Prolonged use of temozolomide leads to increased anxiety and decreased content of aggrecan and chondroitin sulfate in brain tissues of aged rats

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Abstract. Chemotherapy with temozolomide (TMZ) is an essential part of anticancer therapy used for malignant tumors (mainly melanoma and glioblastoma); however, the long-term effects on patient health and life quality are not fully investigated. Considering that tumors often occur in elderly patients, the present study was conducted on long-term (4 months) treatment of adult Wistar rats (9 months old, n=40) with TMZ and/or dexamethasone (DXM) to investigate potential behavioral impairments or morphological and molecular changes in their brain tissues. According to the elevated plus maze test, long-term use of TMZ affected the anxiety of the adult Wistar rats, although no significant deterioration of brain morphology or cellular composition of the brain tissue was revealed. The expression levels of all studied heparan sulfate (HS) proteoglycans (HSPGs) (syndecan-1, syndecan-3, glypican-1 and HSPG2) and the majority of the studied chondroitin sulfate (CS) proteoglycans (CSPGs) (decorin, biglycan, lumican, brevican, neurocan aggrecan, versican, Cspg4/Ng2, Cspg5 and phosphacan) were not affected by TMZ/DXM, except for neurocan and aggrecan. Aggrecan was the most sensitive proteoglycan to TMZ/DXM treatment demonstrating downregulation of its mRNA and protein levels following TMZ (-10-fold), DXM (-45-fold) and TMZ-DXM (-80-fold) treatment. HS content was not affected by TMZ/DXM treatment, whereas CS content was decreased 1.5-2.5-fold in the TMZ- and DXM-treated brain tissues. Taken together, the results demonstrated that treatment of adult Wistar rats with TMZ had long-term effects on the brain tissues, such as decreased aggrecan core protein levels and CS chain content and increased anxiety of the experimental animals.

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Introduction

Temozolomide (TMZ) is the main chemotherapeutic drug for the treatment of malignant gliomas (1-3) and melanoma (4-6); it is also proposed for colon cancer chemotherapy (7) and as a second line gastroenteropancreatic neuroendocrine carcinoma treatment (8). Being used as a system therapy, TMZ treatment is accompanied by a number of short- and long-term side effects, including fatigue, nausea, vomiting, thrombocytopenia, neutropenia (9), myelosupression (10) and rarely myelodysplastic syndrome or aplastic anaemia (11). In addition, previous data have been published on the effects of TMZ on quality of life, depression and increased anxiety; however, published results on these topics are contradictory.

According to previous studies, TMZ chemotherapy does not affect cognitive function and emotional functioning (12) in patients receiving standard chemotherapy (13,14). Glioblastoma (GBM) treatment can aggravate the wellbeing of patients (15) and causes depression, particularly in the 3rd month of the treatment, which requires additional specialized treatment (16).

By contrast, TMZ administration has been demonstrated to cause behavioral impairments in animal models *in vivo*. In adult mice, TMZ treatment leads to anxiety- and depression-like behavior both with a short administration of 3 days (17) and with a longer administration of 5-15 weeks (18-20). Several studies have shown that TMZ chemotherapy disrupts hippocampus-dependent learning (21) and impairs social recognition (17) and spatial and episodic memory (22).

An important issue is that the majority of the studies have been conducted on young animals, whereas GBM and other malignant tumors occur mainly in adult and elderly patients. It was proposed that elderly patients aged 70 years and older (who are considered eligible for combined modality treatment) should receive a short-course radiotherapy with concomitant and adjuvant TMZ treatment up to 12 cycles to reduce the negative impact on health-related quality of life (23,24). However, the effects of TMZ on the behavior of adult and elderly animals have not been sufficiently studied. In a previous conducted by the authors, it was revealed that long-term treatment with TMZ to adult Wistar rats resulted in a significant decrease in their locomotor activity (25), whereas its effects on the anxiety of the animals remained unknown.

Anti-GBM chemotherapy is accompanied with dexamethasone (DXM) treatment in the majority of cases to prevent brain edema (26). Such treatment can potentially interfere with TMZ effects; therefore, in the present study, a single TMZ treatment regimen was used with DXM treatment (alone or in combination with TMZ). This concept was supported by previously published data on the effect of DXM influence on long-term behavioral characteristics of experimental animals. It was shown that DXM causes affective changes in humans, such as depression and anxiety (27,28) and elevated levels of other glucocorticoid (GC) cortisol may contribute to anxiety of patients (29). Prolonged use of GC during clinical practice in adult patients with glioma results in negative consequences, including psychiatric symptoms (30). In an animal model, adult 9-10-week-old C57BL/6J mice that were injected daily with DXM demonstrated a variety of depression-like behaviors and activated stress-related genes in the prefrontal cortex and hippocampus (31). A different study indicated a different effect of DXM on the behavior of animals depending on the dose. Anxiolytic effects were noted in lower doses and anxiogenic effects were observed at a high dose (32).

Various experimental models are used to assess the molecular mechanisms of the effect of chemotherapeutic drugs on the development of malignant gliomas (33). The majority of the studies investigating TMZ have been focused on the role of extracellular matrix (ECM) in the development of malignant gliomas (34-36) and the development of TMZ resistance (37,38). The main components of brain ECM are complex glycosylated proteoglycan (PGs) molecules, which are composed of the various core proteins with covalently attached polysaccharide chains of glycosaminoglycans (GAGs) (36). The effect of TMZ on the expression of PGs and GAGs in normal brain tissues has been studied inadequately. It has been revealed that TMZ exhibits no significant effect on PG expression in the hippocampus and cerebral cortex of 2-month-old Wistar rats; however, it demonstrates age- and brain zone-specific effects on the content of such GAGs, such as heparan sulfate (HS) and chondroitin sulfate (CS) (39). In brain tissues derived from 10-week-old mice, TMZ did not affect the expression of PG core proteins, whereas it reduced the CS content in the subcortical brain structures of severe combined immunodeficiency mice (40). Concomitantly, no data have been reported on the potential influence of long-term TMZ administration on glycosylated macromolecules in adult brain tissues and this research area should be investigated further.

The aim of the present study was to investigate the effect of long-term administration of TMZ and/or DXM on the development of anxiety of adult Wistar rats and on the content of the PGs and GAGs in the brain tissue of these animals.

Materials and methods

Animals. Nine-month-old male Wistar rats (Crl:WI) weighing 400-500 g were used at the beginning of the experiment (40 animals in total). The animals were obtained from the Institute of Cytology and Genetics (Novosibirsk, Russia) and housed in polycarbonate cages (36x50x28 cm) with free access to food and water; the temperature was maintained at 25±1˚C and the humidity range was 50-60%. The animals were weighed once a day and maintained at a light/dark 12/12 cycle. The number of animals per cage was 3 or 4. The animals were sacrificed by decapitation using a guillotine according to the American Veterinary Medical Association guidelines for the euthanasia of animals (2013). All efforts were made to minimize animal suffering and to reduce the number of animals used. All procedures were conducted in accordance with the European Communities Council Directive 2010/63/EU and in compliance with the Federal Research Center for Fundamental and Translational Medicine Ethical committee (approval no. N3/2017 from 23.06.2017; Novosibirsk, Russia).

TMZ and DXM administration. Rats were randomly assigned to 4 groups (control, TMX, DXM and TMZ + DXM; 10 animals/group). Simple randomization using random number generation was used. TMZ-based drug (TMZ; Teva Pharmaceutical Industries, Ltd.) was administered orally (150 mg/m^2) per day as a water suspension. The synthetic GC agonist (Dexamethasone; KrkA) was administered intraperitoneally at a dose of 2.5 mg/kg twice a week. The animals received 5 cycles of TMZ in the TMZ and TMZ/DXM groups for 5 consecutive days, with intermissions of 16 days between cycles. The control group received saline injections/peroral administration of the same volume as the experimental groups. During the experiment, the weight of the animals was monitored. The animals were sacrificed by decapitation and the brain from each animal was collected; one hemisphere was collected in RNALater solution (Invitrogen; ThermoFisher Scientific, Inc.) for reverse transcription-quantitative PCR (RT-qPCR) analysis. The other hemisphere was incubated in a 10% neutral buffered formalin for 48 h at room temperature and used to prepare paraffin blocks.

Elevated plus maze (EPM) test. EPM test was employed for studying mechanisms underlying anxiety and anti-anxiety drugs (41,42). The maze apparatus was composed of polyvinylchloride and comprised two opposing closed arms (50x10x40 cm) and two opposing open arms (50x10 cm). The maze was elevated at a height of 50 cm above the floor. Each rat was placed in the center of the maze, facing an open arm, and allowed to freely explore the maze for 5 min. The percentages of time spent in the center, in open arms and during head dipping in open arms, were used as an indication for anxiolytic behaviour. The percentages of time spent in closed arms and stretching in closed arms and the number of faecal boli were used as indicators for anxiolytic behavior. The EPM test was

Table I. Sequences of primers used in reverse transcription-quantitative PCR analysis.

Protein	Gene	Primer sequence (5'→3')
Syndecan-1	*Sdc1*	F: GAACCCACCAGCAGGGATAC
		R: CACACTTGGAGGCTGATGGT
Syndecan-3	*Sdc3*	F: TGCTCGTAGCTGTGATCGTA
		R: TGTCGGGCTTCTGGTATGTG
Glypican-1	*Gpc1*	F: GCCAGATCTACGGGGCTAAG
		R: AGACGCAGCTCAGCATACAG
Heparan sulfate proteoglycan 2/perlecan	*Hspg2*	F: TGATGACGAGGACTTGCTGG
		R: ACACCACACTGACAACCTGG
Decorin	*Dcn*	F: AATGCCATCTCCGAGTGGTG
		R: TTGTCGTGGAGTCGAAGCTC
Biglycan	*Bgn*	F: GAACAGTGGCTTTGAACCCG
		R: CCTCCAACTCGATAGCCTGG
Lumican	*Lum*	F: AATTTGACCGAGTCCGTGGG
		R: GCCTTTCAGAGAAGCCGAGA
Brevican	*Bcan*	F: AGGGGACCTCACAAGTTCTTC
		R: ATTTGACTCGGGGAAAGCCC
Neurocan	*Ncan*	F: AACCTGTGCGAGAAGGACAC
		R: GGGAGTGGACACTTGTCAGG
Aggrecan	*Acan*	F: CAGATGGCACCCTCCGATAC
		R: GACACACCTCGGAAGCAGAA
Versican	*Vcan*	F: ATGTGGATCATCTGGACGGC
		R: GTTTCGATGGTGGTTGCCTC
Chondroitin sulfate proteoglycan 4/Ng2	*Cspg4/Ng2*	F: ATCTGGGAGGGGGCTATTGT
		R: GTACGCCATCAGAGAGGTCG
Chondroitin sulfate proteoglycan 5	*Cspg5*	F: CTCCCATCCAAATGACATGGA
		R: CTCGAGTTTGGGTGACATGGA
Proteintyrosine phosphatase receptor type Z1/phosphacan	*Ptprz1*	F: TGTGTCATCGGAAGGATCGG
		R: GTCCGCATCGAAGCAGTAGA
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	F: ATGGCCTTCCGTGTTCCTAC
		R: TCCAGGGTTTCTTACTCCTTGG

F, forward; R, reverse.

performed at the same time of the day at 13.00-15.00 pm. The first behavioral test was performed prior to the initiation of the drug administration (rats were naive) and the second test following the end of drug administration (rats had already been trained).

RT-qPCR analysis. Coronal sections of the mouse brain (~1.5 mm thick) were obtained from the bregma (5±0.5 mm) and used for RT-qPCR analysis. Total RNA was extracted from the brain samples using the QIAzol reagent (Qiagen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The analysis was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) and real-time BioMaster HS-qPCR SYBR Blue Mastermix (2x) (Biolabmix) under the following conditions: 95˚C for 2 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. The total reaction volume was 25 μl. The relative amount of mRNA was normalized against GAPDH mRNA, and the fold-change for each mRNA was calculated by the $2^{-\Delta\Delta Cq}$ method (43). The primer sequences used for the rat genes are presented in Table I.

Histological analysis. To study the morphology of the rat brain, one hemisphere of the rat brain tissue was fixed in 10% neutral formalin for 48 h at room temperature, dehydrated in a series ethanol solution of increasing concentration and embedded in paraffin. Serial 3-4 μm coronal sections of the mouse brain were obtained from the bregma (5±0.5 mm), stained with hematoxylin and eosin (H&E) for 2 min, and analysed using light microscope LeicaDM 4000B (Leica Microsystems, Inc.) with the LeicaDFC 320 camera (Leica Microsystems, Inc.). The cerebral cortex was studied within the primary and secondary motor cortex (M1, M2) and primary somatosensory (S1HL, S1FL, S1DZ, S1BF) cortical zones.

Immunohistochemical (IHC) analysis. For immunohistochemistry, 3-mm sections of formalin-fixed, paraffin-embedded tissue samples were deparaffinized in xylene and ethanol series. Tissue sections were stained using Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (cat. no. ab64264; Abcam) according to the manufacturer's instructions. Sections were incubated with Hydrogen Peroxide Block (cat. no. ab64264; Abcam) for 10 min at room temperature, and then exposed to Protein Block (cat. no. ab64264; Abcam) for 10 min at room temperature. The antigen was retrieved following treatment with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20) at 95-98˚C for 20 min. The following primary antibodies were used for immunostaining: mouse monoclonal anti-CS (1:100; cat. no. C8035; Sigma-Aldrich; Merck KGaA), rabbit polyclonal anti-aggrecan (1:100; cat. no. ab36861; Abcam), rabbit polyclonal anti-decorin (1:100; cat. no. ab175404; Abcam), and rabbit polyclonal anti-brevican (1:100; cat. no. ab111719; Abcam) for 1 h at room temperature; mouse monoclonal anti-GFAP (ready-to-use; cat. no. MS-1376-R7; Thermo Fisher Scientific) and mouse monoclonal anti-Olig2 (ready-to-use; cat. no. 211F1.1; Cell Marque) for 30 min at room temperature The staining patterns for CS, aggrecan, decorin and brevican were visualized with Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (cat. no. ab64264; Abcam). The sections were counterstained with hematoxylin and observed by light microscopy using an AxioScopeA1 microscope (Zeiss AG). Quantitative analysis was performed with ZENblue 2.3 software (Zeiss AG). A total of ten fields for each section were analyzed.

Dot-blot analysis. Coronal brain tissue sections 1.5-mm thick were obtained from the bregma (5±0.5 mm), lysed with RIPA-buffer (Thermo Fisher Scientific, Inc.), containing complete protease inhibitor cocktail (Roche Diagnostics), sonicated (20 kHz, 3 times for 10 sec) and centrifuged for 15 min at 14,000 x g. The protein concentration was quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). A total of 1 mg total protein was dot-blotted onto polyvinylidene fluoride membranes at a volume of 2 ml. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with mouse anti-CS primary antibody (1:500; cat. no. C-8035; MilliporeSigma), mouse anti-HS primary antibody (1:500; cat. no. MAB2040; MilliporeSigma) overnight at 4˚C followed by incubation with secondary peroxidase-conjugated antibodies goat anti-mouse IgG (1:2,000; cat. no. ab6823; Abcam) for 1 h at room temperature. GAGs were detected with an Optiblot ECL Detection Kit (Abcam) according to the manufacturer's instructions. The blot images were captured using ChemiDoc (Bio-Rad Laboratories, Inc.) and analyzed semi-quantitatively using ImageJ software (v.1.52; National Institutes of Health).

Statistical analysis. One-way ANOVA analysis with Fisher's Least Significant Difference post hoc test and Kruskal-Wallis' test was performed to determine the statistical significance between the studied groups $P<0.05$ was considered to indicate a statistically significant difference. The number of the repeats of the experiments was three. The data are expressed as the mean ± standard deviation. Statistical analysis was performed using Origin Pro 8.5 software (OriginLab).

Results

Study design. To model anti-GBM chemotherapy in an experimental animal model *in vivo*, adult Wistar rats were treated with TMZ and/or DXM for 4 months. The effects of TMZ and/or DXM on animal anxiety, morphology of the cerebral cortex, PG expression and GAG content in the rat brain tissue were investigated.

TMZ treatment results in anxiety-like behavior of the experimental animals. The effects of TMZ and/or DXM on anxiety were investigated in adult rats using the EPM test. The main monitored parameters included the following: Percentage of time spent in open and closed arms as well as in the center of the arena, head dipping in open-arms, stretching in closed arms and the number of fecal boli (Fig. 1A-F).

Long-term TMZ treatment of the adult rats resulted in a decrease in the percentages of time and head dipping in open-arms and an increase in stretching in closed arms in the EPM test (Fig. 1A, B and D, respectively), which indicated a significant increase in the anxiety of the animals. The combination of TMZ with DXM did not demonstrate significant differences with TMZ alone, suggesting a negligible effect of DXM on the animal anxiety. The observation was further supported by the DXM administration as a mono-regimen, which did not affect the majority of the studied behavioral parameters except for a reduction in head dipping in open-arms (Fig. 1B).

These results demonstrated that the long-term use of TMZ can affect anxiety of the adult Wistar rats in the experimental system *in vivo*.

TMZ does not affect the morphology of the cerebral cortex. To look for potential morphological basis of the demonstrated behavioral changes, paraffin sections of the brain of the rats were stained with H&E (Fig. 2A).

The cerebral cortex of the Wistar rat (neocortex) has a typical six-layer structure in all groups (Fig. 2A). During the morphological analysis of the tissue of the cerebral cortex, the architectonics of the cerebral cortex were not disturbed; in addition, circulatory disorders and other pathological changes, including necrobiosis and necrosis of cellular elements and inflammatory infiltration, were not detected. The H&E slides of the cortex tissue of the animals (control and experimental groups) were characterized by usual tinctorial properties of neurons and neuropil with minimal visible variations. The most prominent in the cortex of all experimental groups was the fifth layer, which contained comparatively large neurons of pyramidal shape. It is important to note that in all experimental groups in this layer (and to a lesser degree in the third layer) the attention was attracted on the moderately pronounced eosinophilia of the cytoplasm of pyramidal neurons without signs of cyto-destruction or other structural reactions, such as gliosis, peri-cellular infiltration, edema and destruction of blood vessels (Fig. 2A).

The number of astrocytes and oligodendrocytes in the cerebral cortex tissues of the Wistar rats was analyzed by IHC

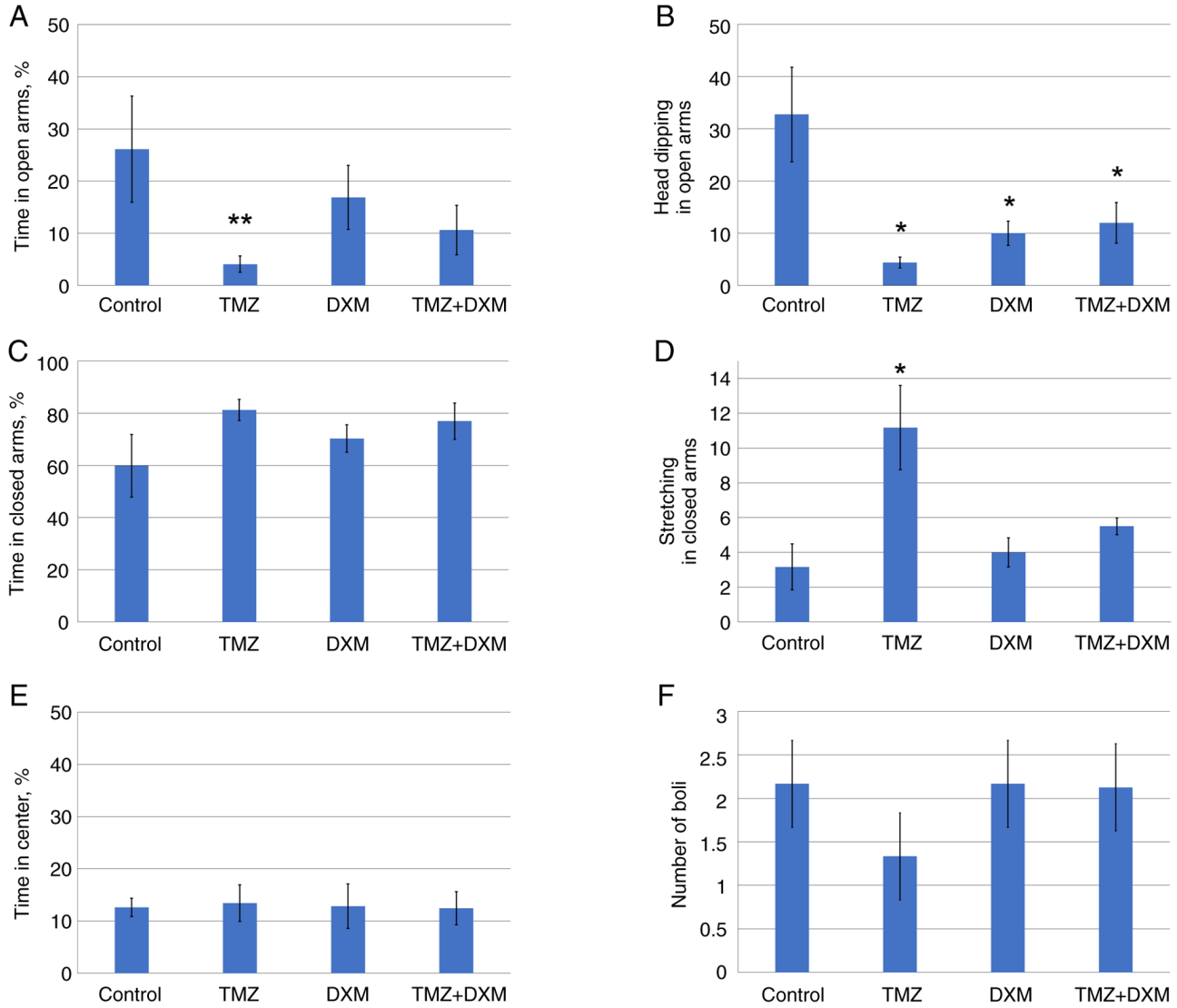

Figure 1. Anxiety of rats in the elevated plus maze following TMZ and/or DXM treatment. (A) Percent of time spent in open arms. (B) Head dipping in open arms. (C) Percentage of time spent in closed arms. (D) Stretching in closed arms. (E) Percentage of time spent in the center. (F) Number of boli. *P<0.05 and **P<0.01 compared with control. All data are expressed as mean ± CI/2 (Origin 8.5 software). TMZ, temozolomide; DXM, dexamethasone.

staining of paraffin sections with antibodies to glial fibrillary acidic protein (GFAP; marker of astrocytes) and nuclear protein oligodendrocyte transcription factor 2 (OLIG2; marker of oligodendrocytes and astrocytes). A tendency towards an increase in the content of astrocytes derived from the brain tissues of TMZ-treated animals was observed. However, the trend was not statistically significant (Fig. 2B). The number of astrocytes and oligodendrocytes in the TMZ-treated brain tissues did not demonstrate an evident difference compared with that of the control cells (Fig. 2B).

Taken together, the obtained data did not reveal significant deterioration of astroglia and oligodendroglia derived from the cerebral cortex of the brain tissue of TMZ-treated adult animals.

TMZ inhibits aggrecan expression in rat brain tissues. Despite the unaltered morphological structure and cellular composition of the brain tissue, certain molecular changes may occur following TMZ/DXM treatment to the brain ECM. The main components of the ECM are PGs. As the first step, transcriptional profiling of the core proteins of the main PGs investigated was performed by RT-qPCR (Fig. 3).

The expression levels of all HSPGs (syndecan-1, syndecan-3, glypican-1, and perlecan) and the majority of the studied CSPGs (decorin, biglycan, lumican, brevican, versican, Cspg4/Ng2, Cspg5 and phosphacan) were not significantly affected by the long-term treatment with TMZ and/or DXM (except for neurocan and aggrecan). Aggrecan was the most sensitive to TMZ/DXM treatment and its expression was downregulated following TMZ (-10-fold), DXM (-45-fold), and TMZ-DXM (-80-fold) treatment. Neurocan expression demonstrated -1, and 8-fold decreases following single treatment with DXM.

To confirm the validation of these results at the protein level, IHC staining was performed on the brain sections derived from these animals with antibodies to aggrecan, decorin and brevican (Fig. 4).

The protein content of aggrecan, decorin and brevican corresponded to transcriptional data obtained by RT-qPCR (Fig. 3), supporting the result obtained on the significant

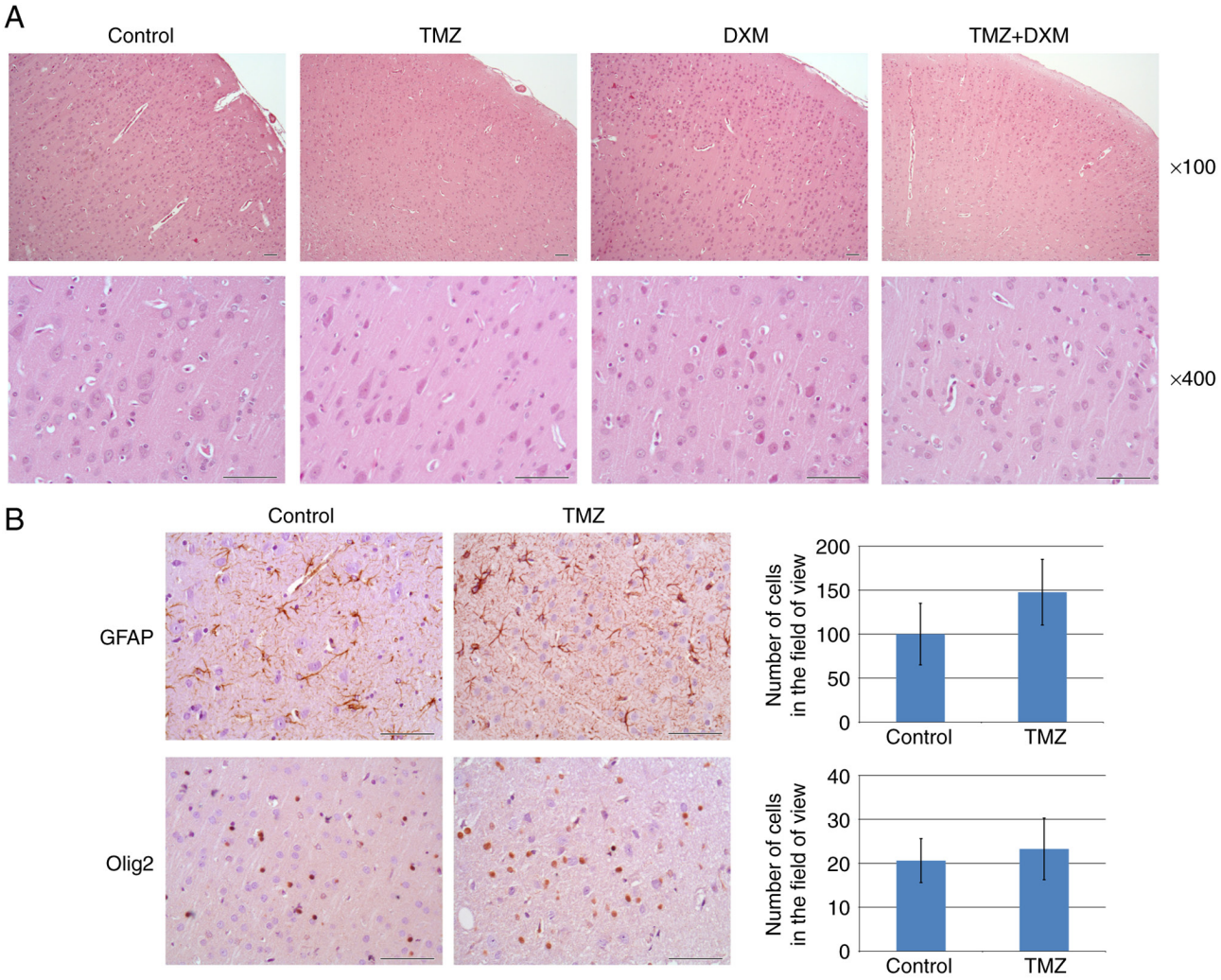

Figure 2. Histological analysis of rat brain tissues in the control, TMZ, DXM and TMZ + DXM rat groups. (A) Hematoxylin and eosin staining (magnification, x100; scale bar, 50 μm). (B) Immunohistochemical analysis of GFAP and OLIG2 expression in the TMZ rat group. Quantitative analysis of the DAB signal was performed with ZENblue software (magnification, x400; scale bar, 50 μm). The bars represent the mean ± standard deviation from triplicate experiments (Origin 8.5; Kruskal-Wallis' test). Control, non-treated rat brain tissues; TMZ, temozolomide, DXM; dexamethasone; GFAP, glial fibrillary acidic protein; OLIG2, oligodendrocyte transcription factor 2; DAB, 3,3'-diaminobenzidine.

decrease of aggrecan expression both at the mRNA and protein levels.

TMZ reduces CS content in rat brain tissues. Since PGs are complex protein-carbohydrate molecules, it was important to assess whether changes occurred in the content of carbohydrate chains of HSPGs and CSPGs. The total HS and CS contents in the control and TMZ/DXM-treated brain tissues were investigated by dot-blot analysis with anti-HS and anti-CS primary antibodies specific for the polysaccharide HS and CS epitopes, respectively (Fig. 5).

It was revealed that the HS content was not affected by TMZ and/or DXM treatments (Fig. 5A), whereas the CS content was decreased by 1.5-fold in the brain tissues derived from the TMZ- and DXM-treated groups (Fig. 5B). IHC staining supported the results of dot-blot analysis, demonstrating a significant (2-2.5-fold) decrease of the CS content in the brain cerebral cortex following long-term TMZ exposure both as a mono-regimen and as a combination treatment with DXM (Fig. 6).

Discussion

In the present study, the effects of long-term administration of TMZ and/or DXM on the anxiety of adult Wistar rats were assessed. Moreover, the content of the PGs and GAGs in the brain tissues of these animals were studied. The dose and regimen of TMZ and DXM treatments were selected to maximally mimic a chemotherapeutic anti-GBM treatment.

Patients with GBM receive radiotherapy plus TMZ daily (75 mg/m^2), followed by six cycles of TMZ (150-200 mg/m^2 for 5 days during each 28-day cycle) (1). For animals, the TMZ amount is calculated based on the surface area of the animal's body, which is further calculated by the following formula: S=(K*W2/3)/10,000, where S is the surface area (m^2), W is the body weight obtained on the day of dosing (g), and K ¼ 9.0 (constant for estimating surface area) (44,45).

The dose of DXM used to treat patients with GBM is not clearly regulated. The maximum dose of DXM is 16 mg daily, administered in 4 equal doses and recommended for symptomatic patients (46). The dosage range used in experimental

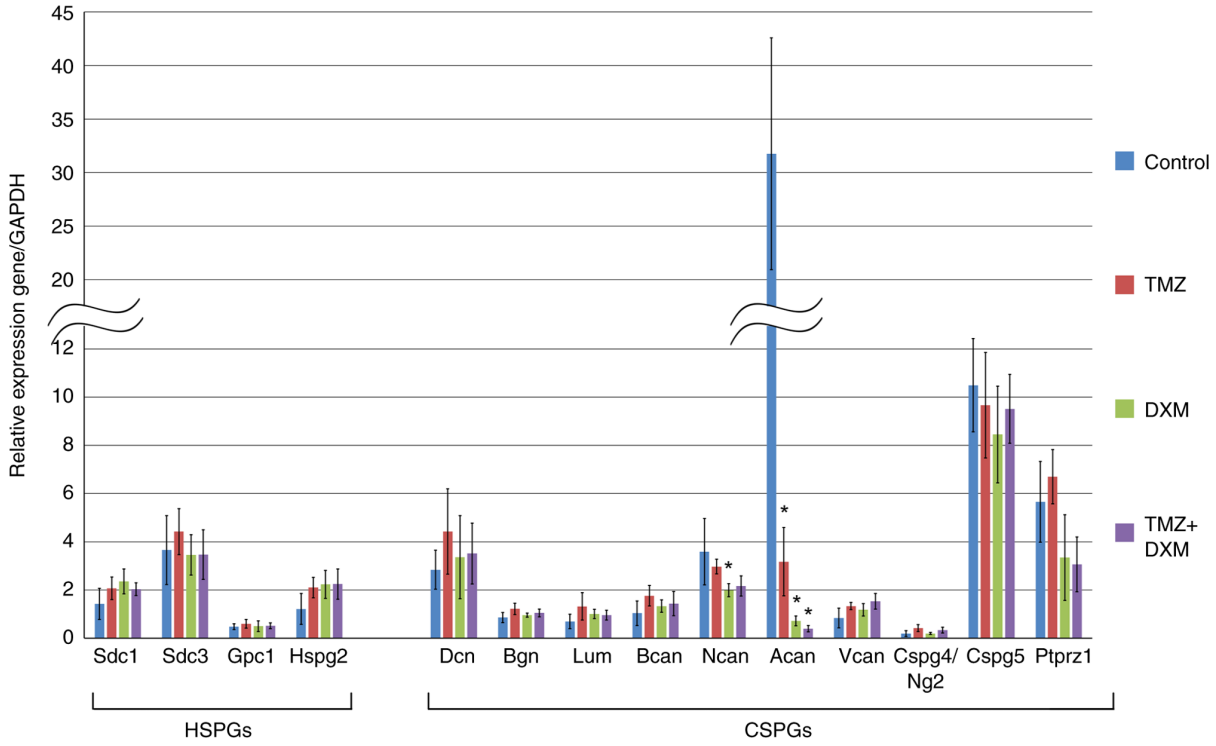

Figure 3. Expression of HSPGs and CSPGs core proteins in rat brain tissues of the control, TMZ-treated, DXM-treated and TMZ + DXM-treated experimental animals. Reverse transcription-quantitative PCR, expression normalized to that of GAPDH (OriginPro 8.5). The bars represent the mean ± standard deviation from triplicate experiments (OriginPro 8.5). ANOVA + Fisher's Least Significant Difference test; *P<0.05. Control, non-treated rat brain tissue; TMZ, temozolomide; DXM, dexamethasone; HSPG, heparan sulfate proteoglycan; CSPG, chondroitin sulfate proteoglycan; Sdc1, syndecan-1; Sdc3, syndecan-3; Gpc1, glypican-1; Hspg2, heparan sulfate proteoglycan 2/perlecan; Dcn, decorin; Bgn, biglycan; Lum, lumican; Bcan, brevican; Ncan, neurocan; Acan, aggrecan; Vcan, versican; Cspg4/Ng2, chondroitin sulfate proteoglycan 4; Cspg5, chondroitin sulfate proteoglycan 5; Ptprz1, proteintyrosine phosphatase receptor type Z1/phosphacan.

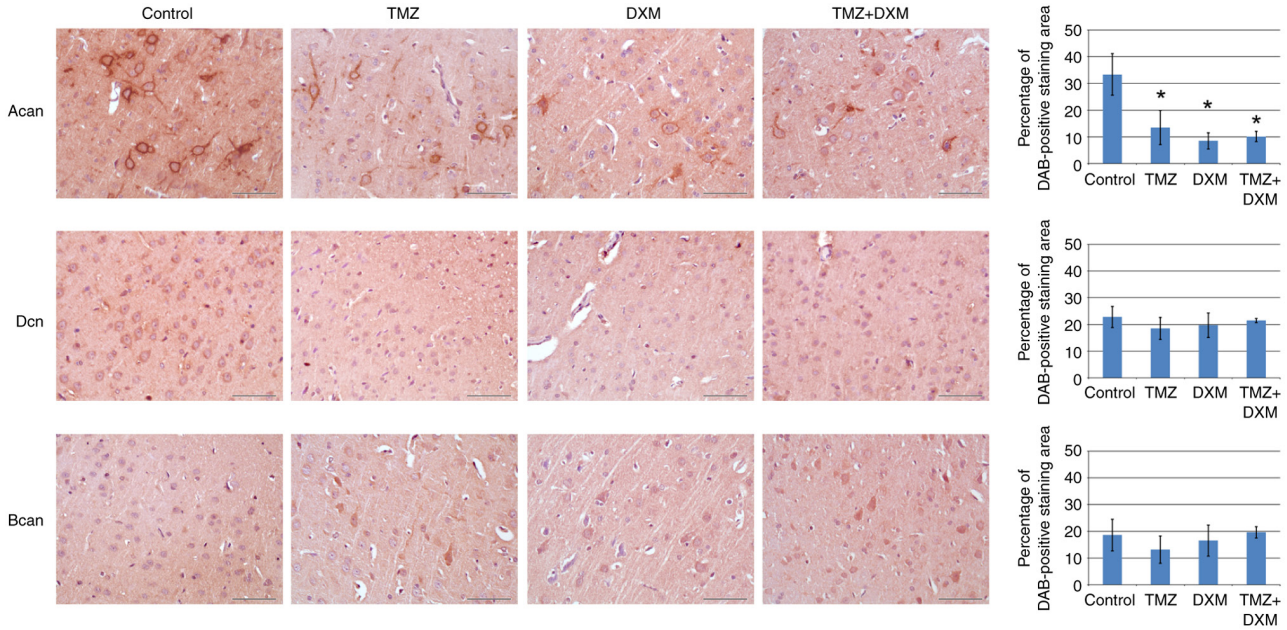

Figure 4. Immunohistochemical analysis of aggrecan, decorin and brevican content in the control and TMZ/DXM-treated experimental rats. Quantitative analysis of aggrecan, decorin and brevican content was performed with ZENblue software (magnification, x400; scale bar, 50 μm). The bars represent the mean ± standard deviation from triplicate experiments (OriginPro 8.5). ANOVA + Fisher's Least Significant Difference test; *P<0.05. Control, non-treated rat brain tissue; TMZ, temozolomide; DXM, dexamethasone; Acan, aggrecan; Dcn, decorin; Bcan, brevican; DAB, 3,3'-diaminobenzidine.

models is considerably wide ranging from 0.1 to 50 mg/kg. A dosage of 2.5 mg/kg was selected in the present study based on literature data (47-52) and experimental conditions. Due to the fact that the animals received the drug for a long time

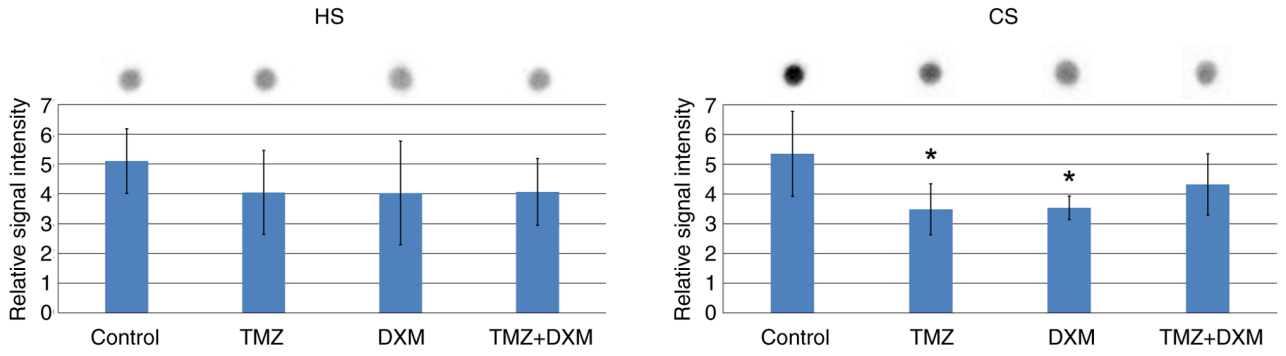

Figure 5. Dot-blot analysis of the total CS and HS content in rat brain tissues. Original representative dot blots and semi-quantitative analysis of the dot-blots (ImageJ version 1.52 software; National Institutes of Health). The bars represent the mean ± standard deviation from triplicate experiments (OriginPro 8.5). ANOVA + Fisher's Least Significant Difference test; *P<0.05. Control, non-treated rat brain tissue; TMZ, temozolomide; DXM, dexamethasone; CS, chondroitin sulfate; HS, heparin sulfate.

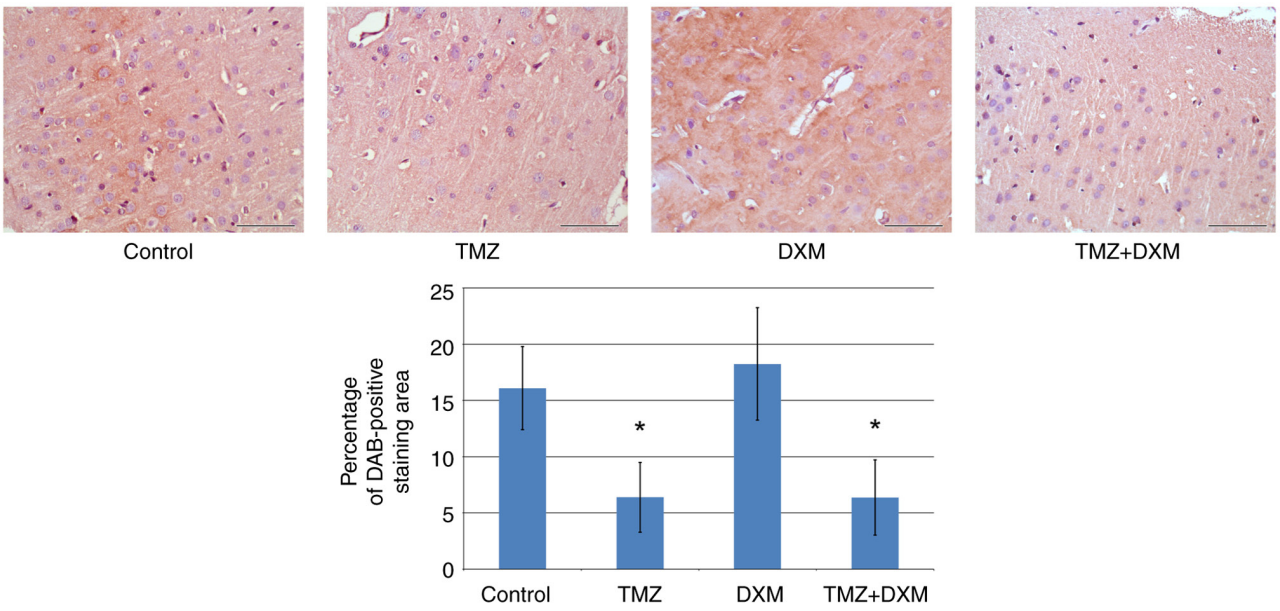

Figure 6. Immunohistochemical analysis of the CS content in the control and TMZ/DXM-treated experimental rats. Quantitative analysis of the DAB signal was performed with ZENblue software (magnification, x400; scale bar, 50 μm). The bars represent the mean ± standard deviation from triplicate experiments (OriginPro 8.5). ANOVA + Fisher's Least Significant Difference test; *P<0.05. Control, non-treated rat brain tissue; TMZ, temozolomide; DXM, dexamethasone; CS, chondroitin sulfate; DAB, 3,3'-diaminobenzidine.

(4 months, 2 times/week), the moderate DXM dose (2.5 mg/kg) was selected to reduce toxicity.

In the present study, it was shown that long-term use of TMZ led to an increase in the anxiety of adult rats. The data are consistent with the results of the study, where TMZ administration to adult mice (once daily) for three days resulted in the decrease of the time spent by the mice in open arms and consequently increased anxiety (17). Treatment of adult two-month-old mice with TMZ and radiotherapy three times per week for 6 weeks also led to an increase in anxiety and neurogenesis deficit in the mice of the EPM test (20). An additional study indicated that following 5 and 15 weeks of radiochemotherapy, anxiety-like behavior and anxiety- and depression-like behavior were observed in 6-month-old mice, respectively (18).

The present study indicated that the long-term use of TMZ and/or DXM therapy in a Wistar rat model leads to changes in the brain ECM and increase of anxiety in elderly rats. These data are in agreement with previous studies reporting that elderly patients aged 70 years and older who are considered eligible for combined modality treatment should receive a short-course of radiotherapy with concomitant and adjuvant TMZ treatment up to 12 cycles (24); in addition, 6 courses of TMZ for elderly patients appear to be sufficient for an optimal treatment response (53). Taken together, these data may suggest the reduction in the number of TMZ cycles that can in turn reduce the toxicity and side effects of chemotherapy.

Previous studies have suggested the presence of different neurophysiological mechanisms of behavioral disorders during and following TMZ chemotherapy that are responsible for reducing neurogenesis (17,19). Moreover, a decrease in theta activity in the hippocampal tissues of adults (21) and changes in markers of oxidative stress (catalase, superoxide dismutase, lipoxygenase and reduced glutathione) in the

hippocampal and frontal cortex regions of the brain have been proposed as additional mechanisms (22). In the present study, it was hypothesized that one of the mechanisms responsible for the behavioral disturbance may be attributed to the changes in the expression levels of PG core proteins and their CS carbohydrate chains.

According to the findings of the present study, long-term administration of TMZ resulted in the decrease of the expression of aggrecan core proteins present in the cerebral cortex of adult rats, which could cause impairments in neurogenesis and plasticity (54). These results are hard to compare with those reported from previous studies since the information on this matter is very scarce. It has been revealed that in 2-month-old Wistar rats, TMZ did not affect PG core protein expression in the brain tissue at the mRNA level, although certain changes were noted in the decorin and syndecan-1 protein contents as well as in the HS/CS content (39). It can be hypothesized that aggrecan becomes sensitive to long-term TMZ administration in elderly patients suggesting that this PG can be a potential target for brain protection at the elder age.

The effects of TMZ and DXM on the polysaccharide CS chains were studied with dot-blot analysis and IHC. According to the data of dot-blot analysis, the CS content is decreased after TMZ and DXM treatment, according to IHC analysis CS content is decreased after TMZ and TMZ/DXM treatments. As for DXM, there are differences in the presented data on CS content coming from the methodological basis of dot-blots and IHC. These methods were performed with the use of different tissue samples-whole tissue lysates for dot-blot and paraffin-embedded tissue for IHC. Additionally, methodology includes different approaches to the registration of the changes observed-quantification of the total CS signal (dot-blot) and microphotography of the brain cortex (IHC). The effects of TMZ on the polysaccharide CS chains have been investigated in previous studies. In younger (2-month-old) Wistar rats, TMZ differentially affected CS in different brain zones increasing the CS content in the hippocampus but not in the brain cortex (39). Concomitantly, TMZ administration (by three cycles of 5 consecutive days) to 10-week-old mice decreased the CS-AC content in the cerebral cortex (40). The results demonstrated in the current study complement the previous data reported on the CS content of the brain tissue following TMZ/DXM treatment and contribute to the identification of a molecular mechanism of CS sensitivity to systemic chemotherapy.

Taken together, the obtained results demonstrated that long-term use of TMZ and/or DXM causes behavioral disorders in adult rats, which are accompanied by changes in the expression of CSPG aggrecan core protein and in the CS content. This may be a novel molecular mechanism of long-term side-effects of TMZ. Prevention of aggrecan loss may be a novel strategy for neuroprotection and can potentially improve the quality of life of elderly patients with cancer receiving long-term treatment with TMZ.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

EVG and AVS conceptualized the study. EVK, VSU, NVM and SVA developed methodology. AVS and OPM performed software analysis. AVS, DKS and VSU validated data. DKS, OPM, EVK, NVM, GMK and EEK conducted formal analysis. DKS, EVK, VSU, MOP, NVM, GMK, EEK and SVA conducted investigation. EVG provided resources. AVS performed data curation. AVS prepared the original draft. EVG reviewed and edited the manuscript. AVS, GMK and SVA conducted data visualization. AVS and EVG supervised the study. AVS conducted project administration. AVS and EVG acquired funding. All authors have read and approved the final version of the manuscript. VSU and AVS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Institute of Molecular Biology and Biophysics, Federal Research Center of Fundamental and Translational Medicine (FRC FTM; approval no. N3/2017 from 23.06.2017; Novosibirsk, Russia).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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