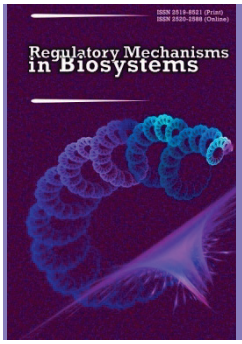

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Downregulation of SIRT1, SIRT3, and IGF-1 in ApoE-deficient mice exacerbates neuronal damage induced by chronic cerebral hypoperfusion

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Chronic cerebral hypoperfusion is a widespread pathological condition caused by chronically reduced cerebral blood flow leading to brain damage, but the specific molecular mechanisms that regulate these phenomena remain poorly understood. In this study, we investigated brain damage and neuronal DNA injury in a vulnerable region of the brain, the hippocampus, as well as the involvement of apolipoprotein E (ApoE), sirtuins of 1 (SIRT1) and 3 (SIRT3) types and insulin-like growth factor 1 (IGF-1) in pathogenetic mechanisms in mice with chronic cerebral hypoperfusion caused by the permanent occlusion of the left unilateral common carotid artery. Male C57/6j (C57, wild type) and ApoE(-/-) mice were divided into four experimental groups (10 mice per group): sham-operated C57, C57 with chronic cerebral hypoperfusion, sham-operated ApoE(-/-) mice, ApoE(-/-) mice with chronic cerebral hypoperfusion. Our results showed that the number of damaged neurons in the hippocampus at 8 weeks after surgical manipulation increased in both groups of mice with chronic cerebral hypoperfusion, with more pronounced rates in ApoE(-/-) mice than in C57 mice. However, ApoE deficiency in moderate chronic cerebral hypoperfusion was accompanied by a higher level of undamaged DNA (class 0) and a low level of maximally damaged DNA (class 4) in brain cell nuclei in contrast to group C57. In ApoE-deficient mice, reduced expression of SIRT1, SIRT3, and IGF-1 was found. In chronic cerebral hypoperfusion, expression of sirtuins was preserved, but IGF-1 expression was significantly reduced in ApoE(-/-) mice in comparison to C57. The obtained results indicate that ApoE deficiency leads to downregulation of SIRT1, SIRT3 and IGF-1 in the brain; this lack of cytoprotection is enhanced in chronic cerebral hypoperfusion and may participate in the mechanisms of neuronal damage.

Keywords: chronic cerebral hypoperfusion; ApoE(-/-); DNA damage; SIRT1; SIRT3; IGF-1; hippocampus.

Introduction

Chronic cerebral hypoperfusion is a widespread pathogenic factor associated with development of cerebrovascular diseases including neuronal demyelination, aging-related neurodegeneration, brain atherosclerosis etc. Insufficiency of blood supply may be caused by diseases of large and small vessels of the head and neck, which appear as hypoxic and metabolic neuronal disorders, vascular cognitive deficits, and is one of the major causes of acute ischaemic stroke (He et al., 2023). The decrease in cerebral blood flow due to carotid artery disease leads to cognitive decline which is proportional to the degree of vascular stenosis (Buratti et al., 2014). Occlusive and stenotic pathology in the carotid artery has a high prevalence and morbidity, the clinical spectrum of which varies from an asymptomatic course to severe stroke or death (Mortimer et al., 2018). Therefore, there is great clinical significance in deeply studying the pathogenesis of chronic cerebral ischemia for the prevention and treatment of ischemic cerebrovascular diseases (Yamashita & Abe, 2016).

Apolipoprotein E (ApoE) is an important risk factor for neurodegenerative and cardiovascular diseases as one of the key regulators of lipid metabolism. ApoE is the main ligand for the low-density lipoprotein receptor, which transports cholesterol and other lipids through the cerebrospinal fluid and plasma (Yin & Wang, 2018). Information on the involvement of ApoE in the mechanisms of brain damage in acute and chronic ischemic lesions is ambiguous (Martínez-González & Sudlow, 2006; Kumar et al., 2016; Lindgren & Maguire, 2016). The involvement of the ApoE gene polymorphism in the development of stroke has been shown (Zhong et al., 2018), but the role of ApoE was negligible in the chronic experiment (Kitagawa et al., 2005). Most studies of ApoE implicate it as a genetic risk factor for Alzheimer's disease and vascular dementia (Montagne et al., 2020; Onyango et al., 2021; Palmer et al., 2021). The effect of ApoE genotype on ApoE modification, lipidation or their levels, can change the resistance of neurons to pathogenic factors and change the duration and intensity of neuroinflammation (Flowers & Rebeck, 2020), which is the pathway to any cerebrovascular disease.

All these processes are accompanied by DNA damage (Li et al., 2018), which can be prevented by cytoprotective molecular pathways. Sirtuins (Sirtuins, Silent Information Regulator 2 proteins, SIR2) are a class of regulatory proteins that carry out epigenetic control of many biological processes, in particular, metabolism, cell growth, apoptosis, autophagy, aging, and regulate the response to cellular stress in pathological conditions (Li et al., 2018). Seven animal homologues of sirtuins (SIRT1-7) have been identified, they are localized in the nucleus (SIRT1, SIRT6, SIRT7), mitochondria (SIRT3, SIRT4, SIRT5), or cytoplasm (SIRT2). In the brain, high levels of SIRT1 are expressed in the nucleus, and a small amount in the mitochondria. SIRT3 is primarily localized in mitochondria (Michishita et al., 2005), which suggests their participation in the mechanisms of diseases of the central nervous system. Data point to the involvement of SIRT1 and SIRT3 in aging-related diseases (Jiao et al., 2020; Park et al., 2020; Zhao et al., 2021). Despite intensive study of sirtuins, their role in the chronic cerebral hypoperfusion caused by occlusive-

stenotic pathology of the brachiocephalic arteries is still unclear. Insufficiency of blood supply in the brain can result in tissue hypoxia. Therefore hypoxia-inducible insulin-like growth factor IGF-1 is of interest among other neuroprotective factors. Recent studies indicate that this protein can exert neurotrophic and regenerative effects in the central nervous system, stimulate protein synthesis, neuronal and synaptic plasticity, promote the survival of neurons, glia, oligodendrocytes, prevent apoptosis and glutamate-mediated excitotoxicity (Bianchi et al., 2017; Lu et al., 2020; Bhalla et al., 2022; Ge et al., 2022). IGF-1 has been shown to be involved in neuroprotection in brain ischemic stroke, cerebral trauma, Alzheimer's disease, amyotrophic lateral sclerosis, epilepsy, neurological dysfunctions, and, in vessels, can prevent atherosclerotic lesions (Bianchi et al., 2017; Lu et al., 2020; Bhalla et al., 2022; Ge et al., 2022; Wang et al., 2022; Sukhanov et al., 2023). However, the role of IGF-1 and its receptors in chronic cerebral hypoperfusion has been investigated only in a few works (Yousef et al., 2020; Chen et al., 2021; Kim et al., 2021) and needs clarification.

Thus, all of the above points to the fact that chronic cerebral hypoperfusion is a public health concern that constitutes one of the most common serious conditions worldwide with chronic cerebral blood flow reduction, which causes brain damage and cell DNA injury. Therefore, the molecular pathways of brain damage or protection (in particular, role of ApoE, sirtuins of 1 and 3 types, and IGF-1) in chronic cerebral hypoperfusion caused by chronic pathology of the brachiocephalic arteries require detailed study. Based on the previous information, the first aim of the present study was to investigate the effect of chronic cerebral hypoperfusion induced by unilateral common carotid artery occlusion in C57/6j mice and ApoE(-/-) mice on damage to one of brain areas vulnerable to global cerebral ischemia, particularly the hippocampus (Gao et al., 2019), and on injury to DNA of neurons. The further aim of the work was to evaluate the possible role of SIRT1, SIRT3 and IGF-1 in these events.

Materials and methods

Experimental animals and experimental design. The animal studies were performed in accordance with the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines 2.0 (Percie du Sert et al., 2020) and were approved by The Local Ethics Committee at Bogomoletz Institute of Physiology (Kyiv, Ukraine) (protocol No. 13, 12.05.2020), as investigations conducted according to requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and the current legislation of Ukraine on protection of experimental animals (No. 3447-IV, 21.02.2006).

Experiments were performed on 20 male C57/6j (C57) mice and 20 male ApoE(-/-) mice 6 weeks old (body weight 15–18 g). The animals were randomized into the following groups: 1) sham operated C57 mice (control C57, n = 10); 2) C57 mice with chronic cerebral hypoperfusion (n = 10); 3) sham operated ApoE(-/-) mice (control ApoE(-/-)); 4) ApoE(-/-) mice with chronic cerebral hypoperfusion (n = 10). The animals were kept under standard vivarium conditions on a 12 h light/dark cycle for a period of 8 weeks of the experiment with free access to standard mouse chow and water. At the end of the experiment, the animals were anesthetized with ketamine (60 mg/kg, i.p.), and tissue samples were removed for further examination.

Surgical model of chronic cerebral hypoperfusion. The mice underwent a left common carotid artery occlusion surgery, as previously described, to elicit chronic cerebral hypoperfusion (Zuloaga et al., 2016). Briefly, under ketamine (60 mg/kg, i.p.) anesthesia, the left common carotid artery was ligated with two 6–0 silk sutures. The sham surgery consisted of exposing the carotid artery without ligation.

Tissue sample preparation and histological examination. At 8 weeks after surgery, the mice were anesthetized with ketamine. The brain was quickly removed after decapitation and washed three times with a cooled PBS solution (NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO_4 •$12H_2O$ 2.8 g/L, KH_2PO_4 0.2 g/L, pH 7.4, 4 °C). Then brain tissues were fixed with 10% neutral-buffered formalin solution for 24 h. The paraffin-embedded brain blocks were sliced at a thickness of 2 μm to the brain sections including the hippocampus. The obtained sections were stained with hematoxylin and eosin solutions (H&E) for histopathological examination using XSP-139-TP microscope (×400) (China, 2018) equipped with digital camera Levenhuk M1400 PLUS (USA, 2020). Three images were used in each area of interest and separated into ipsilateral and contralateral hemispheres. The viable and dead cells were counted using ImageJ software (USA, 2022).

DNA-comet assay (alkaline). To detect DNA single-strand breaks of neurons, the method of alkaline gel electrophoresis of isolated cells was used (DNA-comet assay) (Sribna et al., 2019; Afanasieva et al., 2010). Electrophoresis of preparations was performed after stabilization for 20 minutes in alkaline electrophoresis buffer using the device MultiphorII ("LKB", Sweden, 2000) at a voltage of 24 V and a current of 100 mA for 30 minutes. The DNA comet analysis on electrophoregrams painted by Hoechst 33342 (700 μmol/L) was performed visually using a luminescent microscope LUMAM I-1 (Russia, 1995) using a water-immersion lens (×30). In each micropreparate up to 400 separately located DNA comets were analyzed. By the ratio of DNA in the "head" and "tail", the comets were divided into 5 classes (0–4) (Collins, 2004) (Fig. 1), and their number was separately counted.

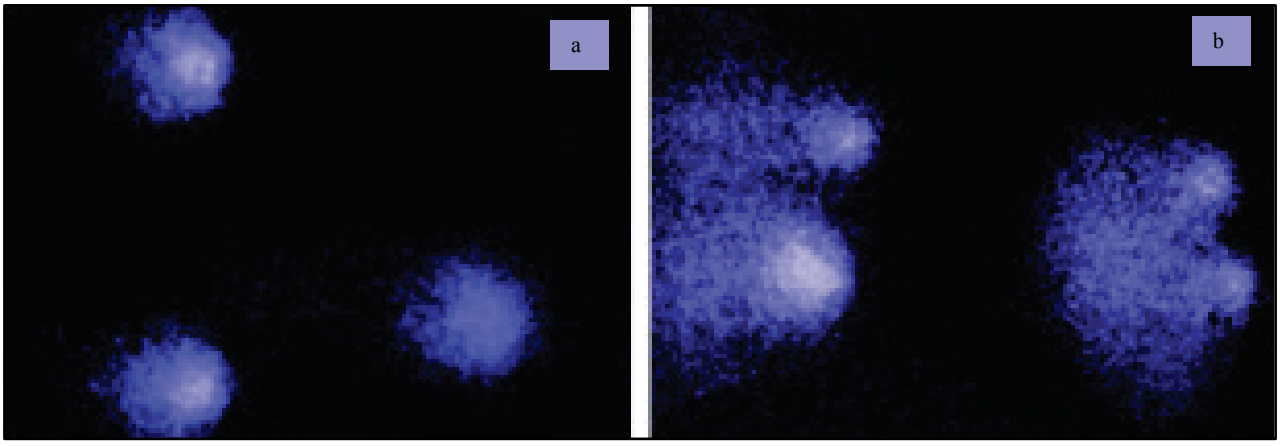

Fig. 1. Comet shape changes during the degradation process of nuclear DNA in brain of mice under 8 weeks of the chronic cerebral hypoperfusion: *a* – 1–2 classes of DNA comets, *b* – 3–4 classes of DNA comets

RNA Isolation, Reverse Transcription and Real-Time PCR. For gene expression determination, total RNA was isolated from frozen brain hemisphere samples with Trizol reagent (Invitrogen, USA). RNA concentration was determined using the NanoDrop spectrophotometer ND1000 (NanoDrop Technologies Inc, USA, 2010). cDNA was synthesized from 5 μg of total RNA by reverse transcription with 10 mM Tris-HCl (pH 9.0), 5 mM $MgCl_2$; 1 mm dNTPs; 20 U Ribo-Lock, Random hexamer primers (0.5 μg/μL) and 200 U RevertAid H Minus M-MuLV Reverse Transcriptase. Quantitative PCR was performed using the 7500 Fast Real-time PCR (Applied Biosystems, USA, 2015) with TaqMan® Gene Expression Assays ("Applied Biosystems") for SIRT1 (Mm01168521_m1) and SIRT3 (Mm00452131_m1). Gene expression

in each probe was normalized by GADPH expression, using TaqMan Rodent GADPH Control Reagent (VIC™Probe, lot number 0608014). The thermal cycles of PCR amplification were the following: initial denaturation step at 95 °C for 20 s, followed by 60 cycles of treatment at 95 °C for 3 s, and at 60 °C for 30 s using 7500 Fast Real-time PCR (Applied Biosystems, USA, 2015). Analysis of expression data was carried out with 7500 Fast Real-time PCR Software.

Western blot analysis. Protein expression was assayed in frozen brain hemisphere samples by immunoblotting as previously described (Portnychenko et al., 2023). Briefly, tissue lysates (100 μg of protein) were subjected to SDS-PAGE and transferred onto PVDF membrane (Sigma). Blots were blocked with PierceTM Clear Milk Blocking Buffer (37587, ThermoScientific), and incubated overnight with anti-IGF-1 (MA5-12247, Invitrogen; 1:500) and anti-GADPH (G8795, Sigma; 1:5000) antibodies. After washing, the blots were treated with peroxidase-conjugated anti-mouse IgG (31432, Pierce; 1:2500) and stained using 1-Step™ TMB-Blotting Substrate Solution (34018, Thermo Scientific). Densitometric values were evaluated with ImageJ (NIH Image, USA, 2022) and normalized to expression of house-keeping GADPH protein.

Statistical analysis. All data are presented as x ± SE (means ± standard error). Data were analyzed using GraphPad Prism version 8.1.0.325 for Windows (GraphPad Software, USA, No GPS-1461670-TEQH-6AC22). The Shapiro-Wilk test was used to evaluate the distribution normality of data. To detect the homogeneity of variance among groups Levine's test was used. Two-way ANOVA with Tukey's post hoc test was used to determine the significant differences between the groups. $P < 0.05$ was considered as statistically significant difference.

Results

Effect of chronic cerebral hypoperfusion on neuronal damage. The data on hyppocampal neuronal damage under chronic cerebral hypoperfusion are presented in Figure 1. In C57 mice, chronic cerebral hypoperfusion caused changes in the qualitative composition of neurons in the CA1 area of the hippocampi, the number of damaged neurons increased by almost two times (Fig. 2a, 2b, 3).

In ApoE(-/-) mice, neuronal damage caused by chronic cerebral hypoperfusion was more pronounced compared to C57 mice (Fig. 2, 3). At the same time, in ApoE(-/-) mice, the number of damaged neurons increased by two times compared to the group control (Fig. 2, 3), i.e., the changes of the indicators were similar to the changes in C57 mice ($P < 0.05$).

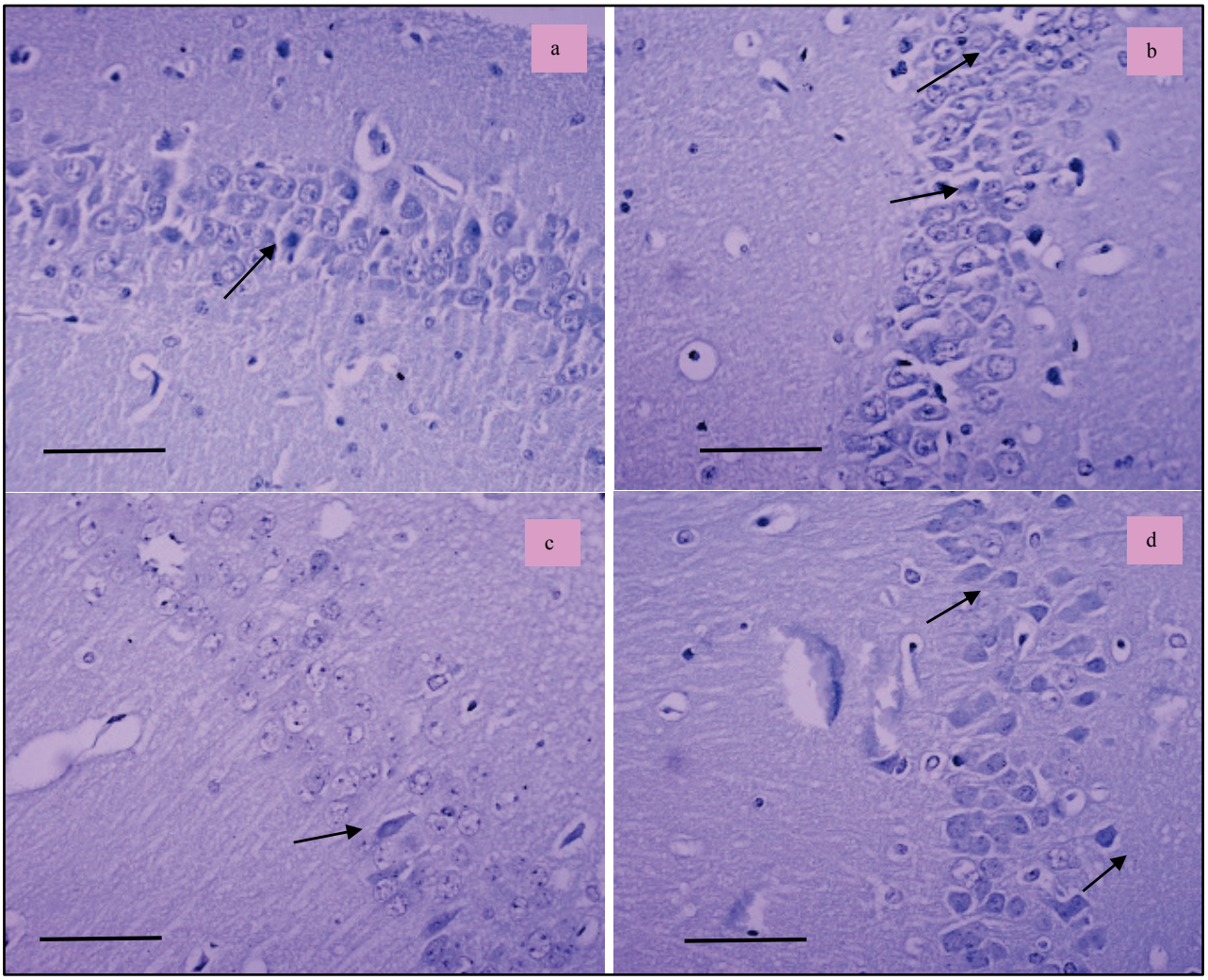

Fig. 2. Photomicrograph of hippocampus at the bregma by hematoxylin-eosin staining in 8 weeks after left common carotid artery occlusion in mice: *a*, *b*, *c*, *d* – photomicrographs of the C1 area of the hippocampi in control C57 mice (*a*), C57 mice with chronic cerebral hypoperfusion (*b*), control ApoE(-/-) mice (*c*), ApoE(-/-) mice with chronic cerebral hypoperfusion (*d*); n = 10 mice per group; scale bar indicated the distance of 100 μm; arrows indicated injured neurons of the C1 area of the hippocampi

Effect of chronic cerebral hypoperfusion on neuronal DNA damage in mice. The data about distribution of the classes of neuronal DNA comets under the chronic cerebral hypoperfusion are presented in Table 1. The DNA-comet assay showed that for the damaged neurons in the control group of C57 mice the total proportion of cells with nuclei of classes 0–1 (the lowest level of neuronal DNA damage) was 94.0 ± 1.3%, and the total proportion of cells with nuclei of 3–4 classes (the highest level of neuronal DNA damage) was 2.5 ± 0.9%.

The number of cells with DNA damage increased under the conditions of chronic cerebral hypoperfusion. Thus, the proportion of cells with

nuclei of classes 0–1 was 68.9 ± 1.3% (P < 0.05), and the proportion of cells with nuclei of classes 3–4 grew to 17.2 ± 0.9%, increasing almost 7 times (P < 0.05, Table 1). The level of cells of the second class (a borderline condition in which a cell can pass into groups with less or more damage) increased in chronic cerebral hypoperfusion to 13.9%, increasing by four times compared to the control (P < 0.05, Table 1).

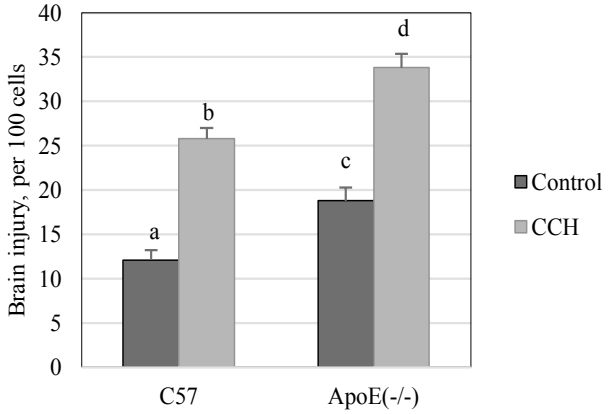

Fig. 3. Neuronal damage in the C1 area of the hippocampi of C57 or ApoE(-/-) mice, control and at 8 weeks with chronic cerebral hypoperfusion; each bar represents the number of injured neurons per 100 cells in the C1 area of the hippocampi of 10 mice per experimental group; CCH – chronic cerebral hypoperfusion; x ± SE; different letters indicate statistical samples that are significantly different from each other according to one-way ANOVA with Tukey's post hoc test results (P < 0.05)

It was shown, that in the control group of ApoE(-/-) mice, the proportion of cells with nuclei of classes 0–1 was 92.6 ± 1.3%, and the proportion of cells with nuclei of classes 3–4 was 2.0 ± 0.9%, there was no difference with the control group of C57 mice (Table 1).

The level of cells with DNA damage increased in ApoE(-/-) mice under chronic cerebral hypoperfusion. Thus, the proportion of cells with nuclei of classes 0–1 was 84.7 ± 1.3% (P < 0.05), and the proportion of cells with nuclei of classes 3–4 increased to 6.1 ± 0.9%, and mainly at in 3rd class cells; relative to the control, the proportion of cells of classes 3–4 increased by three times (P < 0.05, Table 1). The level of cells of the 2nd class increased in chronic cerebral hypoperfusion to 9.1%, rising compared to the control by two times (P < 0.05, Table 1), at the same time, this indicator was lower compared to the chronic cerebral hypoperfusion C57 group (P < 0.05, Table 1). In the absence of ApoE, the proportion of cells with nuclei of class 0 (without DNA damage) increased, and those of classes 3 and 4 (with DNA damage) decreased compared to the chronic cerebral hypoperfusion C57 group (P < 0.05, Table 1), i.e., DNA damage generally diminished.

Table 1

Distributions of DNA comets from nuclei of brain cells in the groups under the mild chronic cerebral hypoperfusion (%, x ± SE, n = 10 mice per group)

Animal groups	Classes of DNA comets				
	0	1	2	3	4
(1) Control sham operated C57 mice	86.0 ± 1.1[a]	8.0 ± 1.2[a]	3.5 ± 1.1[a]	2.0 ± 0.9[a]	0.5 ± 0.0[a]
(2) Chronic cerebral hypoperfusion of C57 mice	55.4 ± 1.7[b]	13.5 ± 1.1[b]	13.9 ± 1.1[b]	8.1 ± 0.9[b]	9.2 ± 0.8[b]
(3) Control sham operated *ApoE(-/-)* mice	84.4 ± 1.3[a]	8.2 ± 1.1[a]	5.4 ± 0.9[a]	1.3 ± 0.8[a]	0.8 ± 0.1[a]
(4) Chronic cerebral hypoperfusion of *ApoE(-/-)* mice	70.4 ± 1.8[c]	14.3 ± 1.2[b]	9.1 ± 0.8[c]	4.4 ± 0.5[c]	1.7 ± 0.1[c]

Notes: the effect of chronic cerebral hypoperfusion on the damage of DNA in nuclei of the left temporal lobe of brain of mice in the comet assay estimated 8 weeks after surgical modeling of chronic cerebral hypoperfusion; 0–4 – classes of DNA comets; different letters indicate statistical samples that are significantly different from each other according to one-way ANOVA with Tukey's post hoc test results (P < 0.05).

Thus, the obtained results indicate the pathogenetic role of ApoE in the neuronal DNA damage in moderate chronic cerebral hypoperfusion.

Changes of the expression of sirtuins and IGF-1 in the brain under chronic cerebral hypoperfusion. In the control, both types of sirtuins (SIRT1 and SIRT3) are expressed in the brain of C57 mice, while the expression of SIRT3 was twofold higher than that of SIRT1 (Fig. 2). Chronic cerebral hypoperfusion caused a dramatic fall in the expression of SIRT1 and SIRT3 in the brain by almost 10 times compared to the control group (P < 0.05, Fig. 2). The obtained data indicate the inhibitory effect of blood circulation changes on the expression of SIRT1 and SIRT3 genes in the brain.

We found a significant difference in the expression of SIRT1 and SIRT3 in the brain of ApoE(-/-) mice compared to C57 mice. Both types of sirtuins (SIRT1 and SIRT3) were expressed at low levels in the brain in the control (Fig. 4). The expression of SIRT1 in the control group of knockout ApoE mice was almost 30 times lower compared to C57 mice, and the expression of SIRT3 was reduced to a lesser extent – by 9 times (P < 0.05, Fig. 4).

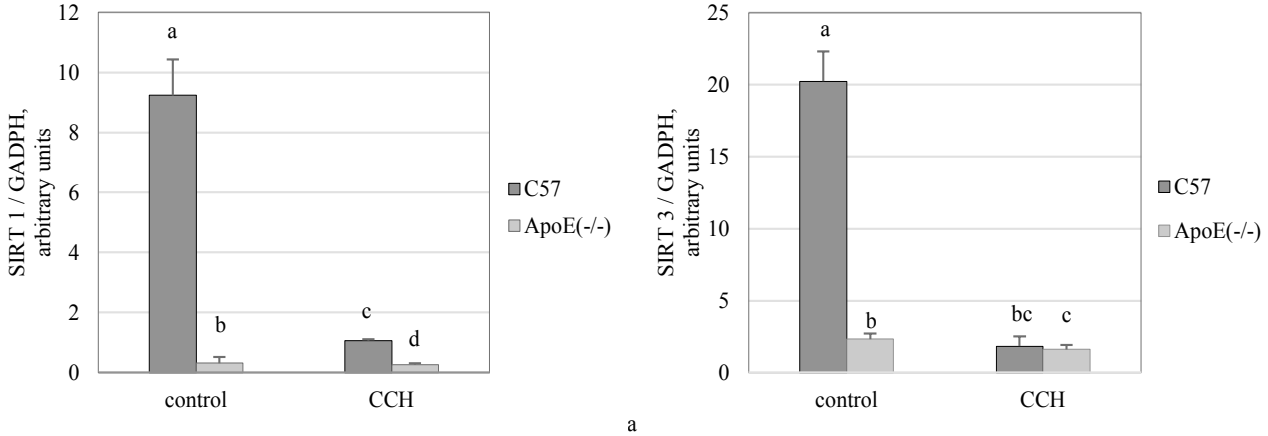

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Fig. 4. Relative values of mRNA expression of SIRTs (*a* – SIRT1, *b* – SIRT3) in the brain of C57 or ApoE(-/-) mice estimated 8 weeks after surgical modeling of chronic cerebral hypoperfusion: each bar represents the mRNA expression of SIRTs in the left temporal lobe of the brain expressed in arbitrary units normalized to GADPH expression level; 10 mice per experimental group; CCH – chronic cerebral hypoperfusion; x ± SE; different letters indicate statistical samples that are significantly different from each other according to one-way ANOVA with Tukey's post hoc test results (P < 0.05)

Similar to the changes in C57 mice, chronic cerebral hypoperfusion caused a fall in SIRT1 and SIRT3 expressions in the brain, but these changes were not as critical. Thus, the expression level of SIRT1 decreased by 15%, and the expression level of SIRT3 – by 31% compared to the control group (P < 0.05, Fig. 4).

The obtained data demonstrate the fall in expression of SIRT1 and SIRT3 in the brain in chronic cerebral hypoperfusion. However, in ApoE(-/-) mice with chronic cerebral hypoperfusion ,this reduction occurs to a lesser extent than in C57, with a greater effect on the expression level of SIRT3.

Regarding IGF-1 protein expression, we found another pattern of changes (Fig. 5). Unlike SIRTs, chronic cerebral hypoperfusion caused a tendency to elevation of IGF-1 protein expression in wild type C57 mice, but in ApoE-deficient mice the changes were opposite ($P < 0.05$). Herewith, in ApoE(-/-) mice with or without chronic cerebral hypoperfusion, IGF-1 expression was markedly lower than in C57 mice ($P < 0.05$, Fig. 5).

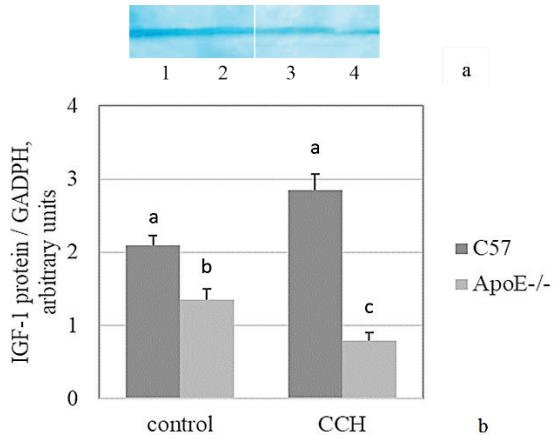

Fig. 5. IGF-1 protein expression in the brain of mice estimated 8 weeks after surgical modeling of chronic cerebral hypoperfusion: *a* – representative blot; *b* – relative densitometric values of IGF-1 protein expression in the left temporal lobe of the brain expressed in arbitrary units normalized to GADPH in brain tissue; CCH – chronic cerebral hypoperfusion; 1 – C57; 2 – C57+CCH; 3 – ApoE(-/-); 4 – ApoE(-/-) + CCH; 10 mice per experimental group; x ± SE; different letters indicate statistical samples that are significantly different from each other according to one-way ANOVA with Tukey's post hoc test results ($P < 0.05$)

Taken together, the data of SIRTs and IGF-1 expression demonstrated the failure of these cytoprotective pathways in ApoE-deficient mice in comparison to wild type animals in response to chronic cerebral hypoperfusion.

Discussion

Chronic ischemic brain damage is the subject of many experimental studies, but brain damage in moderate chronic cerebral hypoperfusion, which develops over time, is much less studied, despite its relevance for chronic cerebrovascular pathology of any genesis. The main mechanisms of brain damage in chronic hypoperfusion are associated with dysfunction of the vascular endothelium, damage to the blood-brain barrier, oxidative stress and neuroinflammation, a violation of the transmission of neurotransmitters and energy metabolism, which causes apoptosis, damage to the white matter of the brain, synapses, and the structure of neurons, and ultimately leads to to cognitive dysfunction (Choi et al., 2016; Du et al., 2017). To the best of our knowledge, we are the first to reveal the possible relationship between ApoE, cellular DNA damage, and the expression of SIRT1 and SIRT3 in the brain in moderate chronic cerebral hypoperfusion.

The present study revealed that the total number of damaged neurons in the C1 area of the hippocampi in ApoE(-/-) mice was greater in all groups compared to C57 mice. This means that chronic cerebral hypoperfusion causes a more pronounced decrease in cerebral blood flow in ApoE(-/-) mice, and as a result, leads to a greater influence on CA1 hippocampal neurons. These changes indicate a greater susceptibility of ApoE(-/-) mice to this type of damage than C57 mice. However, cell death in chronic cerebral hypoperfusion may be mediated by apoptosis, pyroptosis, which is known as inflammatory apoptosis, and which has partial characteristics of both cell apoptosis and necrosis (Bertheloot et al., 2021), and possibly also ferroptosis (Mao et al., 2022).

To the best of our knowledge, DNA damage has not been previously studied in the model of chronic cerebral hypoperfusion. The obtained results of the effect of chronic cerebral hypoperfusion on DNA damage in brain cells of ApoE(-/-) mice have shown the difference from the results in C57 mice. In ApoE(-/-) mice, the revealed chronic cerebral hypoperfusion effect on brain cell DNA was unexpected: class 3–4 cell DNA damage was lower than in C57, while class 4 DNA damage was almost completely absent in contrast to C57 mice, in which the level of DNA damage increased precisely due to the comet class 4. This may indicate the involvement of ApoE in the mechanisms of brain damage due to adverse effects on DNA. It was also unexpected that the level of class 0 DNA damage in ApoE(-/-) mice with chronic cerebral hypoperfusion was greater than in the appropriate group of C57 mice, and the reduced level of class 2 DNA damage compared to the corresponding group of C57 mice has been established.

In ApoE(-/-) mice, the described effects were accompanied by partial insensitivity of the expression of SIRT1 and SIRT3 genes to chronic cerebral hypoperfusion in comparison with C57 mice, in which the expression levels of SIRT1 and SIRT3 were sharply reduced compared to the control. Most likely, one of the mechanisms of the protective effect in ApoE(-/-) mice may be the preservation of the expression of SIRT1 and SIRT3, the epigenetic regulation of which is one of the neuroprotective mechanisms in pathological conditions due to the effects on cellular metabolism, DNA repair mechanisms, inflammation, apoptosis, and mitochondrial function (Verma et al., 2019; Shi et al., 2021). Our investigation of the changes in the expression of SIRT1 and SIRT3 in C57 mice with chronic cerebral hypoperfusion showed its sharp decrease. SIRT1 and SIRT3 are known to be NAD^+-dependent deacetylases, and therefore a decrease of NAD^+ affect their function. Intense oxidative stress during hypoxia leads to a decrease in NAD^+ levels, which suppresses SIRT1 and SIRT3 (Nogueiras et al., 2012; Zhao et al., 2021). We have shown that in ApoE(-/-) mice, chronic cerebral hypoperfusion had a moderate inhibitory effect on the expression of SIRT1 and SIRT3 genes in the brain, while these changes were less pronounced compared to C57 mice, and were more related to SIRT3 expression. Although the changes in SIRT3 expression were more pronounced, its expression remained at a sufficient level, and it is known that SIRT3 is mainly localized in mitochondria and regulates mitochondrial proteins through post-translational modification, which is involved in the regulation of energy homeostasis, mitochondrial function and lipid metabolism (Hirschey et al., 2010; Vassilopoulos et al., 2014; Wang et al., 2020; Zhao et al., 2021).

Preserved function of mitochondria in mice with chronic cerebral hypoperfusion, and the preserved endogenous expression of SIRT1 and SIRT3 can protect the brain from inflammatory reactions and the mitochondrial ROS for reduces oxidative stress (Zhang et al., 2020; Cao et al., 2022), in particular, due to the improvement of mitochondrial antioxidant protection and the activation of SOD2 (He et al., 2019). The reduction of DNA damage, which we also observed in our study, may indirectly indicate a reduction of oxidative stress (Li et al., 2018; Puente-Bedia et al., 2022). Therefore, the activation of antioxidant protection is important in conditions of impaired metabolism and hypoxia of brain, which can be supported by the activation of SIRT1 and SIRT3.

Considering the fact of mutual regulation of mitochondria and sirtuins (with SIRT1 and SIRT3, and the cross-relationship between them) (Chen et al., 2018; Wang et al., 2020; Zhao et al., 2021; Yang et al., 2023), it is quite possible that the low levels of expression of SIRT1 and SIRT3 registered by us in the brain of ApoE(-/-) mice at the time of the beginning of the experiment indicate an already existing reduction in the energy metabolism of mitochondria. Low initial levels of SIRT1 and SIRT3 expression may indicate a low content of NAD^+, which is necessary for the normal functioning of SIRTs, which also indicates insufficient production of NAD^+ by mitochondria, and indirectly confirms our assumption about their already existing dysfunction. Mitochondrial dysfunction, in turn, triggers the mechanism of cell death, which is potentiated when modeling chronic cerebral hypoperfusion. Thus, the described changes in the energy metabolism of the brain may be associated with oxidative stress before the modeling of chronic cerebral hypoperfusion, which can explain the high degree of cell damage in ApoE(-/-) mice. In turn, the presence of oxidative stress can activate the mechanisms of neuroprotection, which to some extent can explain the decrease in the severity of DNA damage in brain cells, which we observed in our study in chronic cerebral hypoperfusion in ApoE (-/-) mice when compared with C57 mice. At the same time, the results of histological examination and DNA-comet assay reflected the neuronal injury in different ways: the total number of cells that gradually

die from the moment of carotid artery occlusion increases with duration of cerebral hypoperfusion, which, taking into account the changes described above, indicates brain metabolic insufficiency (Somrednggan & Thong-Asa, 2018). In turn, DNA-comet assay reflects the degree of DNA damage in living cells at the time of the study.

In general, it is likely that the described effect of ApoE deficiency on DNA damage in chronic cerebral hypoperfusion is realized through the changes of SIRT1 and SIRT3 expression and its influence on mitochondrial metabolism (Hirschey et al., 2010; Keeney et al., 2015; Tang, 2016; Walker et al., 2018). In addition, the -219G/T and +113G/C polymorphisms of the ApoE promotor are known to be predictors of ischemic injury, suggesting that quantitative rather than qualitative changes in ApoE are associated with ischemia, which may observed in our study (Abboud et al., 2008).

The several studies of chronic cerebral hypoperfusion on experimental models have shown the beneficial role of IGF-1 in neuroprotective effects. In rats with right carotid artery ligation and hypoxic exposure, administration of exogenous IGF-1 reduced the manifestations of periventricular leukomalacia, increased the number of surviving mature neurons in the cerebral cortex (Kim et al., 2021). When reproducing bilateral carotid artery stenosis in mice, white matter injury, an increase in IGF-1 expression, and the involvement of the LMP7 immunoproteasome in the regulation of TGFβ/Smad-mediated neuroinflammation, oligodendrocyte remyelination and cognitive impairment were demonstrated (Chen et al., 2021). Antagonists or IGF-1R silencing reversed neuroprotection against white matter damage, apoptosis, and oligodendrocyte demyelination in chronic cerebral hypoperfusion in mice and its induced vascular dementia (Yousef et al., 2020), demonstrating a protective role of IGF-1-mediated signaling pathways in damage to white matter and the development of cognitive deficits. Our results demonstrate that the hippocampus tissue is also characterized by a clear tendency to increase in IGF-1 expression, which indicates that these protective mechanisms are triggered in other areas of the brain when there is a lack of blood supply.

Since IGF-1 has multiple effects, including survival, antiapoptotic effects, stimulation of cell growth, differentiation, and anabolic remodeling of metabolism, it is important to distinguish the cellular effect in chronic cerebral hypoperfusion. In our study, the increase in neuronal damage in C57 mice was accompanied by a tendency to increase in the expression of IGF-1, which indicates the intensification of protective mechanisms in the brain. SIRT1 gene silencing has been shown in cultured neuronal cells to induce activation of IGF-1-mediated signaling pathways that provide neuroprotection, including cell survival, in apoptotic insult (Sansone et al., 2013). Taken together, these results suggest that a decrease in SIRT1 expression in chronic cerebral hypoperfusion could up-regulate IGF-1 to promote neuronal survival in C57.

In ApoE-deficient mice, we observed low levels of SIRT1 and SIRT3, which only slightly decreased in chronic cerebral hypoperfusion. At the same time, this was not accompanied by the induction of IGF-1 in these mice, and the lack of blood flow was even associated with a decrease in the expression of this protein. Therefore, ApoE may be involved in the coordination of SIRT1/IGF-1 neuroprotective mechanisms, or affect the hypoxic sensitivity of neurons. It was shown that administration of exogenous IGF-1 in ApoE(-/-) mice reduced oxidative stress (Sukhanov et al., 2007), which links mitochondrial ways, including sirtuins, lipid homeostasis and cytoprotective effect of IGF-1 in common regulatory pathways. The lack of endothelial IGF-1 receptor (IGF1R) in such mice potentiated atherosclerotic vascular injury (Higashi et al., 2020), and macrophage-specific IGF-1 overexpression prevented such damage in ApoE deficiency (Snarski et al., 2022), which highlights the new role of IGF-1 in the pathogenesis of vascular diseases.

A combination of SIRT1 and IGF-1 reduction was also observed in experimental metabolic syndrome, with restoration of IGF-1 expression achieved by administration of the sirtuin activator resveratrol (Shamardl et al., 2023).

Recent studies have hypothesized that an age-related reduction in IGF-1 expression may increase vulnerability to Alzheimer's disease, the risk of which is associated with the presence of the ApoE ε4 allele (Galle et al., 2020). In patients with alternative genetic isoforms of ApoE, a reduction in the level of IGF-1 and downstream signaling molecules was observed (Keeney et al., 2015), which may indicate a key role of ApoE in maintaining IGF-1-mediated neuroprotection and supports the role of this mechanism in the prevention of Alzheimer's disease. Combined with our results, these data indicate that chronic cerebral hypoperfusion contributes to SIRT1 deficiency and IGF-1-mediated neuroprotection, which is significantly intensified in the background of ApoE deficiency and may be a factor in the accelerated development of atherosclerosis, vascular dementia and Alzheimer's disease.

Conclusions

Our results showed for the first time the involvement of ApoE in SIRT1, SIRT3 and IGF-1-mediated neuroprotection in moderate chronic cerebral hypoperfusion. Deficiency of ApoE in mice resulted in increased numbers of damaged neurons in the hippocampus 8 weeks after reproducing unilateral cerebral hypoperfusion, although neuronal DNA injury was limited. ApoE deficiency was also accompanied by a decrease in cytoprotective SIRT1, SIRT3 and IGF-1 in the tissue of the damaged brain hemisphere. In chronic cerebral hypoperfusion, sirtuin expression remained low, and IGF-1 expression continued to decrease in ApoE(−/−) mice compared with wild-type C57 mice. Thus, ApoE deficiency results in downregulation of SIRT1, SIRT3, and IGF-1 in the brain; this reduction of mitochondrial and neuronal protective pathways is exacerbated by chronic cerebral hypoperfusion and may be involved in mechanisms of neuronal damage.

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