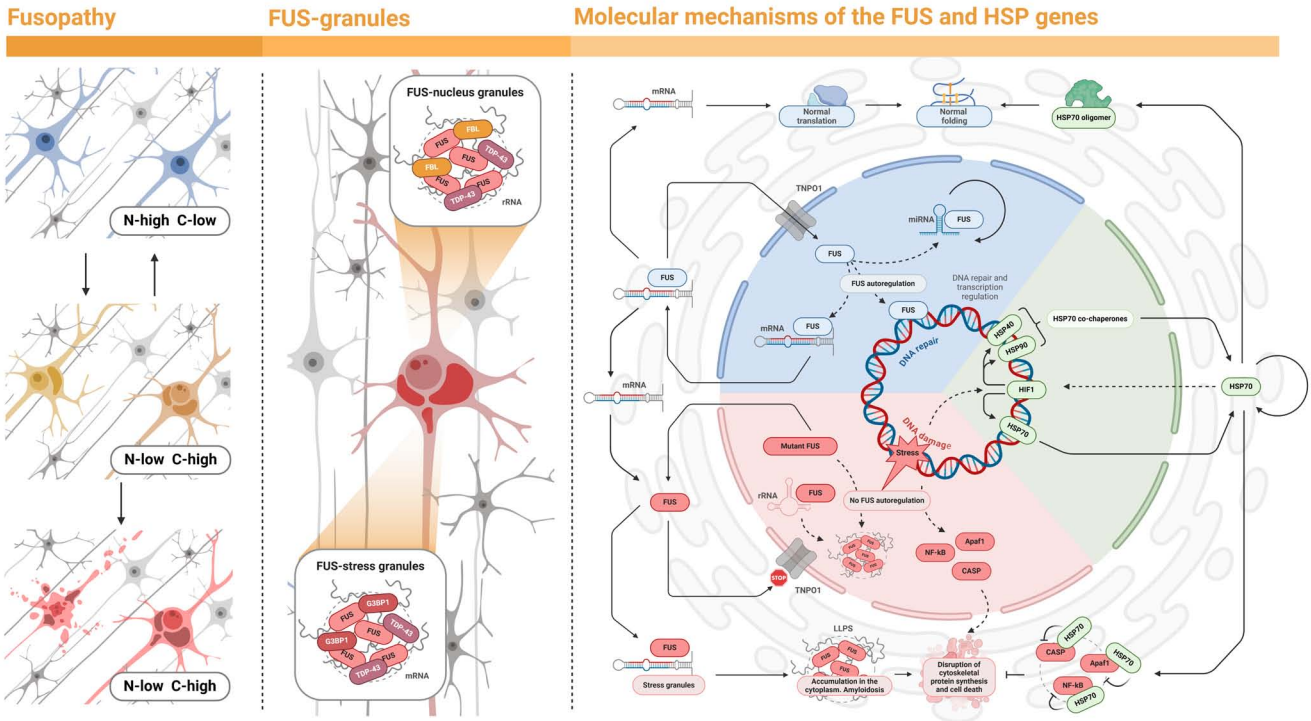

FIG. 8. Mechanisms of FUS-HSP70 interaction in cells.
Left: localization of fused-in-sarcoma (FUS) protein in the nucleus (blue). Loss of the nuclear localization signal leads to the migration of FUS into the cytoplasm (yellow). "N" – nucleus, "C" – cytoplasm.
Center: in the nucleus, FUS, together with fibrillarin (FBL) and the TDP-43 protein, binds rRNA to form nuclear granules. In the cytoplasm, stress granules containing mRNA with the G3BP1 marker are present. FUS in the cytoplasm associates with these granules.
Right: FUS activity (blue sector) supports DNA repair and transcriptional regulation, with autoregulation of FUS activity. FUS located outside the nucleus returns through the nuclear pore with the help of TNPO1 protein, while HSP70 chaperones ensure proper translation and folding. HSP70 activity (green sector) requires interaction with HSP40 and HSP90. Damage induces the synthesis of HSP70, for example through hypoxia-inducible factor 1 (HIF1). Stress-induced DNA damage can lead to mutations in the FUS gene. As a result, mutant FUS loses its nuclear localization signal. The accumulation of FUS in the cytoplasm and its aggregation disrupts liquid-liquid phase separation (LLPS) mechanisms, causing amyloidosis, and together with the activation of apoptosis (CASP; Apaf1; NF-κB) leads to impaired cytoskeleton repair and cell death.

Further research perspectives

We assume that it is a challenging task to establish the molecular mechanisms contributing to the positive impact of HSP70 overexpression on slowing neurodegenerative manifestations in the FUS-related ALS. Recording electrical changes in limbic system structures will allow a more complete description of the double transgenic murine models.

CONCLUSION

In the present study, we explored quantitative parameters of neurodegenerative changes in the limbic system in mice with overexpression of HSP70 in the form of HSPA1A and co-expression of mutant human *FUS[1-359]* protein related to ALS manifestation. According to data obtained, the co-expression of HSPA1A protein together with the mutant *FUS[1-359]* in the cytoplasm of nervous tissue cells in the limbic system is accompanied by fewer neurodegenerative changes compared to the monoexpression of the mutant FUS. Moreover, pathological changes are most significantly reduced in co-expression of FUS and intracellular HSPA1A overexpressed with HSP70 secretion out of cells. This study indicates HSPA1A overexpression as a promising approach to slow the progression of FUS-related ALS.

AUTHOR CONTRIBUTION

Gennadii A. Piavchenko and Sergey L. Kuznetsov developed the idea and design of the study. Egor A. Kuzmin and Ksenia S. Pokidova carried out the experiments with animals, made histological slides, and provided statistical calculations. Artem A. Venediktov carried studied the literature sources and wrote a draft text of the article. Egor A. Kuzmin and Ksenia S. Pokidova designed graphical elements of the work. Gennadii A. Piavchenko and Sergey L. Kuznetsov edited the article. All authors approved the final version of the article.

ВКЛАД АВТОРОВ

Г.А. Пьявченко и С.Л. Кузнецов сформулировали идею и разработали дизайн исследования. К.С. Покидова и Е.А. Кузьмин провели эксперименты с животными, изготовили гистологические препараты и выполнили статистическую обработку результатов. А.А. Венедиктов изучил литературные источники и написал текст статьи. К.С. Покидова и Е.А. Кузьмин оформили иллюстрации. Г.А. Пьявченко и С.Л. Кузнецов провели научную редактуру статьи. Все авторы статьи утвердили окончательную версию статьи.

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Vascular endothelial growth factor attenuates enhanced spontaneous transdifferentiation of classical and intermediate monocytes in patients with ischemic cardiomyopathy

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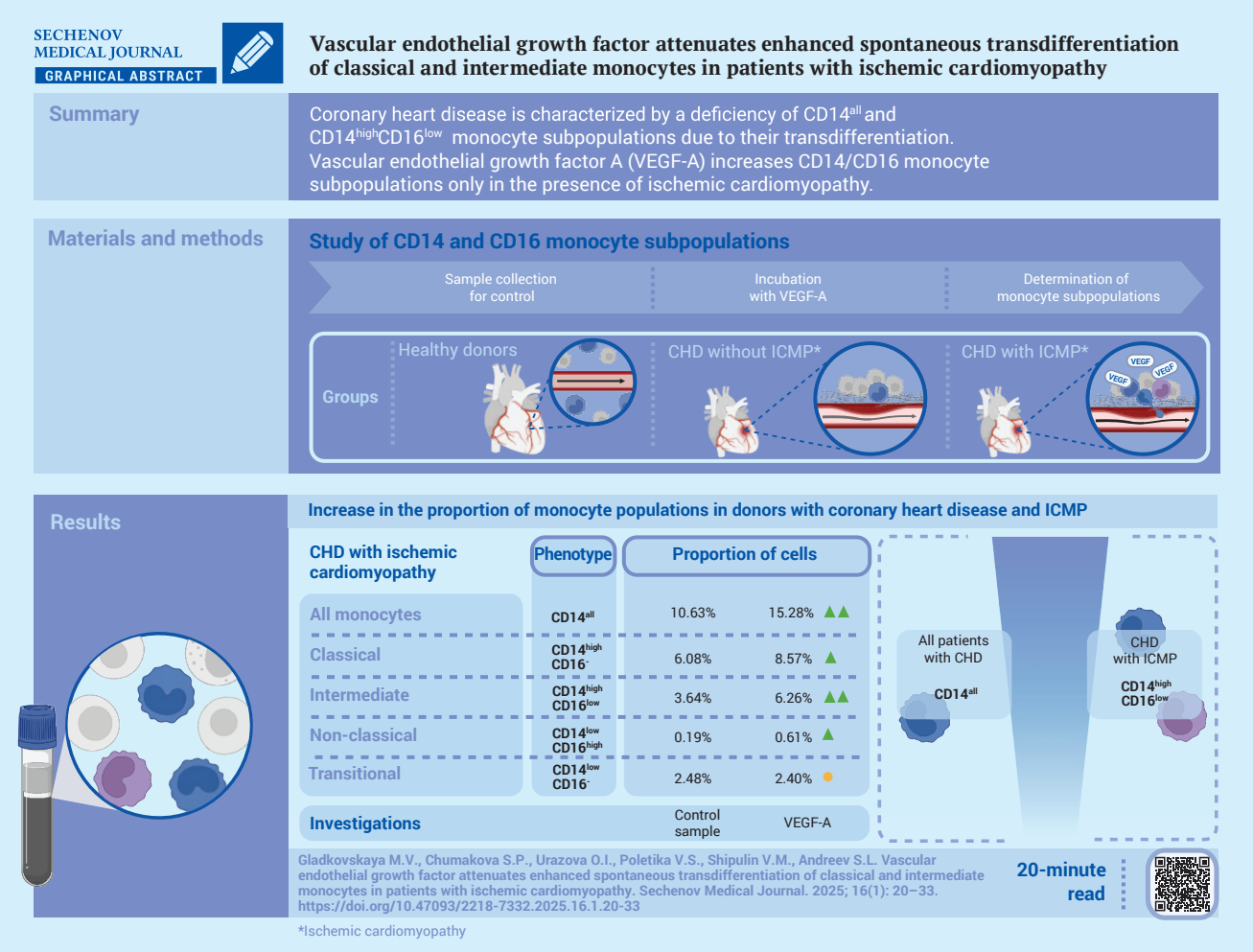

Abstract

Aim. To evaluate the effect of vascular endothelial growth factor A (VEGF-A) on the subpopulation composition of monocytes in the blood mononuclear cell culture of patients with coronary heart disease (CHD), with and without ischemic cardiomyopathy (ICMP).

Materials and methods. A single-center, experimental *in vitro* study was conducted. The study included 22 patients with CHD: 11 with ICMP, 11 without ICMP, and 10 healthy donors. Blood mononuclei were isolated from venous blood by immunomagnetic separation for CD14 and CD34 antigens, then incubated with and without the addition of VEGF-A 50 ng/mL (control and stimulated samples). After 6 days, the total monocyte content, the proportion of classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$, non-classical $CD14^{+}CD16^{++}$, and transitional $CD14^{+}CD16^{-}$ monocytes were assessed using flow cytofluorimetry.

Results. In groups of patients with CHD and in those groups where the patients were considered relatively healthy, a decrease in the content of $CD14^{++}CD16^{+}$ in the control and stimulated samples was shown. Only in the CHD group with ICMP relative to the control sample, after VEGF-A stimulation, a statistically significant increase in all $CD14^{+}$ was found: 10.63% (6.80; 17.64) vs. 15.28% (8.75; 27.99), $p < 0.01$, and their subpopulations: $CD14^{++}CD16^{-}$: 6.08% (1.76; 8.84) vs. 8.57% (3.51; 16.8), $p < 0.05$, $CD14^{++}CD16^{+}$: 3.64% (2.03; 8.59) vs. 6.26% (3.87; 10.3), $p < 0.05$. In the same group, a tendency towards an increase in $CD14^{+}CD16^{++}$ was noted after stimulation: 0.19% (0.18; 1.11) vs. 0.61% (0.37; 1.58), $p = 0.062$. No differences in the content of all monocytes and their subpopulations after VEGF-A stimulation were found in the CHD without ICMP group nor in the healthy group. The content of $CD14^{+}CD16^{-}$ in all groups in the control and stimulated samples did not differ.

Conclusion. CHD is characterized by a deficiency of all $CD14^{+}$ cells and intermediate monocytes due to their transdifferentiation. VEGF-A affects the subpopulation composition of monocytes in CHD only in the presence of ICMP by increasing the content of all $CD14^{+}$ cells, and in their intermediate and classical forms without exceeding the indicators in healthy donors.

Keywords: VEGF-A; differentiation; angiogenesis; classic monocytes; $CD14^{++}CD16^{-}$; intermediate monocytes; $CD14^{++}CD16^{+}$; non-classic monocytes; $CD14^{+}CD16^{++}$; transient monocytes; $CD14^{+}CD16^{-}$

MeSH terms:

MYOCARDIAL ISCHEMIA – BLOOD
MYOCARDIAL ISCHEMIA – PHYSIOPATHOLOGY
CARDIOMYOPATHIES – BLOOD
CARDIOMYOPATHIES – PHYSIOPATHOLOGY
VASCULAR ENDOTHELIAL GROWTH FACTOR – ANALYSIS
VASCULAR ENDOTHELIAL GROWTH FACTOR – PHARMACOLOGY
MONOCYTES-DRUG EFFECTS
LYMPHOCYTE SUBSETS-DRUG EFFECTS

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Ethics statements. The studies were conducted in accordance with the ethical principles set out in the Helsinki Declaration (1975) and with the permission of the local ethics committee of the Siberian State Medical University of the Ministry of Health of the Russian Federation (protocol No. 9299 dated November 28, 2022). Informed consent to participate in the study was obtained from all examined individuals.
Data availability. The data confirming the findings of this study are available from the authors upon reasonable request. Data and statistical methods used in the article were examined by a professional biostatistician on the Sechenov Medical Journal editorial staff.
Conflict of interest. The authors declare that there is no conflict of interests.
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Фактор роста эндотелия сосудов нивелирует усиление спонтанной трансдифференцировки классических и промежуточных моноцитов при ишемической кардиомиопатии

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Аннотация

Цель. Оценить влияние фактора роста эндотелия сосудов А (VEGF-A) на субпопуляционный состав моноцитов в культуре мононуклеаров крови у пациентов с ишемической болезнью сердца (ИБС) в зависимости от наличия ишемической кардиомиопатии (ИКМП).

Материалы и методы. Проведено одноцентровое экспериментальное исследование *in vitro*. В исследование включены 22 пациента с ИБС: 11 – с ИКМП, 11 – без ИКМП и 10 здоровых доноров. Мононуклеары крови выделяли из венозной крови иммуномагнитной сепарацией по антигенам CD14 и CD34, инкубировали без и с добавлением VEGF-A 50 нг/мл (контрольная и стимулированная пробы). Через 6 суток методом проточной цитофлуориметрии оценивали общее содержание моноцитов, долю классических $CD14^{++}CD16^{-}$, промежуточных $CD14^{++}CD16^{+}$, неклассических $CD14^{+}CD16^{++}$, переходных $CD14^{+}CD16^{-}$ моноцитов.

Результаты. У пациентов с ИБС в обеих группах относительно здоровых доноров показано снижение содержания $CD14^{++}CD16^{+}$ в контрольной и стимулированной пробах. Только в группе ИБС с ИКМП относительно контрольной пробы после стимуляции VEGF-A установлено значимое увеличение всех $CD14^{+}$: 10,63% (6,80; 17,64) vs. 15,28% (8,75; 27,99), $p < 0,01$, и их субпопуляций: $CD14^{++}CD16^{-}$: 6,08% (1,76; 8,84) vs. 8,57% (3,51; 16,8), $p < 0,05$, $CD14^{++}CD16^{+}$: 3,64% (2,03; 8,59) vs. 6,26% (3,87; 10,3), $p < 0,05$. В этой же группе отмечена тенденция к увеличению $CD14^{+}CD16^{++}$ после стимуляции: 0,19% (0,18; 1,11) vs. 0,61% (0,37; 1,58), $p = 0,062$. В группах ИБС без ИКМП и здоровых доноров не установлено различий содержания всех моноцитов и их субпопуляций после стимуляции VEGF-A. Содержание $CD14^{+}CD16^{-}$ во всех группах пациентов в контрольной и стимулированной пробах не различалось.

Заключение. Для ИБС характерна недостаточность всех $CD14^{+}$ клеток и промежуточных моноцитов ввиду их трансдифференцировки. VEGF-A влияет на субпопуляционный состав моноцитов при ИБС только при наличии ИКМП, увеличивая содержание всех $CD14^{+}$ клеток, их промежуточных и классических форм без превышения показателей у здоровых доноров.

Ключевые слова: VEGF-A; дифференцировка; ангиогенез; классические моноциты; $CD14^{++}CD16^{-}$; промежуточные моноциты; $CD14^{++}CD16^{+}$; неклассические моноциты; $CD14^{+}CD16^{++}$; переходные моноциты; $CD14^{+}CD16^{-}$

Рубрики MeSH:
ИШЕМИЧЕСКАЯ БОЛЕЗНЬ СЕРДЦА – КРОВЬ
ИШЕМИЧЕСКАЯ БОЛЕЗНЬ СЕРДЦА – ПАТОФИЗИОЛОГИЯ
КАРДИОМИОПАТИИ – КРОВЬ
КАРДИОМИОПАТИИ – ПАТОФИЗИОЛОГИЯ
КРОВЕНОСНЫХ СОСУДОВ ЭНДОТЕЛИАЛЬНЫЙ ФАКТОР РОСТА – АНАЛИЗ
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ЛИМФОЦИТОВ ПОДГРУППЫ – ДЕЙСТВИЕ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ

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КОНТАКТНАЯ ИНФОРМАЦИЯ:
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Соответствие принципам этики. Исследования проводились в соответствии с этическими принципами, изложенными в Хельсинкской декларации (1975), и с разрешения локального этического комитета ФГБОУ ВО СибГМУ Минздрава России (протокол №9299 от 28.11.2022). У всех обследованных лиц было получено информированное согласие на участие в исследовании.
Доступ к данным исследования. Данные, подтверждающие выводы этого исследования, можно получить у авторов по обоснованному запросу. Данные и статистические методы, представленные в статье, прошли статистическое рецензирование редактором журнала – сертифицированным специалистом по биостатистике.
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Abbreviations:

VEGF-A – Vascular Endothelial Growth Factor A
VEGFR – Vascular Endothelial Growth Factor Receptors
CHD – Coronary Heart Disease
ICMP – Ischemic Cardiomyopathy

HIGHLIGHTS

Patients with CHD exhibit a reduction in the number of intermediate monocytes *in vitro* due to their spontaneous transdifferentiation which is most pronounced in ICMP.

VEGF-A demonstrates an *in vitro* protective effect in patients with ICMP, preventing excessive loss of mononuclear cells with classical, intermediate, and, to some extent, non-classical immunophenotypes.

The effect of VEGF-A on the subpopulation composition of monocytes is not associated with an excessive accumulation of intermediate and classical forms, regardless of the presence of ICMP. This suggests a potential use of this cytokine in CHD treatment without the risk of exacerbating atherogenesis.

Cardiovascular diseases remain one of the leading causes of mortality worldwide [1]. Despite significant advancements in conservative and surgical treatments, the search for novel therapeutic strategies for patients with atherosclerosis continues. Among these strategies, the induction of angiogenesis has emerged as a promising approach both for patients with ischaemia and for individuals who have undergone endovascular or open surgical interventions to prevent stent or graft restenosis [2]. Vascular endothelial growth factor A (VEGF-A) has become one of the most extensively studied signalling proteins for stimulating angiogenesis in patients with atherosclerosis. To date, not only recombinant VEGF-A but also a drug for prolonged *in vivo* synthesis – Neovasculgen® – has been successfully used in the treatment of critical limb ischaemia [3].

VEGF-A is a highly conserved secretory signalling protein that binds to type 1 and type 2 VEGF-tyrosine kinase receptors (vascular endothelial growth factor receptors – VEGFR) on the surface of endothelial cells. VEGFR2 stimulates endothelial cell proliferation, migration, and survival, while VEGFR1 can act as a decoy receptor for VEGF-A [4]. Additionally, VEGF-A can increase vascular permeability, leading to the infiltration of the vascular wall by monocytes, or mediate the development of collateral vessels by recruiting and activating endothelial cells and monocytes [4].

Currently, four immunophenotypically distinct subpopulations of monocytes have been identified in humans: classical, intermediate, non-classical, and transitional cells. The majority of monocytes in circulation are classical $CD14^{++}CD16^{-}$ monocytes, which exhibit pronounced phagocytic and pro-inflammatory properties. These characteristics are also shared by $CD14^{++}CD16^{+}$ monocytes (intermediate cells). Non-classical $CD14^{+}CD16^{++}$ monocytes possess limited phagocytic capacity but promote reparative processes by secreting anti-inflammatory cytokines and growth factors [5]. Transitional $CD14^{+}CD16^{-}$ monocytes remain poorly studied; they are likely precursors of classical monocytes or may differentiate from them [6]. It has been demonstrated that the development of coronary heart disease (CHD) and atherosclerosis is associated with an increase in the proportion of intermediate monocytes and a decrease in the number of classical forms. This imbalance is further exacerbated in acute coronary syndrome and myocardial infarction [7, 8]. In contrast, in ischaemic cardiomyopathy (ICMP), there is anergy of monocyte differentiation with a deficiency of non-classical forms [9].

Despite existing evidence on the involvement of monocyte subpopulations in the development of ischaemic disorders in atherosclerosis, as well as the practical application of VEGF-A for the treatment of such conditions, the influence of VEGF-A on the differentiation of various monocyte subpopulations possessing angioprotective or pro-inflammatory properties remains unexplored. This effect could have a significant impact on disease progression during VEGF-A therapy.

Aim of the study: To evaluate the influence of VEGF-A on the subpopulation composition of monocytes in peripheral blood mononuclear cell cultures from patients with CHD, depending on the presence of ICMP.

MATERIALS AND METHODS

A single-centre, experimental *in vitro* study was conducted. Consecutive sampling of patients was carried out from those admitted to the Research Institute of Cardiology – a branch of the Tomsk National Research Medical Centre – between 1 December 2022 and 31 May 2023. The required number of patients in the subgroups was determined during the experimental planning stage using Mead's resource equation, aiming to achieve a degree of freedom for error equal to 20. During the experiment, the sample size was increased in accordance with Lehr's formula, based on a pilot analysis of data obtained from studying the initial number of patients determined by Mead's resource equation.

Patient recruitment

The patient inclusion flowchart is presented in Figure 1. A total of 28 patients were assessed for participation in the study. Exclusion criteria were identified in 6 patients. The study included 22 patients with CHD (19 men and 3 women) aged between 54 and 70 years, of whom 11 had ICMP and 11 did not. During the first 4 months, 11 CHD patients with ICMP and 8 patients with ICMP were recruited. Over the following 2 months, only patients with ICMP were included in the study to increase the sample size to the calculated value (n = 11).

Inclusion criteria:

- Age: 18 to 70 years;
- Signed informed consent to participate in the study;
- History of myocardial infarction more than 6 months prior;
- Additional criterion for the ICMP group: left ventricular systolic dysfunction (ejection fraction less than 40%), accompanied by one or more of the following:
  - Myocardial infarction or myocardial revascularisation at least 6 months prior;
  - Stenosis of the left main coronary artery > 75%;
  - Stenosis of two or more coronary arteries > 75% [10].

Exclusion criteria:

- Acute infectious diseases within 3 weeks prior to the study (n = 2);
- Autoimmune or allergic diseases in the acute phase (n = 2);
- Malignant neoplasms (n = 1);
- Viral hepatitis (n = 1);
- Syphilis (n = 0);
- Human immunodeficiency virus (HIV) infection (n = 0);
- Erythropoiesis-stimulating (n = 1) or immunosuppressive therapy (n = 1) within 3 weeks prior to the study.

The control group consisted of 10 generally healthy donors (7 men and 3 women, median age 57.5 [48.0; 65.5] years) without any cardiovascular diseases or related complaints, who provided informed consent to participate in the study (Fig. 1).

Isolation of blood mononuclear cells

The study material consisted of 30 ml of blood collected in a single draw from the cubital vein in the morning on an empty stomach, prior to physical activity and diagnostic or therapeutic procedures. The blood was stabilised with heparin (25 IU/ml).

Blood mononuclear cells were isolated using density gradient centrifugation with Ficoll (density 1.077 g/cm^3) (LLC NPO "PanEco", Russian Federation). After washing the mononuclear cells twice with 0.5% PBS (pH = 7.2), immunomagnetic separation was performed using CD14 MicroBeads and CD34 MicroBead Kit ("Miltenyi Biotec B.V. & Co. KG", Germany), MS separation columns ("Miltenyi Biotec B.V. & Co. KG",

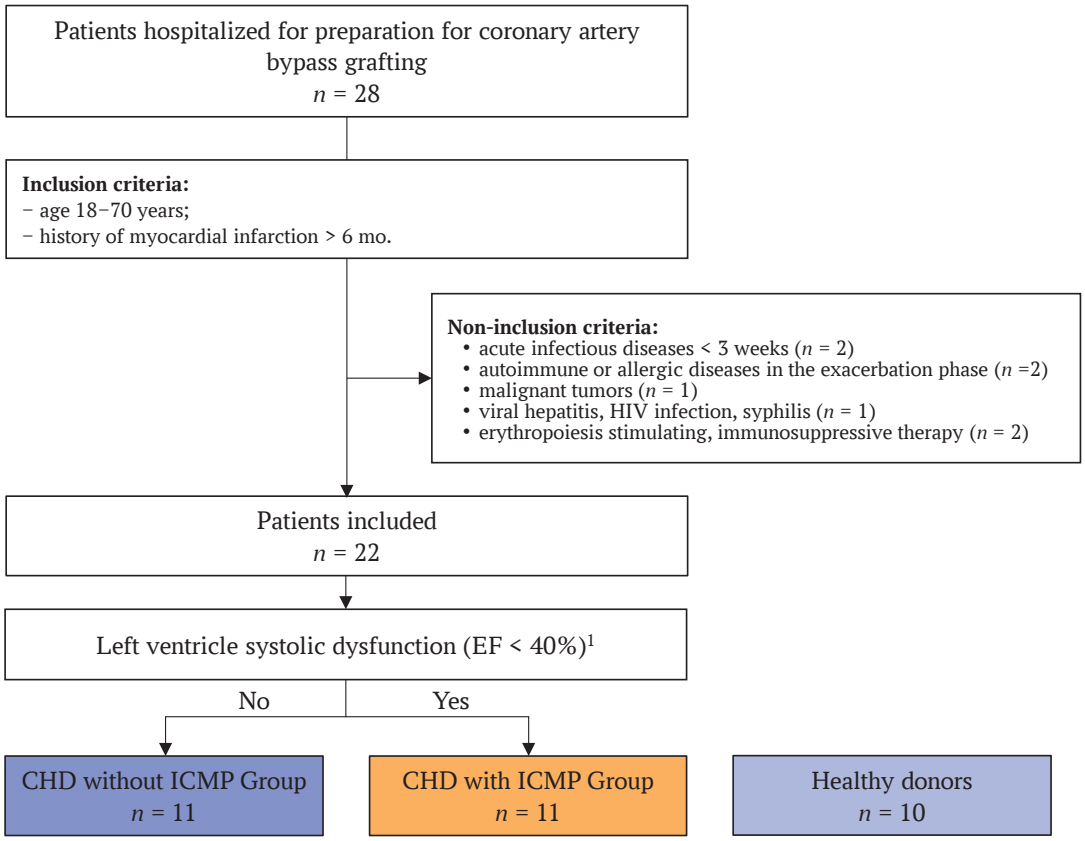

FIG. 1. Study flowchart.

Note: HIV – human immunodeficiency virus; CHD – coronary heart disease; ICMP – ischemic cardiomyopathy; EF – ejection fraction.
[1] With ≥ 1 feature: myocardial revascularization > 6 mo.; left main coronary artery stenosis > 75%; stenosis of two or more coronary arteries > 75%.

Germany), and a MiniMACS magnet ("Miltenyi Biotec B.V. & Co. KG", Germany) according to the manufacturer's instructions. The proportion of $CD14^+$ cells (all monocytes) and $CD34^+$ cells (stem and progenitor haematopoietic cells, as potential precursors of monocytes, also present in the blood) in the culture was 80-85% and 3–5%, respectively.

Cell viability was assessed using a 0.1% trypan blue test (LLC NPO "PanEco", Russian Federation). Cells with a viability of at least 96% were seeded into 2 wells of a 24-well plate at 10^6 cells per well. The cells were incubated for 6 days under 5% CO_2 in complete culture medium (RPMI-1640 medium (LLC NPO "PanEco", Russian Federation), foetal bovine serum, L-glutamine, penicillin-streptomycin) with the addition of 50 ng/ml recombinant human VEGF-A ("Cloud-Clone Corp.", USA) to one of the wells. After 3 days of incubation, a partial medium change was performed, and the stimulant was re-added at the same dose. The sample with recombinant VEGF-A was considered stimulated, while the sample without VEGF-A served as control. After 6 days, the cells were detached from the plate surface by incubation with 500 µl of 0.05% trypsin-EDTA solution (LLC NPO "PanEco", Russian Federation) per well for 5 minutes at 37 °C. After washing the cells with 500 µl of 0.5% PBS, the pellet was resuspended, and the cells were used for flow cytometry (Fig. 2).

Immunophenotyping of monocyte subpopulations

Fluorescence intensity was measured using a "CytoFLEX" flow cytometer ("Beckman Coulter International S.A.", USA) with the "CytExpert 2.3" software application ("Beckman Coulter International S.A.", USA). The boundaries for positive fluorescence signals were established using FMO (Fluorescence Minus One) controls, as a third antibody (not presented in this publication) was also used in the study. However, the determinants CD14 and CD16 were evaluated independently of the expression of its ligand. The proportion of cells positive for each marker was assessed as a percentage of the total number of events, excluding the region of small objects (FSC less than 100×10^4).

Statistical data analysis

For the presentation of results, the median and interquartile range (25th and 75th percentiles) were calculated. The normality of the distribution in samples

was assessed using the Kolmogorov-Smirnov test. Given the deviation of the sample data from a normal distribution, comparative analysis was performed using Mann-Whitney test (for independent samples) and Wilcoxon test (for dependent samples), with Benjamini-Hochberg correction for multiple comparisons. Statistical significance of differences in relative indicators was evaluated using Pearson's chi-square test. Spearman's correlation coefficients were calculated. The strength of association was assessed using Chaddock's scale: a correlation coefficient of 0–0.3 was considered very weak, 0.3–0.5 as weak, 0.5–0.7 as moderate, 0.7–0.9 as strong, and 0.9–1 as very strong. The results of the statistical analysis were considered significant at a level of $p < 0.05$. Statistical data analysis was performed using "Statistica 10.0" software (StatSoft Inc., USA).

RESULTS

Baseline Patient Characteristics

Patients with CHD showed no significant differences in age, sex, or prescribed therapy between the studied groups (with and without ICMP) (Table 1). Calcium channel blockers were not administered to patients with ICMP due to their negative inotropic effects. The majority of patients in both groups presented with angina pectoris of functional class II-III and heart failure of functional class II-III, as classified by the New York Heart Association (NYHA).

The Effect of VEGF-A on Monocyte Subpopulation Composition

The addition of VEGF-A to the mononuclear cell culture of healthy donors did not alter either the total monocyte count or the ratio of their subpopulations. Notably, the total monocyte count in the culture remained at approximately 40%, irrespective of the presence of the stimulant (Table 2).

In samples from patients without ICMP, a threefold reduction in the proportion of intermediate $CD14^{++}CD16^{+}$ monocytes was observed compared to the healthy donor group, while the percentages of other monocyte immunophenotypes and the total monocyte count remained comparable. The addition of VEGF-A to the mononuclear cell culture in these patients, as in healthy donors, had no effect on the total monocyte count or their subpopulation composition (Table 2).

In patients with ICMP, similar to those without ICMP, a deficiency in the number of intermediate $CD14^{++}CD16^{+}$ monocytes was observed in the mononuclear cell culture, regardless of the presence of the stimulant. In unstimulated samples from patients with ICMP, a profound deficit in the total monocyte count (fourfold) and intermediate $CD14^{++}CD16^{+}$ monocytes (tenfold) was identified compared to the healthy donor group. These two parameters showed a statistically significant increase under the influence of VEGF-A relative to the control sample, although they did not reach the levels observed

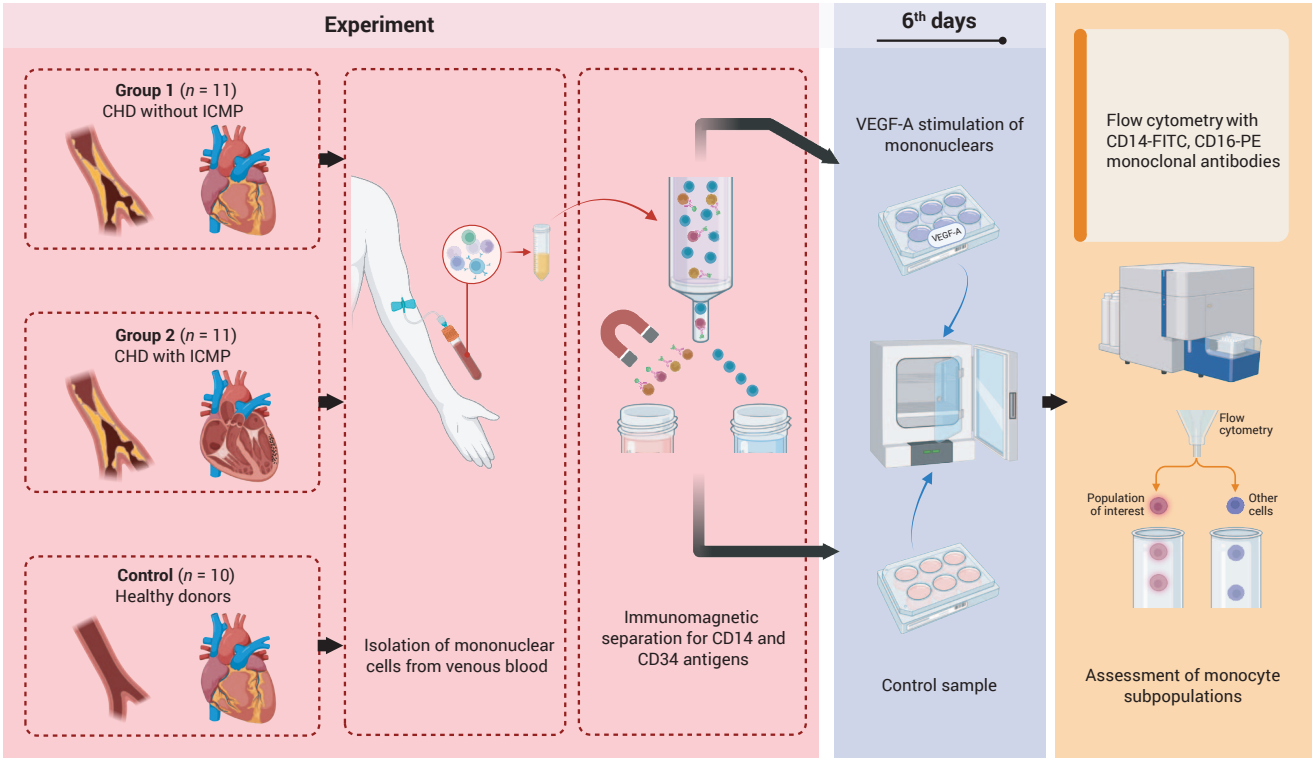

FIG. 2. Experimental design.

Note: CHD – coronary heart disease; ICMP – ischemic cardiomyopathy; VEGF-A – vascular endothelial growth factor A.

in healthy donors. Unlike in patients without ICMP, the proportion of classical $CD14^{++}CD16^{-}$ monocytes in patients with ICMP significantly increased after incubation with VEGF-A compared to the unstimulated sample, reaching levels comparable to those in the healthy donor group (Table 2).

No statistically significant differences in the number of non-classical $CD14^{+}CD16^{++}$ monocytes and transitional $CD14^{+}CD16^{-}$ monocytes were observed between the control and VEGF-A-stimulated samples across the studied groups. However, only in patients with ICMP was there a notable trend ($p = 0.062$) towards an increase in the proportion of non-classical $CD14^{+}CD16^{++}$ monocytes in the cell culture containing VEGF-A compared to the unstimulated sample (Table 2).

Correlation of Monocyte Subpopulations with Total Monocyte Count

(1) Among All Patients with Coronary Heart Disease

Correlation analysis conducted among all patients with CHD (both with and without ICMP) revealed a strong positive association between the total monocyte count and the proportion of intermediate $CD14^{++}CD16^{+}$ and classical $CD14^{++}CD16^{-}$ monocytes in the control sample (Fig. 3A). The strength of this association remained unchanged following the addition of VEGF-A to the culture (Fig. 3B). A similarly positive, moderate-strength correlation between the total monocyte count and the percentage of transitional $CD14^{+}CD16^{-}$ was observed in the control sample; this correlation strengthened to very strong in the presence of VEGF-A. Additionally, a moderate-strength association between the total monocyte count and the number of non-classical $CD14^{+}CD16^{++}$ monocytes, identified in the unstimulated mononuclear cell culture, weakened to a weak correlation under the influence of VEGF-A (Fig. 3C, 3D).

(2) Depending on the Presence of Ischemic Cardiomyopathy and VEGF-A Stimulation

The analysis of correlations between the content of individual monocyte subpopulations and total monocyte count revealed associations of variable strength, depending both on the presence of ICMP and the addition of VEGF-A to the mononuclear cell culture (Fig. 4).

A common feature found in both groups of patients and in healthy donors was a strong positive correlation between the total monocyte count and the proportion of intermediate monocytes in the control (across all categories of individuals) and VEGF-A-stimulated sample (in the control group and in patients without

Table 1. **Baseline characteristics of patients with coronary heart disease in the studied groups**

Feature	Coronary heart disease		*p* value
	without ICMP (*n* = 11)	with ICMP (*n* = 11)	
Men, *n (%)*	10 (91)	11 (100)	n.s.
Women, *n (%)*	1 (9)	-	n.s.
Age, years	63,5 (58,0; 67,5)	60,5 (56,5; 64,0)	n.s.
Stable angina:			
Class II, *n (%)*	2 (18)	3 (27)	n.s.
Class III, *n (%)*	8 (73)	7 (64)	n.s.
Class IV, *n (%)*	1 (9)	1 (9)	n.s.
Left ventricular ejection fraction, %	59,25 (50,00; 67,50)	30,50 (22,75; 36,50)	< 0,001
NYHA classification of heart failure			
Class I, *n (%)*	2 (18)	1 (9)	n.s.
Class II, *n (%)*	4 (36)	7 (64)?	n.s.
Class III, *n (%)*	5 (46)	3 (27)	n.s.
Medications:			
Long-acting nitrates, *n (%)*	7 (64)	6 (55)	n.s.
β1 blockers, *n (%)*	10 (91)	9 (82)	n.s.
Calcium channel blockers, *n (%)*	7 (64)	0	0,001
ACE inhibitors, *n (%)*	3 (27)	5 (46)	n.s.
Antiplatelet agents, *n (%)*	8 (73)	9 (82)	n.s.
Statins, *n (%)*	9 (82)	10 (91)	n.s.

Note: ACE – angiotensin-converting enzyme; ICMP – ischemic cardiomyopathy; n.s. – not significant, не значимо; NYHA – New York heart association.

Table 2. **Content of monocyte subpopulations in the mononuclear cell culture in the control and in the VEGF-A stimulated samples in all groups studied**

Monocyte content	Coronary heart disease						Healthy donors (*n* = 10)		
	without ICMP (*n* = 11)			with ICMP (*n* = 11)					
	Control	VEGF-A	*p* value	Control	VEGF-A	*p* value	Control	VEGF-A	*p* value
All monocytes $CD14^{+/++}$, %	17,79 (7,15; 35,63)	21,50 (7,15; 38,8)	n.s.	10,63 (6,80; 17,64)[b]	15,28 (8,75; 27,99)[a]	<0,01	40,42 (21,70; 47,62)	41,25 (20,55; 46,69)	n.s.
Classical monocytes $CD14^{++}CD16^{-}$, %	5,45 (2,13; 15,27)	8,45 (3,23; 9,09)	n.s.	6,08 (1,76; 8,84)	8,57 (3,51; 16,8)	<0,05	10,72 (6,73; 2,04)	10,66 (6,37; 12,31)	n.s.
Intermediate monocytes $CD14^{++}CD16^{+}$, %	9,12 (5,23; 23,06)[a]	11,10 (4,60; 23,9)[a]	n.s.	3,64 (2,03; 8,59)[b]	6,26 (3,87; 10,3)[a]	<0,05	30,42 (13,36; 35,77)	34,81 (13,73; 40,85)	n.s.
Non-classical monocytes $CD14^{+}CD16^{++}$, %	0,86 (0,47; 1,28)	1,06 (0,22; 1,81)	n.s.	0,19 (0,18; 1,11)	0,61 (0,37; 1,58)	0,062	0,92 (0,56; 1,27)	0,89 (0,33; 1,45)	n.s.
Transitional monocytes $CD14^{+}CD16^{-}$, %	2,90 (1,49; 4,47)	2,23 (1,58; 4,59)	n.s.	2,48 (1,53; 4,80)	2,40 (1,70; 3,51)	n.s.	2,53 (2,11; 4,78)	2,49 (1,92; 6,204)	n.s.

Note: ICMP – ischemic cardiomyopathy; VEGF-A – vascular endothelial growth factor A; n.s. – not significant;
[a] – $p < 0.05$ in comparison to the same sample in healthy donors; [b] – $p < 0.01$ in comparison to the same sample in healthy donors.

ICMP). A distinctive characteristic of patients with ICMP was a strong positive correlation between the total monocyte count and the percentages of three monocyte subpopulations (classical, intermediate, and transitional) in the absence of the stimulant. However, upon the addition of VEGF-A, the correlation with the number of intermediate monocytes disappeared. Contrastingly, in patients without ICMP, a strong positive correlation of the total monocyte count with the proportion of classical and intermediate cells identified in the unstimulated sample was, after stimulation with VEGF-A, accompanied by an additional strong positive correlation with the level of transitional monocytes (Fig. 4).

DISCUSSION

The study revealed a consistent trend across all three groups of examined individuals – CHD patients without ICMP, patients with ICMP, and healthy donors – characterised by a low proportion of $CD14^{+}$ cells after culturing compared to their initial purity (80-85%) after isolation, regardless of VEGF-A stimulation. In the samples of healthy donors, approximately 40% of $CD14^{+}$ cells remained after 6 days of culturing, whereas in ICMP patients, this proportion dropped to 10–15%, despite maintaining cell viability at ⩾96% (indicating that the cells remained alive but had lost their monocytic identity). This phenomenon can be attributed to the high plasticity of monocytes, which enables them to differentiate into various subpopulations, as well as into macrophages [11], and even fibrocytes and fibroblasts, which lack the CD14 surface marker [12]. Additionally, a small subset of monocytes, specifically $CD14^{+}CD34^{+}VEGFR2^{+}$ endothelial progenitor cells (comprising 1–6% of blood monocytes), can directly differentiate into endotheliocytes when exposed to an endothelial (pro-angiogenic) microenvironment [13, 14].

Notably, in healthy donors, monocytes also undergo spontaneous transdifferentiation during culturing (only 40% of $CD14^{+}$ cells remain). In CHD patients without ICMP, this process tends to be more pronounced (approximately 18% $CD14^{+}$ cells remain), while in patients with ICMP, it is enhanced considerably, reaching statistical significance (only 10% $CD14^{+}$ cells remain). The latter may represent an *in vivo* pathogenetic mechanism contributing to the development of ICMP, which is characterized by diffuse myocardium fibrosis [15], likely driven by the excessive transdifferentiation of monocytes into fibroblasts and fibrocytes.

Analysis of the subpopulation composition of monocytes in native mononuclear cell cultures revealed distinct differences in the content of specific monocyte subsets between the two CHD patient groups. In CHD patients without ICMP, the trend towards a reduction in the total $CD14^{+}$ cells was accompanied by a significant decrease in intermediate $CD14^{++}CD16^{+}$ monocytes. In contrast, ICMP patients exhibited a profound deficit of intermediate $CD14^{++}CD16^{+}$ monocytes and a marked tendency towards a deficiency of non-classical $CD14^{+}CD16^{++}$ monocytes, alongside a statistically significant reduction in total $CD14^{+}$ cells. Intermediate and non-classical monocytes represent activated monocytic forms, and their proportion increases in various disease states [16]. This may explain why their numbers declined most significantly during culturing in both patient groups, influencing the overall monocyte count. Such interpretation is supported by a positive correlation between the total monocyte count and the proportion of intermediate $CD14^{++}CD16^{+}$ monocytes

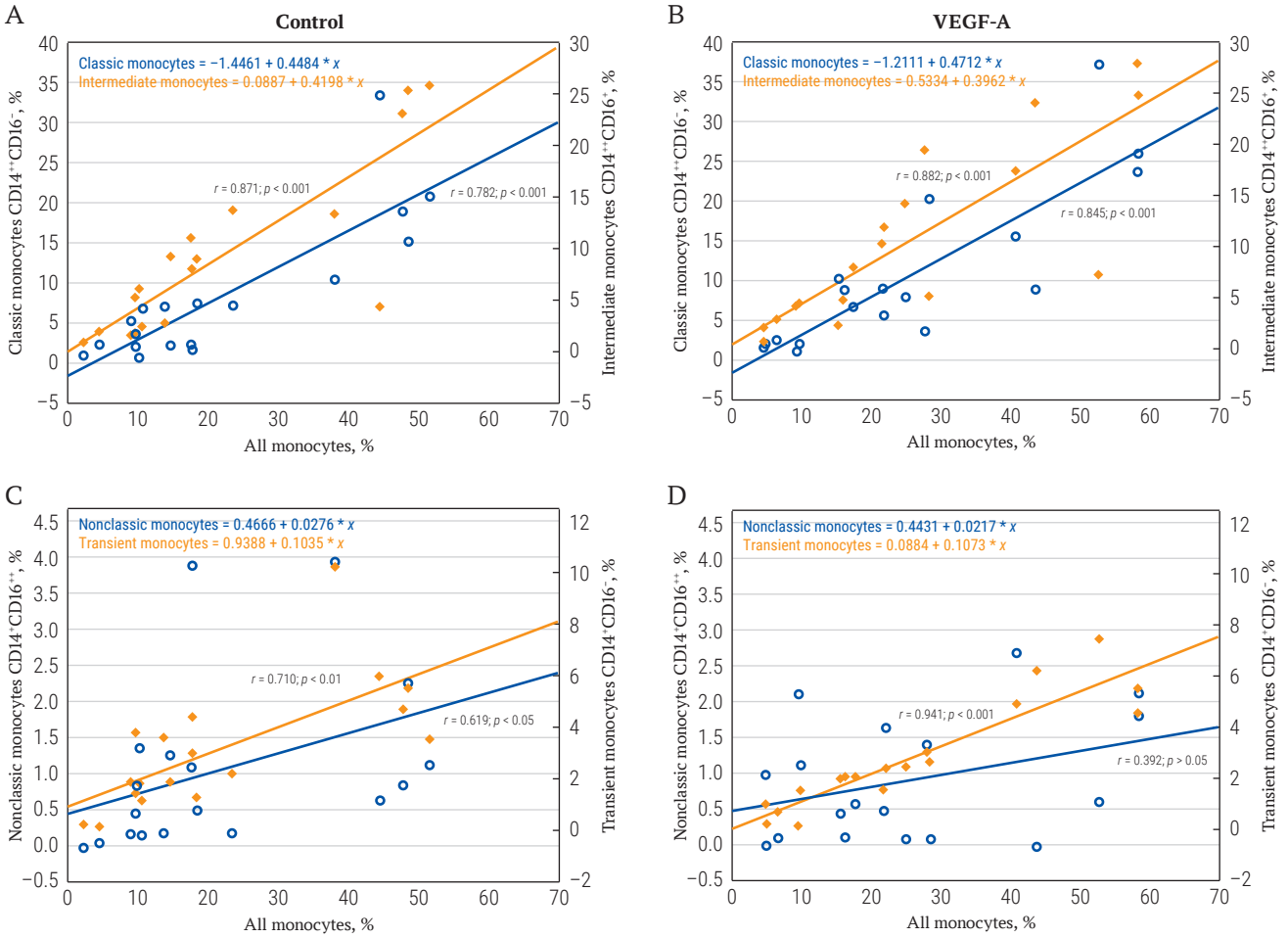

FIG. 3. The correlation of the total number of monocytes in culture with their individual subpopulations in the control and in the VEGF-A stimulated samples in all patients with coronary heart disease.

Note: VEGF-A – vascular endothelial growth factor A.

A. The content of classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes in the control sample.
B. The content of classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes after VEGF-A stimulation.
C. The content of non-classical $CD14^{+}CD16^{++}$ and transitional $CD14^{+}CD16^{-}$ monocytes in the control sample.
D. The content of non–classical $CD14^{+}CD16^{++}$ and transitional $CD14^{+}CD16^{-}$ monocytes after VEGF-A stimulation.

in both patient groups, whereas a correlation with non-classical $CD14^{+}CD16^{++}$ monocytes was observed only in healthy donors. Furthermore, intermediate monocytes are known to produce the highest levels of reactive oxygen species under unstimulated conditions [16], and an elevated count of these cells is linked to an increased risk of cardiovascular diseases [7–9, 17].

The addition of VEGF-A to mononuclear cell cultures from ICMP patients increased both the total monocyte count and the proportion of intermediate $CD14^{++}CD16^{+}$ and classical $CD14^{++}CD16^{-}$ monocytes compared to control sample. In contrast, VEGF-A stimulation had no effect on the studied parameters in CHD patients without ICMP or in healthy donors. Given that the primary cause of the reduction in $CD14^{+}$ monocytes during culturing appears to be their transdifferentiation into $CD14^{-}$ cells (fibrocytes, fibroblasts, and endothelial cells), it is plausible that VEGF-A inhibits this process in ICMP patients, thereby preserving a greater number of intermediate and classical monocytes.

Classical $CD14^{++}CD16^{-}$ monocytes, which participate in innate immune responses following extravasation [11, 18], demonstrate the highest level of plasticity [11]. Consequently, their level should decrease the most during culturing as they differentiate into $CD14^{-}$ cells. However, according to our observations, patients with CHD showed the greatest deficit of intermediate monocytes, while the reduction in classical monocytes was less pronounced. This data indicates that intermediate monocytes exhibit more active transdifferentiation during the culturing process. The comparable number of classical $CD14^{++}CD16^{-}$ monocytes in CHD patients and healthy donors is consistent with their high plasticity, as they represent the predominant population of blood monocytes [9], whereas intermediate $CD14^{++}CD16^{+}$ monocytes are the most abundant population in culture.

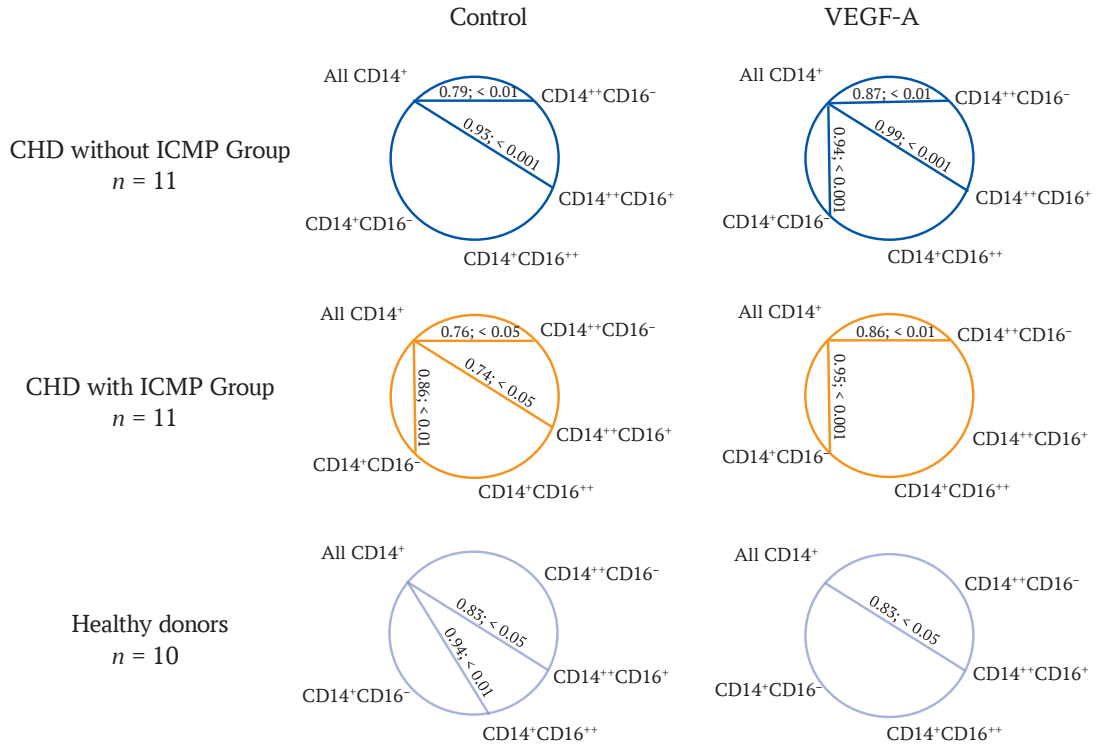

FIG. 4. The correlation of the total number of monocytes in culture with their individual subpopulations in the control and in the VEGF-A stimulated samples in all groups studied.

Note: VEGF-A – vascular endothelial growth factor A; CHD – coronary heart disease; ICMP – ischemic cardiomyopathy; $CD14^{++}CD16^{-}$ – classic monocytes; $CD14^{++}CD16^{+}$ – intermediate monocytes; $CD14^{+}CD16^{++}$ – non-classic monocytes; $CD14^{+}CD16^{-}$ – transient monocytes.

This suggests that, under culturing conditions, classical monocytes initially differentiate into intermediate forms, as observed *in vivo* [19], and subsequently undergo transdifferentiation into non-monocytic cells. The more pronounced reduction in intermediate monocytes compared to classical monocytes is likely due to the fact that the majority of classical monocytes have already transitioned into intermediate forms after 6 days of culturing. Surprisingly, VEGF-A inhibited the differentiation and transdifferentiation of monocytes in patient-derived cultures, despite its primary association with adaptation to hypoxia and angiogenesis rather than to monocytopoiesis and inflammation. This effect can be attributed to the expression of pro-inflammatory VEGFR1 and pro-angiogenic VEGFR2, which are present in 5-10% of monocytes [20]. Notably, the corrective influence of VEGF-A was observed exclusively in patients with ICMP and was absent in CHD patients without ICMP and in healthy donors. This phenomenon may stem from receptor hyperexpression or enhanced intracellular signaling through the 'cytochrome P450 4A/F – 20-hydroxyeicosatetraenoic acid' pathway, which is upregulated by hypoxia [21]; in ICMP, this hypoxia becomes chronic due to widespread myocardial ischemia [14].

Studies have demonstrated that the elimination of VEGF results in mitochondrial fragmentation, suppression of cellular metabolism, and death of autophagic cells [21]. These effects are mediated by the transcription factor FOXO (forkhead box protein), which activates autophagy and promotes the survival of hematopoietic stem cells under metabolic stress. In endotheliocyte cultures, VEGF exposure deactivates FOXO1 and inhibits cell death [22]. While the correlation patterns for classical and intermediate monocytes did not significantly differ in the presence or absence of VEGF-A, the proportion of non-classical and transitional monocytes in the absence of VEGF-A stimulation correlated with the total monocyte count. However, upon the addition of VEGF-A, the correlation between the total monocyte count and transitional monocytes strengthened, whereas the correlation with non-classical monocytes was lost. Furthermore, a notable trend towards a 3-fold increase ($p = 0.062$) in the proportion of non-classical monocytes in cultures from ICMP patients in the presence of VEGF-A underscores VEGF-A's ability to attenuate transdifferentiation and address the deficiency of non-classical monocytes. This effect is advantageous, as non-classical monocytes exhibit protective properties toward the endothelium by removing immune complexes and dead cells from its surface; it is the deficiency of these cells in the blood that is characteristic for patients with ICMP [9].

The observed differences in correlation patterns among the three groups of individuals, depending on the presence or absence of VEGF-A in cultures, indicate that CHD is associated with qualitative impairments in monocyte reactivity, which are most pronounced in ICMP. These disturbances are characterized by a dysregulation of the spontaneous transdifferentiation of classical, non-classical, and transitional monocytes in response to both *in vitro* conditions and VEGF-A stimulation. The ability of intermediate monocytes to undergo spontaneous transdifferentiation is heightened in CHD, irrespective of the presence of ICMP. However, only in ICMP is this phenomenon accompanied by a reduction in the total monocyte count in culture and is mitigated by the presence of VEGF-A.

Limitations of the Study and Directions for Future Research

The findings are applicable to patients from the West Siberian region who have a history of myocardial infarction dating back at least 6 months and who have signs of multivessel coronary artery disease. The data indicating that VEGF-A normalises the subpopulation composition of monocytes suggest that VEGF-A could be utilised for CHD therapy to promote angiogenesis without the risk of exacerbating atherosclerosis. Additionally, insights into the pathologically enhanced transdifferentiation of monocytes in CHD could inform the development of novel therapeutic strategies aimed at regulating this transition.

CONCLUSION

The development of CHD, irrespective of the presence of ICMP, is associated with a reduction in the number of intermediate monocytes *in vitro* due to their spontaneous transdifferentiation. This phenomenon is most pronounced in ICMP and represents a pathogenetic factor of this condition. Stimulation of blood mononuclear cells from CHD patients with the cytokine VEGF-A *in vitro* modulates the subpopulation composition of monocytes exclusively in individuals with ICMP, mitigating the enhanced transdifferentiation and preventing excessive loss of cells with classical, intermediate, and, to some extent, non-classical immunophenotypes, which demonstrates a protective effect of VEGF-A. At the same time, VEGF-A does not promote excessive accumulation of cells with pro-inflammatory properties (intermediate and classical forms). In CHD without ICMP, VEGF-A has no impact on the subpopulation composition of monocytes, suggesting a potential therapeutic opportunity for the use of this cytokine in CHD treatment without the risk of exacerbating atherogenesis.

AUTHORS CONTRIBUTIONS

Margarita V. Gladkovskaya and Svetlana P. Chumakova wrote the manuscript and analyzed the literature on the topic of the study. Svetlana P. Chumakova developed design of the study. Margarita V. Gladkovskay and Vadim S. Poletika prepared the samples and performed the cultural research methods and flow cytometry. Olga I. Urazova made a significant contribution to the correction of the manuscript text. Vladimir M. Shipulin and Sergey L. Andreev provided the clinical material and consulted on the study planning and interpretation of clinical data. All authors approved the final version of the article.

ВКЛАД АВТОРОВ

М.В. Гладковская и С.П. Чумакова осуществляли написание текста рукописи и анализ литературы по теме исследования. С.П. Чумакова разработала дизайн исследования, осуществляла статистическую обработку данных. М.В. Гладковская осуществляла подготовку материала и выполнение культуральных методов исследования и проточную цитофлуориметрию. О.И. Уразова внесла существенный вклад в корректировку текста рукописи. В.М. Шипулин и С.Л. Андреев предоставили клинический материал и осуществляли консультирование по вопросам планирования исследования и интерпретации клинических данных. Все авторы утвердили окончательную версию статьи.

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Bone turnover markers in oral and gingival crevicular fluid in children with end-stage chronic kidney disease

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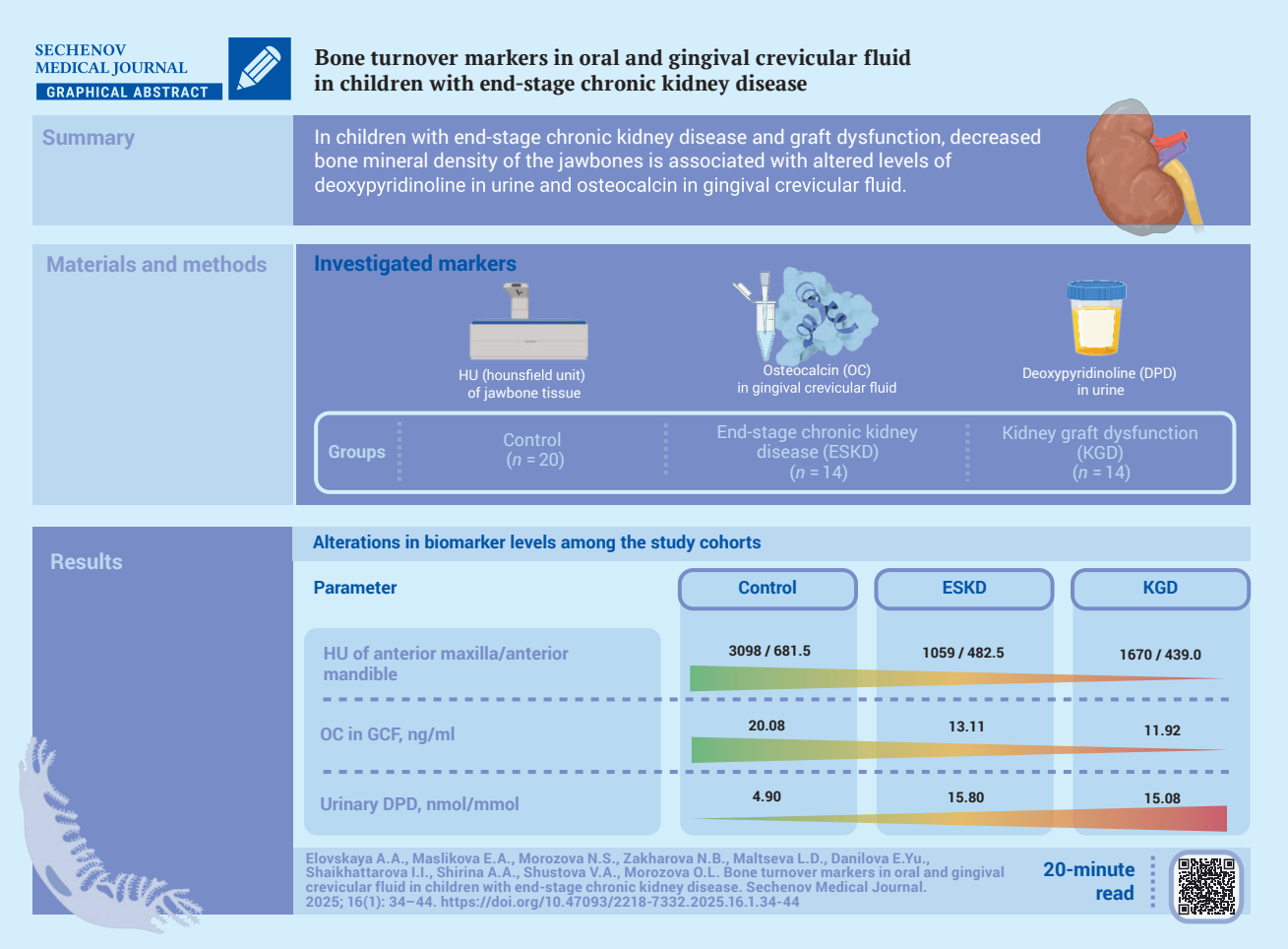

Abstract

Objective. To study bone turnover markers in biological fluids (urine, blood serum, oral fluid (OF) and gingival crevicular fluid (GCF)) at the stage of planning an orthodontic strategy in children with end-stage chronic kidney disease (ESKD).

Materials and methods. Pilot, cross-sectional, multicenter study was conducted. A total of 48 children aged 7 to 17 years were examined and divided into three groups: 14 children with ESCKD, 14 children with renal transplant dysfunction (RTD), 20 almost healthy children. Bone turnover markers were assessed by changes in osteocalcin (OC) in the OF, GCF and blood serum, urinary deoxypyridinoline (DPD), levels of total, ionized calcium and phosphorus in

blood and pH of OF. Bone tissue mineral density was assessed by cone-beam computerized tomography according to the C. Mish classification.

Results. All groups of children were comparable by gender and age. All patients had no significant mineral and bone disorders. Total and ionized calcium did not demonstrate statistically significant differences between the study groups. Serum phosphorus level was higher in ESCKD children compared to RTD children and control group. Urinary DPD, OC in GCF and OF pH were higher in children with CKD compared to healthy children. However, there were no statistically significant changes between the ESCKD group and the RTD group. In the posterior maxilla, the Hounsfield index was higher in the group with RTD compared to the ESCKD group ($p < 0.01$), and similar to the control group. In the anterior maxilla, as well as in the anterior and posterior mandibular regions, the Hounsfield index was higher in the control group than in the ESCKD and RTD groups.

Conclusion. The most prominent changes of bone turnover markers were found in children with ESCKD. Urinary DPD and OC in GCF were associated with the decrease in kidney function and jawbone mineral density.

Keywords: renal transplant dysfunction; mineral and bone disorders in chronic kidney disease; osteocalcin; deoxypyridinoline; orthodontic treatment

MeSH terms:

CHRONIC KIDNEY DISEASE – MINERAL AND BONE DISORDER – PHYSIOPATHOLOGY
ORTHODONTICS, CORRECTIVE – METHODS
BONE DENSITY
OPERATIONS SCHEDULING
BIOMARKERS – ANALYSIS
CHILD

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Ethics statements. This study using biological material was conducted in accordance with the World Medical Association's Declaration of Helsinki on Ethical Principles of Biomedical Research. The study was conducted in accordance with the permission of the Local Ethics Committee of I.M. Sechenov First Moscow State Medical University of the Russian Ministry of Health (Sechenov University), No. 01-22 dated January 20, 2022. Informed voluntary consent for inclusion in the study was obtained from one of the patient's parents or other legal representative.
Data access. The data that support the findings of this study are available from the corresponding authors upon reasonable request. The data and statistical methods presented in the article have been statistically reviewed by the journal editor, a certified biostatistician.
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Маркеры ремоделирования костной ткани в ротовой и зубодесневой жидкостях у детей с терминальной стадией хронической болезни почек

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Аннотация

Цель исследования. Изучить маркеры ремоделирования костной ткани в биологических жидкостях (моче, сыворотке крови, ротовой жидкости (РЖ) и зубодесневой жидкости (ЗДЖ)) на этапе планирования ортодонтической стратегии у детей с терминальной стадией хронической болезни почек (тХБП).

Материалы и методы. Проведено пилотное одномоментное многоцентровое исследование. Обследованы 48 детей в возрасте от 7 до 17 лет, разделенных на три группы: 14 – с тХБП, 14 – с дисфункцией трансплантата почки (ДТП), 20 практически здоровых детей. Определяли маркеры ремоделирования кости: остеокальцин (ОК) в РЖ, ЗДЖ и сыворотке крови, дезоксипиридинолин (ДПИД) в моче, уровень общего, ионизированного кальция и фосфора в крови и pH РЖ. Минеральную плотность костной ткани оценивали по данным конусно-лучевой компьютерной томограммы по классификации C. Mish.

Результаты. Группы детей были сопоставимы по возрасту и полу. Все пациенты были без выраженных минерально-костных нарушений. Уровни общего и ионизированного кальция в крови не различались между исследуемыми группами. Уровень фосфора в крови был выше в группе тХБП по сравнению с группой ДТП и группой контроля. Концентрации ДПИД в моче и ОК в ЗДЖ, а также уровень pH РЖ были выше в группах детей с ХБП по сравнению с контрольной группой, при этом статистически значимых различий между группами тХБП и ДТП не выявлено. В заднем отделе верхней челюсти индекс Хаунсфилда был выше в группе с ДТП по сравнению с группой тХБП ($p < 0,01$) и сопоставим с контрольной группой. В переднем отделе верхней челюсти, а также в переднем и заднем отделах нижней челюсти индекс Хаунсфилда был выше в контрольной группе, чем в группах тХБП и ДТП.

Заключение. Наиболее выраженные изменения маркеров ремоделирования кости выявлены у детей с тХБП. Уровни ДПИД в моче и ОК в ЗДЖ ассоциированы со степенью снижения функции почек и минеральной плотностью челюстных костей.

Ключевые слова: дисфункция трансплантата почки; минерально-костные нарушения при хронической болезни почек; остеокальцин; дезоксипиридинолин; ортодонтическое лечение

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Соответствие принципам этики. Данное исследование с использованием биологического материала проводилось в соответствии с Хельсинкской декларацией Всемирной медицинской ассоциации об этических принципах проведения биомедицинских исследований. Исследование проведено в соответствии с разрешением Локального этического комитета ФГАОУ ВО «Первый МГМУ им. И.М. Сеченова» Минздрава России (Сеченовский Университет) (№ 01-22 от 20.01.2022). Информированное добровольное согласие на включение в исследование было получено у одного из родителей или иного законного представителя пациента.
Доступ к данным исследования. Данные, подтверждающие выводы этого исследования, можно получить у авторов по обоснованному запросу. Данные и статистические методы, представленные в статье, прошли статистическое рецензирование редактором журнала – сертифицированным специалистом по биостатистике.
Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.
Финансирование. Исследование не имело спонсорской поддержки (собственные ресурсы).

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Список сокращений:

ДПИД – дезоксипиридинолин
ДТП – дисфункция трансплантата почки
ЗДЖ – зубодесневая жидкость
ОК – остеокальцин
РЖ – ротовая жидкость
рСКФ – расчетная скорость клубочковой фильтрации
тХБП – терминальная стадия хронической болезни почек
ХБП – хроническая болезнь почек
ХБП-МКН – минеральные костные нарушения при хронической болезни почек

HIGHLIGHTS

- Changes in the level of bone turnover markers (OC in the GCF and DPD in urine) are associated with mineral and bone disorders in children with ESCKD.
- The measurement of OC in the GCF is more informative than in the OF.
- There is an increase in urinary DPD and a decrease in OC levels in the GCF concurrent with a decrease in the Hounsfield index in both anterior and posterior regions of the maxilla and mandible.
- The use of bone turnover markers content is promising for determining orthodontic strategy.

Chronic kidney disease (CKD) is a persistent organ damage for three months or more due to various etiologic factors. The pathologic basis of the disease is the process of replacement of normal anatomical structures by fibrosis, which leads to organ dysfunction. CKD is evidenced by a decrease in estimated glomerular filtration rate (eGFR) and/or albuminuria and other markers of kidney damage [1]. The prevalence of CKD in the world population is more than 800 million people [2]. The global mortality from CKD reached 1.2 million in 2017 and is projected to increase [3].

In the world pediatric population, the prevalence of CKD reaches 18.5–58.3 cases per 1 million children [4]. In Russia since 2012, the overall incidence of CKD in children continues to grow [3]. Approaches to CKD diagnosis are unified for both children and adults. However, due to the predominance of non-glomerular etiology of CKD in children, albuminuria is detected less frequently than in adults [5]. According to the European Pediatric Registry, congenital anomalies of the kidney and urinary tract and genetic diseases are the leading etiologic factors of CKD in children, accounting for 40–60% and 20–30% of detected cases, respectively; glomerulonephritis makes an etiologic contribution in less than 10% of cases [5].

End-stage kidney disease (ESCKD) requires renal replacement therapy (hemodialysis, peritoneal dialysis or renal transplant). ESCKD is associated with life quality decline and unfavorable outcomes [6]. Moreover, ESCKD in children is accompanied by significant mineral and bone disorders (CKD-MBD) [7–9], arising as a result of hyperparathyroidism and impaired calcium-phosphorus (Ca-P) metabolism [10, 11]. In CKD-MBD children are observed with a decrease in growth [12], a high tendency to fractures [13, 14], as well as multiple structural changes in bone tissue, including cortical loss,

demineralization, bone trabeculae rarefaction, which are associated with increased osteoclast activity [13].

Bone turnover markers in CKD-MBD are deoxypyridinoline (DPD) and osteocalcin (OC) [15]. DPD is a compound formed during collagen breakdown, it is released into the bloodstream, and then excreted in the urine. DPD reflects osteoclasts activity; DPD level increasing directly correlates to the severity of renal dysfunction in experimental study on rats [16]. OC is a vitamin K-dependent protein synthesized by osteoblasts, reflects impaired bone mineralization in CKD-associated hyperparathyroidism [17, 18].

CKD patients are prone to various maxillofacial bone changes such as decreased density of cortical bone and increased jawbone porosity [19], shortened mandible branches, increased gonial angle, decreased posterior facial height [8, 20, 21], structural and functional temporomandibular joint changes [22, 23], along with a significant slowdown in teething [22]. These changes require a personalized approach to orthodontic treatment in CKD children and objective markers for making a medical decision.

Today there are still some open questions about optimal timing for initiating orthodontic treatment and its types in CKD children, and monitoring bone remodeling during this treatment. Thus, searching for biomarkers reflecting specific bone changes including maxillofacial bones in CKD patients remains in demand.

Study objective: to investigate bone turnover markers in different biological fluids (urine, blood serum, oral fluid (OF) and gingival crevicular fluid (GCF) at the stage of planning an orthodontic strategy in children with end-stage chronic kidney disease (ESCKD).

MATERIALS AND METHODS

This pilot cross-sectional multicenter study based on Russian Federal Law No. 323-FZ dated 21.11.2021 "On the Fundamentals of Public Health Protection in the Russian Federation", (Legislation Bulletin of the Russian Federation, 2011, No. 48, Art. 6724). The required number of patients in groups was determined before the study. The sample size was sufficient given the power of 80%.

Patient enrollment

The study was conducted from March 1 to June 30, 2024, at the following clinical centers: E.V. Borovsky Institute of Dentistry, Sechenov University; Surgical Department No. 1, Academician V.I. Shumakov National Medical Research Center of Transplantology and Artificial Organs, Ministry of Health of the Russian Federation. A continuous recruitment of patients was carried out from those who applied to the above-mentioned medical institutions.

Inclusion criteria:

- age from 7 to 17 years;
- established diagnosis of CKD (ICD-10 codes[1]: N18 Chronic kidney disease; T86.1 Renal transplant dysfunction);
- dental anomalies, including bite anomalies;
- availability of written informed voluntary consent of parents/legal representatives for the child's participation in the study.

Non-inclusion criteria:

- active/current orthodontic treatment (n = 5);
- concomitant acute/chronic diseases affecting bone metabolism:
  - endocrine and metabolic diseases (n = 10),
  - autoimmune diseases (n = 2),
  - genetic diseases (n = 4),
  - oncological diseases (n = 1),
  - diseases of the gastrointestinal tract (n = 3);
  - chronic liver diseases (n = 7),
  - drug-induced disorders of bone metabolism (n = 5).

A total of 65 children and adolescents were assessed for participation in the study. Exclusion criteria were identified in 37 patients (Fig.). The study included 28 CKD children, who were divided into two groups: Group 1 – 14 ESCKD patients with eGFR according to CKiD U25 with a constant creatinine coefficient ⩽ 25 ml/min/1.73m^2; Group 2 – 14 patients with kidney graft dysfunction (RTD) with eGFR according to the CKiD U25 with a constant creatinine coefficient > 25 ml/min/1.73 m^2.

The control group consisted of 20 practically healthy children and adolescents with no general medical pathology, matched by sex and age to the group of children with CKD, who underwent dental examination at the Department of Pediatric, Preventive Dentistry and Orthodontics in E.V. Borovsky Institute of Dentistry during the study period.

Determination of bone metabolism biomarkers

Biological fluids from patients were taken once in the morning before the breakfast and diagnostic and therapeutic procedures. Blood samples of 5 mL each were collected from cubital vein or from the hand back veins, stabilized with heparin (25 IU/ml), urine sample of 50 mL each, oral fluid sample at least 5 mL.

Biochemical blood analysis was performed by photocolorimetric methods to determine the level of total (Ca) and ionized (Ca^{2+}) calcium, phosphorus (P). Calculation of total blood plasma calcium with correction for albumin was performed according to the formula: measured plasma calcium level (mmol/L) + 0.02*(40 – measured plasma albumin level (g/L).

Urinary DPD was measured by solid-phase chemiluminescent immunoassay.

[1] International Classification of Diseases, 10th revision (ICD-10). Access date: 10.01.2025 . https://mkb-10.com

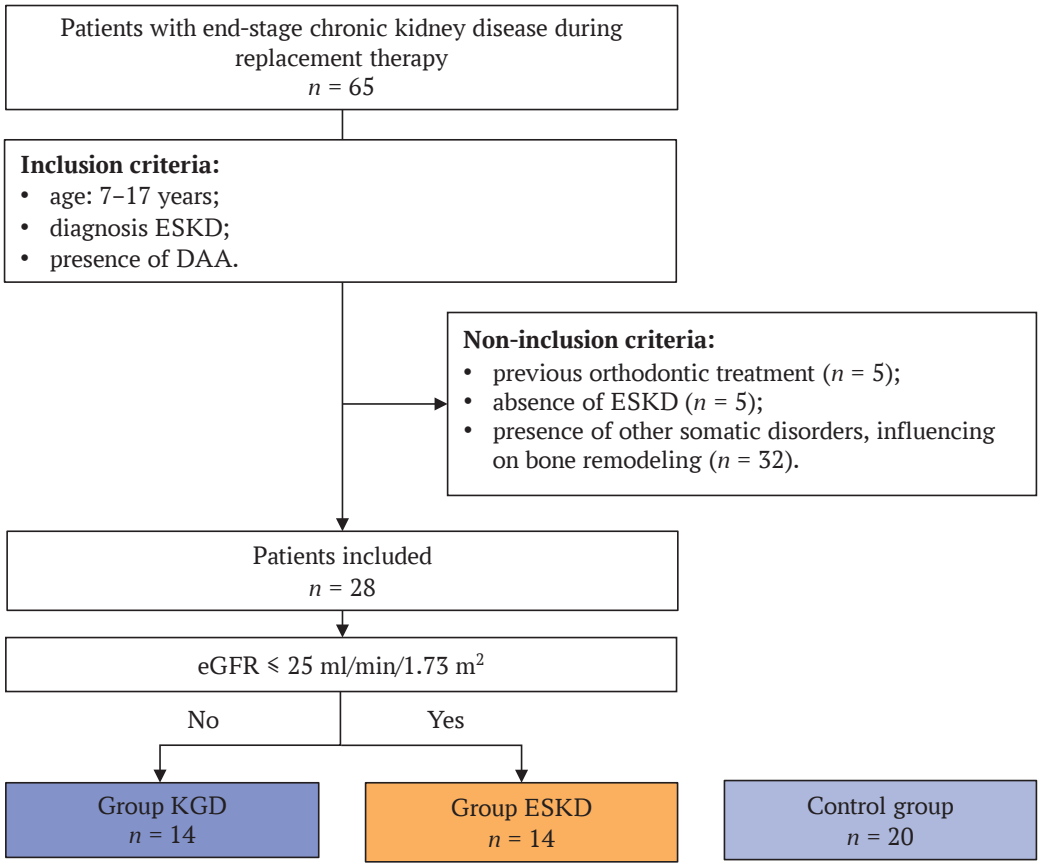

FIG. Study flowchart.

Note: ESCKD – end-stage kidney disease, DAA – dentoalveolar anomalies, RRT – renal replacement therapy, RTD – renal transplant dysfunction, eGFR – estimated glomerular filtration rate (CKiD U25).

OC in serum, GCF and OF was measured by using commercial Osteocalcin ELISA kits for solid-phase enzyme-linked immunosorbent assay (BioVendor, USA).

The Milwaukee PH56 (Milwaukee Instruments, USA) device was used to determine oral pH.

Bone density was assessed using cone-beam computed tomography (CBCT), expressed in Hounsfield units (HU) according to C. Mish classification [24], based on the mathematical reconstruction of X-ray attenuation coefficients assigned to each pixel. The X-ray assessment was performed in four sections: the anterior and posterior sections of the upper jaw; the anterior and posterior sections of the lower jaw.

Statistical analysis

Quantitative features are presented as median and interquartile range, qualitative ones – as proportion. The studied features of patient groups were tested for normal distribution using Shapiro-Wilk test and for homogeneity of variances using Levene test. Variables corresponding to normal distribution and having homogeneous variances are presented as mean values and standard deviation, mean values were compared using one-way analysis of variance (ANOVA). Other variables are presented as median and interquartile range (25th; 75th percentiles), for their comparison the Kruskal–Wallis method was used. For post-hoc analysis the Tukey test was used. The results of statistical analysis were considered significant at $p < 0.05$. The experimental results were processed using Prism 8.0.1 (GraphPad Software, USA) and R language. 4.4.2 in the R-Studio software environment.

RESULTS

The study included patients without pronounced clinical manifestations of CKD-MBD and osteoporosis. The main characteristics of the study groups are presented in Table 1.

Mean age in study groups were 12.7 ± 2.9 years and girls' number in RTD group was lower than in ESCKD group and control, but the differences were not statistically significant (Table 1).

Serum creatinine in ESCKD group was significantly higher and eGFR was lower compared to RTD group and control. ESCKD patients received hemodialysis or peritoneal dialysis treatment for an average from 6 months to 3 years.

Total and ionized serum calcium did not differ between the study groups (Table 1). Serum P was

significantly higher in ESCKD group compared to RTD group and control. While there were not found any statistically significant differences in phosphorus levels in RTD group and control (Table 1).

Summarized markers results in studied groups are presented in Table 2.

Urinary DPD concentration was higher in CKD groups compared to control (Table 2). However, no statistically significant differences were found between urinary DPD concentration in ESCKD and RTD patients.

Serum OC concentration was increased in ESCKD patients compared to control ($p < 0.05$) and did not differ from RTD group. OC in GCF was higher in control compared to ESCKD ($p < 0.001$) and RTD ($p < 0.001$) groups. Meanwhile salivary OC was comparable in all groups (Table 2).

OF pH was statistically significantly higher in both ESCKD and RTD children compared to control (Table 2). Moreover, there were no differences between oral pH in RTD and ESCKD groups, thus oral acidity was similar in these two groups.

Bone density radiographical assessment shows that Hounsfield Index of posterior maxilla was higher in RTD group compared to ESCKD group ($p < 0.01$), and there was no difference with control. Hounsfield Index of anterior maxilla in control was higher than in ESCKD and in RTD groups. A similar pattern was found in Hounsfield index of both anterior and posterior mandible where the control group levels were statistically significantly higher than in ESCKD and RTD patients (Table 2).

DISCUSSION

Study results demonstrated CBCT changes in bone turnover markers and bone density were the most pronounced in ESCKD children. Urinary DPD increase, a decrease in serum and GCF OC and a decrease in the Hounsfield index in both anterior and posterior regions of the maxilla and mandible were revealed. Similar changes were noted in RTD patients, but OC level was reduced only in GCF. Bone density assessment had no significant differences between ESCKD and RTD groups, except Hounsfield index in posterior maxilla.

Kidneys play a crucial role in Ca-P metabolism by almost complete tubular reabsorption of these ions. Ca-P homeostasis in CKD patients is disrupted, so serum P in ESCKD patients had been increasing with eGFR decreasing in our study. It's important to check serum P in CKD-MBD patients for maintaining bone homeostasis, and our results are consistent with Rastogi A. et al. [25]. Serum P in KDG group was close to control group, and inversely correlates with a higher eGFR level, because of kidney filtration improvement after transplantation, but due graft dysfunction P level remained high. These trends are consistent with other studies data on mineral metabolism effect on bone remodeling in patients after kidney transplantation [26, 27].

Our study results showed that serum Ca does not statistically differ between the groups, which indicates the stability of this marker regardless of kidney and graft function. However, ESCKD markers are less homogeneous and it still has a greater spread. No expressed calcium metabolism disorders were detected as well as in Liu J. et al. and Hasanzamani B. et al. studies [28, 29]. It should be noted that the levels of parathyroid hormone and bone fraction of alkaline phosphatase were not taken into account.

Urinary DPD was found as highly sensitive marker of bone metabolism disorders in ESCKD and RTD patients. Thus, we found clear and significant differences in this marker between the study groups. DPD increase in CKD children compared to control may indicate high osteoclast activity and bone resorption activation. DPD changes were recorded simultaneously with a Hounsfield index decrease in jawbones. It indicates bone collagen breakdown, type I mainly, the end products of bone metabolism excretion with urine

Table 1. **Characteristics of study patient groups**

Feature	Chronic kidney disease		Control group (n = 20)	p value (ANOVA)
	ESCKD (n = 14)	RTD (n = 14)		
Age, years	12.1 ± 2.4	13.4 ± 3.0	12.6 ± 3.4	n.s.
Girls, n (%)	11 (79)	6 (43)	13 (65)	n.s.
eGFR ml/min/1.73 m²	10.51 ± 3.25 [a,c]	56.73 ± 15.31 [b,c]	90.01 ± 10.26 [a,b]	<0.0001
Creatinine in serum, μmol/L	477.8 (403.1; 571.6) [a,c]	85.7 (73.2; 131.9) [b,c]	63.0 (50.35; 71.68) [a,b]	<0.0001
Calcium total in serum, mmol/L	2.40 (2.14; 2.62)	2.42 (2.34; 2.46)	2.40 (2.27; 2.49)	<0.01
Total serum calcium adjusted for albumin, mmol/L	2.34 ± 0.28	2.39 ± 0.13	2.37 ± 0.14	n.s.
Calcium ionized in serum, mmol/L	1.16 (0.96; 1.21)	1.18 (1.10; 1.23)	1.21 (1.17; 1.24)	<0.01
Phosphorus in serum, mmol/L	1.751 ± 0.490 [a,c]	1.342 ± 0.266 [s]	1.436 ± 0.195 [a]	<0.005

Note: RTD – renal transplant dysfunction; eGFR – estimated glomerular filtration rate; ESCKD – end-stage chronic kidney disease; [a] – $p < 0.05$ when comparing ESCKD and control groups; [b] – $p < 0.05$ when comparing RTD and control groups; [c] – $p < 0.05$ when comparing RTD and ESCKD.

Table 2. **Bone turnover markers**

Feature	Chronic kidney disease		Control group (*n* = 20)	*p* value (ANOVA)
	ESCKD (*n* = 14)	RTD (*n* = 14)		
Urinary DPD, nmol/mmolCreat	15.80 (12.68; 27.90) [a]	15.08 (10.27; 24.61) [b]	4.90 (2.95; 11.98) [a,b]	<0.001
Serum OC, ng/mL	213.1 ± 55.01 [a]	173.7 ± 86.78	153.9 ± 56.15 [a]	<0.05
Salivary OC, ng/mL	11.78 ± 1.93	12.94 ± 1.76	13.46 ± 3.73	n.s.
OC in gingival crevicular fluid, ng/mL	13.11 ± 3.98 [a]	11.92 ± 3.10 [b]	20.08 ± 4.69 [a,b]	<0.0001
Oral fluid pH	7.080 (6.375; 8.153) [a]	7.240 (6.875; 7.593) [b]	6.250 (5.575; 6.800) [a.b]	<0.001
Hounsfield Index of anterior maxilla	482.5 (394.5; 554.3) [a]	439.0 (396.3; 503.0) [b]	681.5 (449.0; 766.8) [a.b]	<0.0001
Hounsfield Index of posterior maxilla	203.0 (194.8; 238.8) [a.c]	363.0 (248.3; 485.0) [c]	420.0 (329.0; 539.0) [a]	<0.01
Hounsfield Index of anterior mandible	1059 (951; 1451) [a]	1670 (1083; 1985) [b]	3098 (1985; 3538) [a.b]	<0.0001
Hounsfield Index of posterior mandible	824.4 ± 111.0 [a]	826.5 ± 89.5 [b]	1735 ± 377.2 [a.b]	<0.0001

Note: DPD – deoxypyridinoline; RTD – renal transplant dysfunction; ESCKD – end-stage chronic kidney disease; OC – osteocalcin; max – maxilla; man – mandible; [a] – $p < 0.05$ when comparing ESCKD and control groups; [b] – $p < 0.05$ when comparing RTD and control groups; [c] – $p < 0.05$ when comparing RTD and ESCKD.

and it confirms persistent bone metabolism changes in CKD children. However, literary data did not confirm that urinary DPD can reflect bone metabolism in CKD-MBD patients [30]. On the other hand, DPD level is known as one of the leading biochemical markers of bone remodeling and is used in osteoporosis early diagnosis [31]. Thus, urinary DPD determination in CKD patients may become promising for assessing osteoclasts activity and bone resorption and requires further study on a larger CKD patient sample.

Presented study results convincingly demonstrate that OC is an informative marker of osteoblast activity in ESCKD and RTD children, and GCF is the best biological fluid for its determination, since the most significant OC changes are registered in GCF, despite the limited sample. OC decrease in GCF was found in ESCKD and RTD children compared to control, which indicates a violation of bone metabolism. Serum OC increase was detected only in ESCKD group probably due to the limited sample. OC in OF did not statistically significantly differ between three groups possibly due to high protease activity in OF [32]. We didn't find any information about OC measurement in GCF in CKD patients in the reviewed literature. However, Fadli N. et al. used GCF for the assessment of OC and proinflammatory markers [33]. Interest in GCF exertion as a fluid for various markers detection in patients with systemic diseases, including CKD, is growing due to its sufficient informativeness and minimally invasive nature.

OF pH increase with its alkaline tendency may be associated with a disturbance in general metabolism, including a change in the acid-base balance in ESCKD patients [34].

In addition, the obtained data indicate significant disturbances in bone structure in ESCKD and RTD children, which is manifested in a significant decrease in Hounsfield index compared to control, especially in the anterior and posterior mandible. These changes are consistent with previous studies [23], confirming disturbances in bone metabolism and decreased bone mineralization in patients with renal dysfunction.

Limitations and directions for future research

Result interpretations have several limitations due to pilot study design: small sample size, one observation point. The levels of parathyroid hormone and bone fraction of alkaline phosphatase were not taken into account when assessing CKD-MBD. Perhaps due to insufficient study power, there were no statistically significant differences in total serum calcium adjusted for albumin, as well as OC in oral fluid. To draw conclusions on these markers, it is necessary to conduct longitudinal studies on large samples using probability selection of observation units.

AUTHOR CONTRIBUTIONS

Olga L. Morozova and Natalia S. Morozova developed study concept and design and edited the article. Ilsiiar I. Shaykhattarova, Angelina A. Shirina and Violetta A. Shustova performed the scientific literature search. Alina A. Elovskaya and Ekaterina A. Maslikova examined patients, selected and analyzed biomaterial, and wrote the main part of the final version of the article. Natalia B. Zakharova carried out laboratory tests. Larisa D. Maltseva interpreted laboratory data. Elena Yu. Danilova performed statistical analysis. All authors approved the final version of the article.

ВКЛАД АВТОРОВ

О.Л. Морозова и Н.С. Морозова разработали основную концепцию и дизайн исследования, а также проводили редактуру статьи. И.И. Шайхаттарова, А.А. Ширина и В.А. Шустова выполнили научный поиск литературы. А.А. Еловская и Е.А. Масликова проводили осмотр пациентов, отбирали и анализировали биоматериал, а также написали основную часть финальной версии статьи. Н.Б. Захарова осуществляла проведение лабораторных исследований. Л.Д. Мальцева занималась интерпретацией полученных лабораторных данных. Е.Ю. Данилова проводила статистическую обработку данных. Все авторы утвердили окончательную версию публикации.

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Study protocol / Протокол исследования
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A parallel arm randomised controlled trial to achieve remission in patients with type 2 diabetes mellitus through dietary and behavioural interventions: a study protocol

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Abstract

Background. Type 2 diabetes mellitus (T2DM) poses a significant challenge to healthcare, with its prevalence escalating to epidemic proportions. The aging population, coupled with the increasing burden of T2DM, is exerting immense pressure on healthcare systems worldwide. Therefore, there is a critical need to design and validate innovative interventions to mitigate the effects of this disease. This randomised control trial aims to achieve remission in Indian patients aged 18 years and older diagnosed with T2DM through dietary and behavioural interventions.

Materials and methods. A total of 290 participants with T2DM will be recruited from Indira Colony Urban Enclave, the field practice area of the Department of Community Medicine and School of Public Health at Post Graduate Institute of Medical Education and Research, Chandigarh. Participants will be equally allocated into two arms: intervention (*n* = 145) and control (*n* = 145). There will be five measurement timepoints: baseline, 2nd, 4th, 6th and 9th months post-randomisation. The intervention will implement a range of strategies to increase physical activity and promote dietary transitions through behaviour change among patients. The interventions will be designed ensuring a structured approach to behaviour change. Patients from the intervention arm will receive oral hypoglycaemic agents for the first six months of the trial. After this period, medication will be gradually tapered. Patients from the control arm will continue to receive standard care throughout the study. The primary outcome is the number of patients achieving remission of T2DM through behavioural and dietary interventions.

Conclusions. The novelty of this trial lies in its focus on community-based settings, unlike other studies that primarily target clinical or hospital-based environments to achieve clinical outcomes. The intervention integrates dietary and behavioural changes into the community's cultural, socioeconomic, and dietary habits, making it practical and sustainable for patients to adopt and maintain the lifestyle changes needed for remission.

Keywords: community-based intervention; dietary transition; behavioural change; millets; low carbohydrate diet; physical activity

MeSH terms:
DIABETES MELLITUS, TYPE 2 – DIAGNOSIS
DIABETES MELLITUS, TYPE 2 – THERAPY
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Ethics statements. The research protocol was approved by the Ethics committee of Post Graduate Institute of Medical Education and Research, Chandigarh (approval number: INT/IEC/2024/SPL-67 dt 12th February 2024).
Conflict of interests. The authors declare that there is no conflict of interests.

Trial registration information
Note: the numbers in round brackets in this protocol refer to SPIRIT checklist item numbers.[1]

Title (1)	A parallel arm randomised controlled trial to achieve remission in patients with type 2 diabetes mellitus through dietary and behavioural interventions: a study protocol
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Рандомизированное контролируемое исследование в параллельных группах по достижению ремиссии у пациентов с сахарным диабетом 2-го типа через диетические и поведенческие вмешательства: протокол исследования

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Аннотация

Введение. Сахарный диабет 2-го типа (СД2) представляет серьезную проблему для здравоохранения, поскольку его распространенность достигает эпидемических масштабов. Стареющее население и рост бремени СД2 оказывают огромное давление на системы здравоохранения по всему миру, в связи с чем существует острая необходимость разработки и апробации новых мер по уменьшению отдаленных негативных последствий СД2. Цель рандомизированного контролируемого исследования – достижение ремиссии у индийских взрослых пациентов (>18 лет) с СД2 через диетические и поведенческие вмешательства.

Материалы и методы. В исследование планируется включить 290 пациентов с СД2, проживающих в городском анклаве колонии Индира, области полевой практики Департамента общественной медицины и Школы общественного здравоохранения Института постдипломного медицинского образования и исследований, г. Чандигарх. Участники будут распределены в экспериментальную (n = 145) или контрольную (n = 145) группу. Планируется пять контрольных визитов для оценки клинического статуса пациентов: исходный уровень, 2, 4, 6 и 9-й месяцы после рандомизации. Экспериментальное вмешательство включает поэтапное внедрение поведенческих стратегий, направленных на увеличение физической активности и изменение диетических привычек. Пациенты экспериментальной группы будут получать пероральные гипогликемические препараты в течение первых шести месяцев исследования, затем планируется постепенное уменьшение их дозы. Пациенты контрольной группы будут получать стандартную терапию в течение всего исследования. Первичной конечной точкой является количество пациентов, достигших ремиссии СД2 благодаря поведенческим и диетическим вмешательствам.

Заключение. Новизна исследования заключается в том, что для достижения ремиссии СД2 планируется изменение образа жизни сообщества, в отличие от других исследований, ориентированных в первую очередь на медицинские вмешательства, проводимые в госпитальных или амбулаторных условиях. Экспериментальное вмешательство направлено на постепенную интеграцию диетических и поведенческих изменений в культурные, социально-экономические и пищевые привычки пациентов, что позволит длительно поддерживать ремиссию СД2.

[1] SPIRIT 2013 Statement: Defining standard protocol items for clinical trials. Accessed October 04, 2024. https://www.equator-network.org/reporting-guidelines/spirit-2013-statement-defining-standard-protocol-items-for-clinical-trials/

Ключевые слова: вмешательство на уровне сообщества; изменение диеты; изменение поведения; просо; низкоуглеводная диета; физическая активность
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ПОВЕДЕНИЯ КОНТРОЛЬ – МЕТОДЫ
Для цитирования: Сингх А., Тхакур Дж.С. Рандомизированное контролируемое исследование в параллельных группах по достижению ремиссии у пациентов с сахарным диабетом 2-го типа через диетические и поведенческие вмешательства: протокол исследования. Сеченовский вестник. 2025; 16(1): 45–54. Публикация онлайн 18.03.2025. https://doi.org/10.47093/2218-7332.2024.1097.18

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Соблюдение этических норм. Протокол исследования одобрен Этическим комитетом Института постдипломного медицинского образования и исследований, г. Чандигарх (№ INT/IEC/2024/SPL-67 от 12 февраля 2024 г.).
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Информация о регистрации клинического исследования
Примечание: числа в круглых скобках в тексте протокола относятся к номерам пунктов чек-листа SPIRIT[2].

Название (1)	Рандомизированное контролируемое исследование в параллельных группах по достижению ремиссии у пациентов с сахарным диабетом 2-го типа через диетические и поведенческие вмешательства: протокол исследования
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Abbreviations:
HbA1c – glycated haemoglobin
NCD – non-communicable diseases
OHAs – oral hypoglycaemic agents
T2DM – type 2 diabetes mellitus

BACKGROUND (6A)

Non-communicable diseases (NCD) are the leading cause of death globally and disproportionately affect individuals in low- and middle-income countries, where they account for 80% of all deaths and 90% of premature deaths.[3] In absolute terms, out of 56 million global deaths, 38 million (67.8%) are directly attributable to NCD [1].

According to the ICMR-INDIAB study the prevalence of diabetes in India is 7.3% with a wide regional and an urban-rural variation [2]. Given this context, the economic burden of managing NCD and their complications poses a substantial challenge for policymakers when allocating resources and funds for their diagnosis and treatment. The challenge is exacerbated by the need to prioritize funding for traditional public health concerns, such as communicable diseases and maternal and child health, which remain at the top of the agenda. This prioritization further strains the limited resources available [3, 4].

The rationale for conducting the study

As life expectancy rises, India faces a dual health challenge: widespread communicable diseases alongside

[2] SPIRIT 2013 Statement: Defining standard protocol items for clinical trials. Accessed October 04, 2024. https://www.equator-network.org/reporting-guidelines/spirit-2013-statement-defining-standard-protocol-items-for-clinical-trials/
[3] World Health Organization. Global status report on noncommunicable diseases 2014. 265 p. ISBN: 9789241564854. Accessed January 11, 2024. https://www.who.int/publications/i/item/9789241564854

the growing burden of NCD compounded by an aging population and strained healthcare infrastructure [5]. Evidence suggests that lower socioeconomic groups are more prone to alcohol and tobacco use and insufficient consumption of fruits and vegetables, increasing their NCD risk [6].

Management of type 2 diabetes mellitus (T2DM) and other lifestyle diseases has relied on pharmacological interventions, like oral hypoglycaemic agents (OHAs) to achieve normoglycaemia [7]. Along with medication treatment lifestyle changes are integral components of achieving T2DM remission. Recent evidence such as from the DiRECT Trial, highlights the potential for T2DM remission through structured weight management programs [8, 9]. However, these findings need to be validated in a number of diverse settings, particularly in low- and middle-income countries where data is limited to case reports [10]. The study evaluating the effects of dietary and behaviour interventions in achieving remission in patients with T2DM in low- and middle-income countries is acutely needed in order to develop a more efficient strategy for these patients.

The rationale behind choosing dietary and behaviour interventions to achieve remission in patients with T2DM (6b)

Lifestyle changes have a positive impact on managing T2DM. In a retrospective review the complete and partial remission rates in patients 6 years after bariatric surgery were found to be 24% and 26% respectively [11]. By contrast, another study showed that weight loss through calorie restriction can also induce remission of T2DM in a dose-dependent manner, with a 15kg reduction achieving remission in 80% of patients [12]. Moreover, weight loss is cost effective and could significantly reduce out-of-pocket expenses on medications and laboratory tests [13].

In low- and middle-income countries such as India, dietary and behavioural interventions are favoured for achieving remission in patients with T2DM due to their cost-effectiveness, feasibility, and accessibility. Regular physical activity plays a crucial role by enhancing glucose uptake by skeletal muscles, improving insulin sensitivity, and facilitating weight management. Our hypothesis is that a combination of diet and exercises, tailored to the patient's lifestyle and activity level, will contribute to achieving T2DM remission. Our core focus is based on three outcomes: (1) aerobics exercises (brisk walking, jogging) to boost endurance; (2) yoga to improve flexibility [14]; (3) resistance exercises to enhance strength.

The rational for choosing a low carbohydrate diet (6b)

The ICMR (Indian Council of Medical Research) 2018 guidelines for patients with T2DM recommend consuming 55–60% of energy from carbohydrates, prioritizing complex over refined carbohydrates.[4] This dietary approach aims to improve glycaemic control by regulating caloric intake, optimizing macronutrient distribution, and promoting fibre-rich foods. Based on ICMR-NIN (Indian Council of Medical Research National Institute of Nutrition) 2024 guidelines, patients will receive tailored diets focusing on balanced nutrition, with specific energy and macronutrient recommendations for individuals below and above 60 years.[5]

Traditional Indian diets, mainly rice- and wheat-based, should be reconsidered due to the rising burden of T2DM and other NCD in India [15]. Millets such as finger millet (ragi), pearl millet (bajra), and foxtail millet, have a low glycaemic index, leading to a gradual rise in blood glucose levels essential for T2DM management. Rich in fibre, protein, and essential nutrients like magnesium and iron, millets improve satiety, enhance insulin sensitivity, and support gut health [16, 17], establishing their value in T2DM dietary strategies.[6]

The rationale for a 9-month follow-up period

A 9-month follow-up period was selected based on the study design. Weight loss through dietary interventions tends to be slower than achieved via bariatric surgery. Kim et al. showed that sustained lifestyle changes can lower glycated haemoglobin (HbA1c) within 6 months [18]. With regular monitoring and gradual tapering of OHAs based on glycaemic status, significant outcomes are expected within 9 months. This timeframe also balances clinical relevance with practicality, reducing dropout rates while preserving data quality and participant engagement.

Objectives (7)

Primary objective

To evaluate the effect of dietary and behavioural interventions in achieving remission in patients diagnosed with T2DM.

Secondary objectives

1. To assess patients' acceptability of adopting dietary and behavioural interventions through in-depth interviews.

2. To evaluate the impact of dietary interventions on patients with T2DM having co-morbidity such as arterial hypertension.

[4] Indian Council of Medical Research. Guidelines for Management of Type 2 Diabetes. 2018. Accessed January 20, 2024. https://www.icmr.gov.in/icmrobject/custom_data/pdf/resource-guidelines/ICMR_GuidelinesType2diabetes2018_0.pdf

[5] Indian Council of Medical Research. Dietary Guidelines for Indians. 2024. Accessed January 20, 2024. https://www.nin.res.in/dietaryguidelines/pdfjs/locale/DGI07052024P.pdf

[6] International Diabetes Federation. Clinical Practice Recommendations for managing Type 2 Diabetes in Primary Care. 2017. Accessed January 14, 2024. https://idf.org/media/uploads/2023/05/attachments-63.pdf

Trial design (8)

The study is a parallel two-arm randomised control trial (allocation ratio 1:1). Patients will be randomised into an intervention arm (dietary and behavioural intervention) and a control arm receiving standard care (fig.). There will be five measurement timepoints: baseline, 2nd, 4th, 6th and 9th months post-randomisation.

METHODS

Study setting (9)

The 9-month community based randomised control trial will implement dietary and behavioural interventions for adults ⩾18 years with T2DM in the Indira Colony Urban Enclave, the field practice area of the Department of Community Medicine and School of Public Health at Post Graduate Institute of Medical Education and Research Chandigarh [19], Northern India, with a population of 25,000 as per the census conducted in 2011.[7] The sampling frame comprises T2DM cases from a 2018 house-to-house survey (unpublished). Considering changes in morbidity and mortality, investigators will update the database through a new house-to-house survey.

Inclusion criteria (10)

1. Confirmed diagnosis of T2DM per HEARTS-D module criteria.[8]
2. Age ⩾18 years.

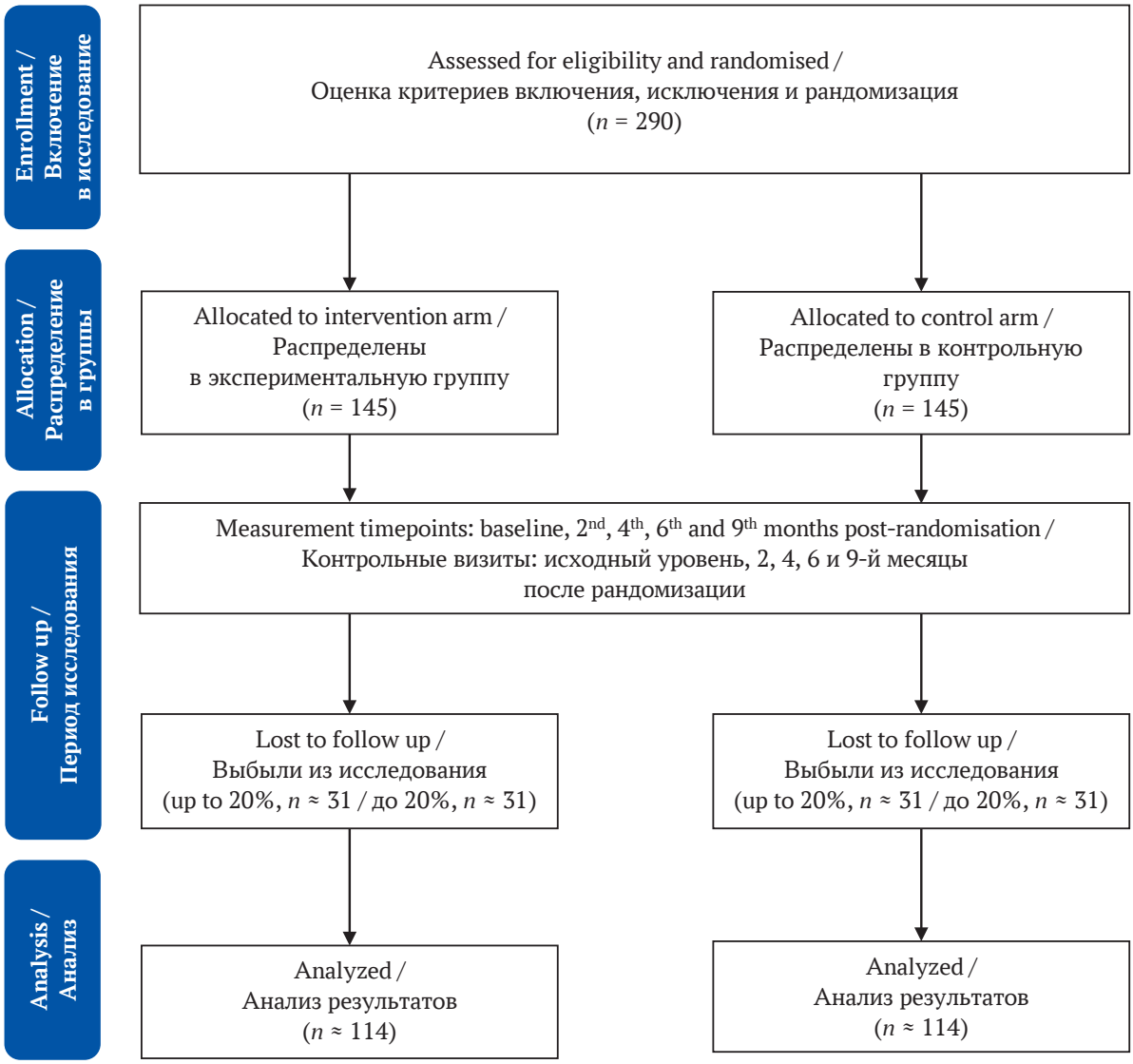

FIG. Study flowchart.
РИС. Потоковая диаграмма исследования.

[7] Census of India 2011 – Chandigarh UT – Series 05 – Part XII A – District Census Handbook, Chandigarh. 2014. Accessed January 15, 2024. https://censusindia.gov.in/nada/index.php/catalog/324

[8] World Health Organization. HEARTS D: diagnosis and management of type 2 diabetes. 2020. Accessed January 11, 2024. https://www.who.int/publications/i/item/who-ucn-ncd-20.1

3. Patients providing informed consent (26a).
4. Patients with systolic blood pressure ⩾140 mm Hg and / or diastolic blood pressure ⩾90mm Hg as per 2016 primary hypertension guidelines by India's Ministry of Health and Family Welfare.[9]

Exclusion criteria

1. Patients not providing informed consent.
2. Patients with type 1 diabetes mellitus.
3. Age < 18 years.
4. Patients with impaired fasting glucose and glucose tolerance.[10]
5. Insulin-treated patients.

Definition of remission

Diabetes remission is defined using specific HbA1c, fasting, and postprandial glucose thresholds maintained without OHAs. The American Diabetes Association classifies remission as complete, partial, or prolonged [20]. The Association of British Clinical Diabetologists and Primary Care Diabetes Society define remission as glycaemia below diagnostic thresholds for ⩾6 months without glucose-lowering therapy [21]. This study adopts the American College of Lifestyle Medicine's definition: HbA1c < 6.5% for at least 3 months without surgery, external devices, or active glucose-lowering medication [22].

Intervention (11a)

Intervention arm

Customised diet plan per Dietary Guidelines[11]

Carbohydrate intake will be restricted to <50% of total calories, replaced with fibre-rich, non-starchy foods and green leafy vegetables, alongside increasing vegetable salads in the diet [23]. Millet will be incorporated flexibly into participants' diets as porridge, chapati (Indian millet flat bread), or traditional Indian millet snacks. While millet will not fully replace other grains, it will be promoted as a healthier alternative, prioritized over refined grains like white rice and refined wheat. The intervention aims to shift dietary patterns toward millet-based options as a primary grain.

Physical activity plan

Physical activity recommendations are tailored to the patient's fitness level, age, and any physical limitations.[12] Exercise plans will be customised according to patient's age. For patients who undertake a moderate amount of activity, the regimen will aim to sustain their activity through aerobic exercises, resistance training, and yoga. For sedentary patients, a gradual initiation into physical activity will be encouraged, starting with light exercises such as walking. Progress will be closely monitored, and plans will be adjusted based on patient response. Daily activity charts will be provided to track time spent on physical exercises.

Standard medication

Patients will receive OHAs for the first six months of the trial. After this period, OHAs will be gradually tapered for patients with HbA1c levels below 6.5%. Patients weaned off OHAs will monitor blood glucose levels using finger-prick tests. If any patient struggles with the reduced dosage, their original medication regimen will be reinstated. The day a patient is completely weaned off OHAs marks the start of a three-month follow-up period. Patients showing symptoms of dysglycaemia will be reviewed and consulted with an endocrinologist to determine whether OHAs should be resumed.

The package of intervention in our study is summarised in Table.

Control arm

Patients in control arm will continue to receive OHAs as prescribed by their physician. No dietary and behavioural interventions will be made in the control arm.

Participant timeline (13)

At each timepoint, HbA1c, waist circumference, weight and body mass index will be measured.

Additionally for patients in the intervention group, investigators will conduct weekly visits to address challenges in adopting lifestyle changes, monitor diet adherence, review self-recorded capillary blood glucose diaries, and provide personalized support. A WhatsApp group will be created for participants in the intervention arm, where investigators will share motivational voice notes and video vlogs to encourage adherence.

Data collection will include a peer-reviewed, Delphi-validated questionnaire designed with input from subject experts to capture demographic profiles and dietary habits through a Food Frequency Questionnaire, assessing daily and weekly consumption patterns [25]. Additional tools include questions to identify barriers and facilitators of healthy dietary and physical activity transitions, the World Health Organisation STEPS (STEPwise approach to NCD risk factor surveillance) questionnaire for dietary and physical history,[13] and the

[9] Ministry of Health & Family Welfare. Screening, Diagnosis, Assessment, and Management of Primary Hypertension in Adults in India. 2016. Accessed January 11, 2024. https://nhm.gov.in/images/pdf/guidelines/nrhm-guidelines/stg/Hypertension_full.pdf

[10] Indian Council of Medical Research. Guidelines for Management of Type 2 Diabetes. 2018. Accessed January 13, 2024. https://www.icmr.gov.in/icmrobject/custom_data/pdf/resource-guidelines/ICMR_GuidelinesType2diabetes2018_0.pdf

[11] Indian Council of Medical Research. Dietary Guidelines for Indians. 2024. Accessed January 20, 2024. https://www.nin.res.in/dietaryguidelines/pdfjs/locale/DGI07052024P.pdf

[12] Fit India Mission. Fitness Protocols and Guidelines for 18+ to 65 Years. Accessed January 18, 2024. https://yas.nic.in/sites/default/files/Fitness%20Protocols%20for%20Age%2018-65%20Years%20v1%20(English).pdf

[13] World Health Organization. The WHO STEPwise approach to surveillance. 2021. Accessed January 15, 2024. https://www.who.int/europe/publications/i/item/WHO-EURO-2021-2446-42201-58182

Table. **Summary of dietary and behaviour interventions**
Таблица. **Резюме диетических и поведенческих вмешательств**

Intervention / Вмешательство	Package under each intervention / Компоненты каждого вмешательства
Dietary[14] / Диета[14]	(a) Introduction of millet-based diet that is low in carbohydrate and energy / Введение диеты на основе проса с низким содержанием углеводов и энергии (b) Encouraging the consumption of five servings of fruits and vegetables daily / Выработка привычки ежедневного потребления пяти порций фруктов и овощей (c) Limiting free sugars intake to less than 10% of total energy consumption / Ограничение потребления легкоусвояемых углеводов до 10% от суточной калорийности рациона (d) Reduction of fat intake to less than 30% of energy consumption / Ограничение потребления жиров до 30% от суточной калорийности рациона (e) Distribution of millet food baskets to patients / Снабжение пациентов продовольственными корзинами с просом
Behavioural / Изменение образа жизни	(a) Community engagement by organising community based events and workshops that promote physical activity, using a support network as a central pillar / Вовлечение сообщества путем организации местных мероприятий и семинаров, пропагандирующих физическую активность, с использованием социальной сети для поддержки (b) Culturally tailored messaging derived through vertical and horizontal communication channels / Информирование с учетом культурных особенностей по вертикальным и горизонтальным каналам связи (c) Addressing barriers to physical activity by conducting focus group discussions and offering targeted solutions to the challenges identified [24] / Устранение препятствий для физической активности путем обсуждений в фокус-группах и разработки решений выявленных проблем [24] (d) Implement an incentive programme to motivate patients to maintain regular physical activity and adhere to dietary interventions / Внедрение мотивационной программы для поддержания регулярной физической активности и соблюдения диетических ограничений (e) Establish community-based yoga groups and promote specific yoga poses known to benefit patients with T2DM / Создание общественных групп йоги для продвижения движений, оказывающих положительное влияние на пациентов с СД2

Note: T2DM – type 2 diabetes mellitus.
Примечание: СД2 – сахарный диабет 2-го типа.

EuroQol EQ-5D-5L questionnaire for quality of life assessment [26].

Criteria for discontinuing the trial (11b)

Parents may withdraw from the trial at any time without prejudice or in case of frequent episodes of hypoglycaemia.

Strategies to improve adherence to the study protocol (11c)

Informative booklets and pamphlets with colourful info graphics will address local dietary habits, cultural beliefs, and misconceptions about T2DM, offering practical guidance on diet and exercise in simple, engaging language. Adherence cards will be provided to each patient in the intervention arm to help track physical activity, monitor dietary patterns, and make adjustments as needed.

To enhance compliance, investigators will conduct weekly visits to review adherence cards, providing constructive feedback to both patients and their family champions on gaps and strategies to achieve targets. This approach emphasizes open, two-way communication. The term family champion refers to a key family member who actively supports the participant during the study in adhering to dietary and behavioural interventions. This role is crucial in fostering accountability, motivation, and creating a supportive home environment, which is essential for sustainable lifestyle changes and effective T2DM management.

The selection of the family champion follows four key principles:

1. Emotional and practical support. The family champion provides encouragement, assists with meal planning, and supports daily routines, improving adherence to lifestyle changes.

2. Close relationship with the patient. Ideally a spouse, sibling, or adult child with a trusting bond, empathy, and open communication to provide reliable support.

3. Consistency and accountability. A consistent presence, the family champion helps maintain adherence by gently reminding patients of the importance of prescribed interventions.

4. Cultural and household dynamics. In Indian households where families play a central role in health decisions, the family champion leverages this cultural strength, embedding support within the broader family network.

The intervention includes focus group discussions to enhance social support by encouraging the sharing of challenges and successes, peer influence through positive behaviour, group norms to establish shared expectations, accountability via regular check-ins, shared goals by

[14] World Health Organization. Healthy diet. Accessed January 15, 2024. https://www.who.int/news-room/fact-sheets/detail/healthy-diet

celebrating achievements, and collaborative problem-solving to address barriers effectively.

Sample Size (14)

The total recruitment target is 228 patients (114 per arm), calculated with a 95% confidence interval and 80% power. The sample size estimation was based on a study reporting remission rates of 57% in patients with T2DM following dietary intervention, compared to 31% in the control group [27]. Accounting for a 20% non-response rate, the adjusted sample size was calculated as 285. However, for simplicity, a sample size of 290 (145 per arm) was chosen. The calculation was performed using OpenEPI software, version 3, for a randomised control trial.

Recruitment of participants (15)

The first step involves baseline recruitment where investigators will visit households to enroll patients in the trial. To prevent contamination bias [28], only patients who provide informed consent will be recruited.

Allocation (16a) and concealment (16b) of participants will be managed through sealed envelopes, with a neutral third party assigning patients to each trial arm. The initial recruitment will include all 290 patients, followed by block randomisation in blocks of 4, 6 and 8 into either of the arm. Varying block sizes was shown to have several advantages over one single block including increased unpredictability of the allocation sequence, thereby reducing selection bias. This approach minimizes risks of manipulation or subversion of the allocation process, enhancing the trial's integrity and robustness.

Outcome measures (12)

Primary outcome

The number of patients achieving remission of T2DM through behavioural and dietary interventions.

Secondary outcomes

a. The number of patients with hypertension as co-morbidity achieving normal blood pressure.

b. Assessment of acceptability and identification of barriers to adopting dietary interventions, particularly the inclusion of millets.

Data collection (18)

Demographic data, medication information, clinical symptoms, data on primary and secondary outcomes, laboratory tests (fasting and post prandial blood glucose, HbA1c), physical examinations (weight, body mass index, waist circumference), and validated questionnaires assessing dietary intake, quality of life will be collected at each timepoints. For promoting data quality outcome measurements will be taken by using pre-formulated standardized protocols. To minimize measurement error, duplicate measurements will be taken where applicable (e.g., blood pressure), and the average of the readings will be recorded.

Statistical methods (20)

Data analysis will be performed using IBM SPSS version 25 (released 2017, IBM Corp., USA).

Statistical methods for analysing primary and secondary outcomes (20a)

Univariate analysis will be conducted to compare the demographic data of patients in the intervention and control groups. McNemar's test or paired t-tests will be performed for pre- and post-intervention comparisons in patients with arterial hypertension. A logistic regression model will be utilized to identify factors associated with blood pressure normalization. Kaplan-Meier survival analysis will be performed to estimate the proportion of patients achieving remission over time, with remission treated as a time-dependent variable at the 9th month.

Methods for additional analyses (20b)

We will conduct focus group discussions and semi-structured interviews to explore patients' perceptions of dietary changes, particularly concerning millet consumption. Content or thematic analysis will be used to identify common barriers, including cost, accessibility, taste, or unfamiliarity with millets.

Subgroup and adjusted analyses

A separate analysis of primary outcome and effect estimates will be performed for physical activity level, gender, age, and fruit and vegetable intake.

Confidentiality (27)

Data confidentiality will be ensured through de-identification and anonymization, with minimal use of identifiers. Prior to collecting any personal data, written informed consent will be obtained from participants. During this process, participants will be informed about the type of the data to be collected, its intended use, and the measures implemented to protect their privacy.

Declaration of interests (28)

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the trial.

Access to data (29)

The principal investigator will be custodian of the data. In the trial, access to the final dataset will be managed to ensure both data integrity and participant confidentiality.

DISCUSSION

Achieving remission in T2DM requires a comprehensive management plan alongside OHAs treatment. This includes the supervised and systematic de-prescription of existing antihyperglycaemic medications and the gradual introduction of lifestyle changes, such as a calorie-restricted diet supplemented with a millet-based component [29]. We propose that T2DM remission is an achievable clinical goal. Our study gains relevance

from its alignment with the declaration of 2023 as the International Year of Millets, focusing on this "wonder grain".[15]

However, challenges exist regarding the acceptance of millets among participants. These include an underdeveloped supply chain, limited availability in retail outlets, higher costs compared to rice and wheat [30], and societal perceptions that undervalue millets as an investment in future health [31]. Despite these barriers, we anticipate significant improvements in participants' quality of life upon achieving remission. Benefits may include enhanced mental and physical health, fewer work-loss days, reduced hospitalizations, and potentially lower risks of complications associated with T2DM [32, 33].

The novelty of this trial lies in its community-based approach, diverging from the typical clinical or hospital-based frameworks. By leveraging accessible and sustainable interventions, it empowers T2DM patients to pursue remission. The intervention integrates dietary and behavioural changes into the cultural, socioeconomic, and dietary contexts of the community, facilitating the adoption and long-term maintenance of lifestyle changes essential for remission.

AUTHOR CONTRIBUTIONS

Jarnail S. Thakur developed the concept and design of the study. Arunjeet Singh drafted the manuscript. Jarnail S. Thakur and Arunjeet Singh took part in editing of the text. All authors approved the final version of the article.

ВКЛАД АВТОРОВ

Джарнаил С. Тхакур разработал концепцию и дизайн исследования. Арунджит Сингх составил текст рукописи. Джарнаил С. Тхакур и Арунджит Сингх принимали участие в редактировании текста. Все авторы одобрили окончательную версию статьи.

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Clinical case with video / Клинический случай с видео
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Microsurgical clipping of aneurysms of the right middle cerebral artery bifurcation, ophthalmic segment of right internal carotid artery using of MIPLATTA approach: a video case report

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Abstract

Microsurgical clipping of aneurysms of the bifurcation of the middle cerebral artery (MCA) and the ophthalmic segment of the internal carotid artery (ICA) requires precise access and minimization of the risk of damage to structures. This video case presents successful clipping of a multiple aneurysm of the above segments using a combined Minimally Invasive Posterolateral Transcavernous Transtentorial Approach (MIPLATTA). A 45-year-old female was admitted with complaints of long-term headache in the occipital and temporal regions, which had been bothering her for 20 years. Computed tomography (CT) angiography revealed saccular aneurysms of the M1–M2 segments of the right MCA and the ophthalmic segment of the right ICA. Complete clipping of the MCA aneurysm was performed, then the ICA aneurysm. Postoperative CT angiography did not reveal any signs of contrasting of the aneurysm necks. The use of MIPLATTA, due to the visualization of important anatomical structures and complete proximal and distal control of the ICA, allows for radical exclusion of aneurysms from the bloodstream without complications.

Keywords: cavernous segment; Parkinson's triangle; sphenoid wing drilling; aneurysm dissection; anterior clinoid process removing

MeSH terms:
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Микрохирургическое клипирование аневризм бифуркации правой средней мозговой артерии, офтальмического сегмента правой внутренней сонной артерии с использованием доступа MIPLATTA: клинический видеослучай

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Аннотация

Микрохирургическое клипирование аневризм бифуркации средней мозговой артерии (СМА) и офтальмического сегмента внутренней сонной артерии (ВСА) требует точного доступа и минимизации рисков повреждения структур. В видеослучае представлено успешное клипирование сочетанной аневризмы указанных сегментов с использованием комбинированного минимально инвазивного заднебокового транскавернозного транстенториального доступа (Minimally Invasive Posterolateral Transcavernous Transtentorial Approach, MIPLATTA). Пациентка 45 лет поступила с жалобами на длительную головную боль в затылочной и височной областях, беспокоящую в течение 20 лет. При компьютерно-томографической (КТ) ангиографии выявлены мешотчатые аневризмы М1–М2 сегментов правой СМА и офтальмического сегмента правой ВСА. Проведено полное клипирование аневризмы СМА, затем аневризмы ВСА. Послеоперационная КТ- ангиография признаков контрастирования шеек аневризм не обнаружила. Применение MIPLATTA за счет визуализации важных анатомических структур, полного проксимального и дистального контроля ВСА позволяет добиться радикального выключения аневризм из кровотока без осложнений.

Ключевые слова: кавернозный сегмент; треугольник Паркинсона; дриллинг крыла клиновидной кости; диссекция аневризмы; удаление переднего наклоненного отростка
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Abbreviations:
MIPLATTA – Minimally Invasive Posterolateral Transcavernous Transtentorial Approach
ICA – internal carotid artery
MCA – middle cerebral artery
ACP – anterior clinoid process

[0:03] We present a microsurgical clipping of aneurysms of the right middle cerebral artery bifurcation, ophthalmic segment of right internal carotid artery using MIPLATTA approach.
[0:14] The patient is a 45-year-old female, presented with complaints of headache for a long time (about 20 years), in occipital and temporal areas, takes paracetamol and nonsteroidal anti-inflammatory drugs for headache relief. Computed tomography angiography of the head was performed. The examination revealed an aneurysm of the right middle cerebral artery bifurcation, ophthalmic segment of right internal carotid artery.
[0:41] After the skull trepanation, the dura matter was opened. Then a sharp dissection of the superficial Sylvian vein was performed.
[0:51] Dissection of the Sylvian fissure.
[0:56] Basal cisterns dissection.
[01:01] Internal carotid artery dissection. Optic nerve, chiasmatic cistern dissection. Internal carotid artery aneurysm dissection.
[01:16] The first step was to begin clipping the middle cerebral artery aneurysm.
Middle cerebral artery dissection. Dissection of the proximal and distal segments of the middle cerebral artery.
[01:35] Aneurysm dissection.
[01:42] Clipping of the aneurysm neck. In this case, a lap-shaped clip was used to clip the neck of the aneurysm, which allowed complete disconnection of the aneurysm from the blood flow.
[01:52] Intra-operative Indocyanine green video-angiography shows that the aneurysm is fully clipped.
[02:00] The next step is to clip the aneurysm of the right internal carotid artery.
Sphenoid wing drilling.
[02:07] Dissection of the small wing of the sphenoid bone. Removal of the small wing of the sphenoid bone.
[02:15] Anterior clinoid process dissection.
[02:23] Anterior clinoid process removal.
[02:27] Peeling of lateral wall of the cavernous sinus.
[02:34] Middle meningeal artery dissection. Middle meningeal artery coagulation and cutting.
[02:41] To improve the dissection, a traction of the dura matter of the temporal lobe was performed. [02:49] Dissection of the cavernous segment of the internal carotid artery in the Parkinson's triangle.
[02:54] Trial temporary clipping of the internal carotid artery in the Parkinson's triangle.
[02:59] Distal opening of the dura matter. Optic nerve decompression by excision of the dura matter. [03:06] Dissection of the clinoid segment of the internal carotid artery by excision of the distal dural ring.
[03:12] Oculomotor nerve decompression.
[03:17] Ophthalmic artery dissection.
[03:22] Proximal temporary clipping of the internal carotid artery in the Parkinson's triangle.
[03:27] Distal temporary clipping of the internal carotid artery.

[03:30] Application of a permanent curved mini-clip. Removal of temporary clips.
[03:36] Intra-operative Indocyanine green video-angiography shows that the aneurysm is fully clipped.
[03:43] Post-operative images: Computed tomography angiography of the head was performed.
No signs of neck contrast were detected.
[03:54] Thank you for your attention.

The video can be found here: https://rutube.ru/video/private/1ae87cb2c464d4a07d18edd6f90e8597/?p=BoTD4duuPN0wEoYNp4NuMA (RuTube).

AUTHOR CONTRIBUTIONS

Albert A. Sufianov performed the surgical procedure described in the submitted publication, made a major contribution to its conception and design, and supervised the writing and editing of the scientific article. Rakhmonzhon R. Rustamov and Rinat A. Sufianov contributed to the conception and design of the publication, prepared materials, wrote and edited the text, and created the illustrations and video. Margarita F. Chakhmacheva participated in the analysis of literature data, processing of illustrations and video materials, and editing of the text. All authors approved the final version of the article and take responsibility for all aspects of the submitted work.

ВКЛАД АВТОРОВ

А.А. Суфианов выполнил хирургическую операцию, описанную в представленном клиническом видеослучае, внес основной вклад в концепцию и дизайн, а также руководил процессом написания и редактирования статьи. Р.Р. Рустамов и Р.А. Суфианов участвовали в разработке концепции и дизайна статьи, подготовке материалов, написании и редактировании текста, а также подготовке иллюстраций и видео. М.Ф. Чахмачева участвовала в анализе данных литературы, обработке иллюстраций и видео, редактировании текста. Все авторы одобрили окончательный вариант статьи и готовы взять на себя ответственность за все аспекты представленной публикации.

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