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V.L. Holubiev,
M.H. Oberemok,
V.A. Tkachenko,
Yu.V. Kharchenko,
O.O. Bondarenko,
A.E. Lievykh,
V.I. Zhyliuk *

Dnipro State Medical University
Volodymyra Vernadskoho str., 9, Dnipro, 49044, Ukraine
Дніпровський державний медичний університет
вул. Володимира Вернадського, 9, Дніпро, 49044, Україна
**e-mail: vzhyliuk@gmail.com*

STUDY OF THE NEUROPROTECTIVE PROPERTIES OF METFORMIN IN RATS WITH TYPE 2 DIABETES MELLITUS AND BRAIN INJURY INDUCED BY INTRACEREBRAL HEMORRHAGE

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Ключові слова: *цукровий діабет 2 типу, внутрішньомозкова гематома, метформін, нейропротекція, оксидативний стрес*

Abstract. Study of the neuroprotective properties of metformin in rats with type 2 diabetes mellitus and brain injury induced by intracerebral hemorrhage. Holubiev V.L., Oberemok M.H., Tkachenko V.A., Kharchenko Yu.V., Bondarenko O.O., Lievykh A.E., Zhyliuk V.I. *The aim of this study was to study the effect of metformin (Met) on the formation of the conditional passive avoidance skills, markers of neurogenesis and oxidative stress in the brain of rats with acute intracerebral hemorrhage (ICH) in the setting of streptozotocin-nicotinamide-induced diabetes. Type 2 diabetes mellitus (T2DM) was induced in rats via the intraperitoneal injection of streptozotocin (STZ) and nicotinamide*

(NA), ICH – by microinjection of bacterial collagenase into the striatum. Rats were randomly divided into four groups: 1 – intact animals (n=8), 2 – T2DM (n=9); 3 – T2DM+ICH (n=7); 4 – T2DM+ICH+Met (n=7). The passive avoidance test was used to evaluate behavioural activity. Advanced oxidation protein products (AOPP) and lactate were measured by spectrophotometry, advanced glycation end products (AGEs) by quantitative fluorescence, level of 8-hydroxy-2-deoxyguanosine (8-OHdG) was assessed by enzyme-linked immunosorbent assay (ELISA). Histopathological examination was performed using general histological staining techniques and immunohistochemical methods for assessment of expression of endothelial NO-synthase (eNOS), Growth Associated Protein 43 (GAP43), Hypoxia-inducible factor 1-alpha (HIF-1α), neural cadherine (N-cadherine) and vascular endothelial cadherine (VE-cadherine). In this study, metformin had nootropic (anti-amnestic) activity and decreased oxidative stress markers (AGEs, AOPPs and 8-OHdG) levels by 29.1% ($p<0.001$), 24.9% ($p<0.015$) and 29.3% ($p<0.05$) respectively, which indicates its positive impact on the course of free radical oxidation reactions intensified by both diabetes and intracerebral hemorrhage. The study provides additional information on neuroprotective properties of metformin and the emphasizes possibility of using metformin in diabetic patients at risk of hemorrhagic stroke. Considering the increase in VE-cadherin expression by the drug, it is possible to predict its positive effect on the function of blood-brain barrier. This study may serve as a reference for the feasibility of studying the clinical efficacy of metformin under these conditions.

Реферат. Вивчення нейропротекторних властивостей метформіну в щурів із цукровим діабетом 2 типу та ураженням головного мозку, спричиненим внутрішньомозковим крововиливом. Голубєв В.Л., Оберемок М.Г., Ткаченко В.А., Харченко Ю.В., Бондаренко О.О., Лєвих А.Е., Жилюк В.І. *Мета – вивчення впливу метформіну (Met) на формування умовної навички пасивного уникнення, стан маркерів нейрогенезу та оксидативного стресу в мозку щурів із гострим внутрішньомозковим крововиливом (ВМК) на тлі стрептозотоцин-нікотинамід-індукованого діабету. Цукровий діабет 2 типу (ЦД2) індукували в щурів внутрішньоочеревинним уведенням стрептозотоцину (СТЗ) та нікотинаміду (НА), ВМК – мікроін'єкцією бактеріальної колагенази в смугасте тіло. Щурів було розподілено на чотири групи: 1 – інтактні тварини (n=8), 2 – ЦД2 (n=9); 3 – ЦД2+ВМК (n=7); 4 – ЦД2+ВМК+Met (n=7). Для оцінювання поведінкової активності використовувався тест пасивного уникнення. Кінцеві продукти окиснення білків (AOPP) вимірювали за допомогою спектрофотометрії, кінцеві продукти глікування (AGEs) – за допомогою кількісної флуоресценції, рівень 8-гідрокси-2-дезоксигуанозину (8-OHdG) оцінювали за допомогою імуноферментного аналізу (ELISA). Патогістологічне дослідження проводили з використанням загальних методів гістологічного фарбування та імуногістохімічних методів з метою оцінювання експресії ендотеліальної NO-синтази (eNOS), пов'язаної зі зростанням білка 43 (GAP43), індукованого гіпоксією фактора-1α (HIF-1α), нейронального кадгерину (N-кадгерину) та кадгерину судинного ендотелію (VE-кадгерину). У цьому дослідженні метформін мав як ноотропну (антиамнестичну) активність, так і знижував рівень маркерів окисного стресу (AGEs, AOPPs і 8-OHdG) на 29,1% ($p<0,001$), 24,9% ($p<0,015$) і 29,3% ($p<0,05$) відповідно, що свідчить про його позитивний вплив на перебіг реакцій вільнорадикального окиснення, що посилюються як при цукровому діабеті, так і при внутрішньомозковому крововиливі. Дослідження надає додаткову інформацію щодо наявності нейропротекторних властивостей та підкреслює можливість застосування метформіну в пацієнтів з ризиком розвитку геморагічного інсульту на тлі діабету. Ураховуючи підвищення експресії VE-кадгерину за умов використання препарату, можна прогнозувати його позитивний вплив на функцію гематоенцефалічного бар'єру. Це дослідження може слугувати зразком для доцільності вивчення клінічної ефективності метформіну за цих умов.*

According to research, in 2019, there were 12.2 million cases of stroke and 6.55 million deaths worldwide, making it the second leading cause of death in the world. (GBD, 2019; WHO, 2022) [1]. Spontaneous intracerebral hemorrhage (ICH) accounts for 10–20% of all strokes and affects more than one million people every year worldwide, and it is the stroke subtype associated with the highest rates of mortality and residual disability. The extravasated blood accumulates and compresses the surrounding brain tissues forming hematoma, the components (especially the blood-derived leukocytes and neutrophils) of which infiltrate the brain parenchyma and break the blood-brain barrier (BBB) leading to inflammatory responses and cerebral edema as well as nerve damage. So far, clinical trials have mainly targeted primary cerebral injury and have substantially failed to improve clinical outcomes. The understanding of the pathophysiology of early and delayed injury after ICH is, hence, of paramount importance to identify potential targets of intervention and develop effective therapeutic strategies [2, 3, 4].

Diabetes mellitus (DM) is one of the most common endocrine pathologies with features of a non-communicable epidemic. Moreover, type 2 diabetes mellitus (T2DM) accounts for almost 90% of the approximately 537 million reported cases of chronic hyperglycemia in patients aged 20-79 years worldwide [1]. It should be noted that the number of patients is steadily increasing, and the trend among children and young people under the age of 40 is particularly alarming [5]. According to epidemiological studies, diabetes mellitus is recognized as an independent and modifiable risk factor for both ischemic and hemorrhagic stroke [6].

Cognitive impairment, including learning and memory disorders, is commonly associated with ICH. However, even today, information on post-ICH cognitive impairment is poorly understood and shows considerable variability, as a certain cohort of patients shows positive dynamics of recovery from acute cognitive decline, while others show persistent changes or further deterioration of cognitive function, which may pose a threat of dementia [3]. Recent studies have shown that near half of the patients had significant cognitive impairment at the acute stage after ICH. Moreover, cognitive impairment was more frequent in the elderly, those with large baseline hematoma volume, and more severe initial neurological deficit. Having a lower MoCA score during the acute phase of ICH was independently associated with an increased risk of delayed cognitive impairment [7]. Experimental studies have also shown that type 1 diabetes mellitus (T1DM) is associated with a significant increase in hematoma volume and deficits in motor, sensorimotor and cognitive behaviour in mice after reproduced ICH. The levels of neuroinflammation, oxidative/nitrosative stress and glial cell activation were increased in diabetic mice after ICH [8].

In our opinion, the impact of comorbidities, in particular DM, on the dynamics of cognitive impairment in patients with ICH as well as the possible preventive efficacy of drugs for DM treatment deserves special attention. DM and ICH patients experience impaired abilities in multiple cognitive domains by relatively comparable mechanisms, which could get exacerbated in the setting of comorbidities [4].

To date, no direct link has been established between effective glucose control and a reduced risk of acute cerebrovascular accident [1]. But careful identification and treatment of concomitant vascular risk factors, especially hypertension and dyslipidemia, along with lifestyle changes, remain the main approaches to preventing cerebrovascular disorders, including stroke, in this population [9, 10]. Although numerous studies have not proven that strict hyperglycemia control reduces the incidence of stroke in people with diabetes, the prevention of any chronic complication of diabetes is based on the correction of carbohydrate metabolism. Metformin has proven cardiovascular benefits in patients with T2DM and is the only antihyperglycemic drug that reduces mortality from cardiovascular risks [10, 11]. Another positive fact is that metformin does not cause hypoglycemia, that is why it is also called an "antihyperglycemic agent" [12, 13]. Metformin can cross the blood-brain barrier and have specific effects on the central nervous system, activate the specific neurons and neuroglia to exert various neurophysiological actions [14, 15]. Metformin can exert potential neuroprotective, neurotrophic, and neurogenesis-stimulated actions; besides, metformin also exerts antiinflammatory effect by inhibiting microglial activation and regulating microglial polarization. These findings indicate there might be extensive pharmacological efficacy and therapeutic insights of metformin in neurological diseases' clinical application [15].

Metformin has been reported in clinical and animal studies to exert neuroprotective effects in many neurological disorders [16, 17, 18, 19, 20, 21], but further validation in different animal models and exploration of its underlying mechanisms are needed. Metformin can improve synaptic transmission, affecting neural circuits and regulating excitation/inhibition balance in neural networks. Further studies are needed to reveal the exact mechanism of its neuroprotective effects [13].

As was demonstrated by Amer et al., histological changes in a brain tissue of the rats with experimental DM included disorganization in all regions and marked ischemic neuronal injury in the granular layer of the Cornu Ammonis characterized by nuclear pyknosis, while enlarged neurons and excess glial cells were seen in the molecular layer. Perineuronal edema and malacic foci associated with aggregation of microglial cells were found in the cerebral cortex of the DM rats. In addition, it was reported that hippocampus of diabetic rats showed significant increase in caspase-3 expression compared to that of control rats [22].

The experiments with neuroprotective effects of metformin under DM, ischemic or traumatic brain injury (TBI) revealed its positive effects on injured brain tissue. In report by Tao et al. metformin significantly ameliorated neurological deficit, cerebral edema and neuronal apoptosis in rats following TBI. However, it was revealed that metformin administration inhibited microglial activation [23]. Furthermore, Karimpour et al. reported significant decrease of leukocyte infiltration, myeloperoxidase activity and malondialdehyde level in samples from the rats with metformin pretreatment before the cerebral ischemia. In summary, metformin's both pre- and post-treatment reduced infarct size compared with the intact animals [24].

Upregulation of HIF1α was observed in some works in ipsilateral brain cortex after injury. For example, Li et al. reported the striking changes of HIF-1α expression in neurons, but not in astrocytes, microglia, or oligodendrocytes. Consequently, application of TUNEL indicated that HIF-1α was associated with neuronal apoptosis following TBI [25].

In several reports focused on TBI study, the alterations in pre-synaptic GAP43 adaptor protein were found. Correspondingly, Hulsebosch et al. demonstrated increased GAP43 expression in agranular, forelimb and hindlimb cortices as well as in hippocampus, areas

that are associated with impairments in locomotor and/or memory associated tasks [26].

As it is well known, the permeability of the endothelial barrier is determined by the integrity of the tight junctions (TJs) and adherens junctions (AJs) between endothelial cells under physiologic conditions. Among junction proteins, vascular endothelial (VE)-cadherin is a key component of the AJs and claudin-5 is important in TJs. Phosphorylation of the tyrosines in VE-cadherin, especially Tyr731, is associated with weak junctions and impaired barrier function *in vitro* and *in vivo*, whereas a decrease in claudin-5 is linked to breakdown of the BBB both *in vitro* and *in vivo*. Impairment or modulation of these junction proteins after TBI could thus lead to BBB breakdown and the development of brain edema [27]. Eventually, other endothelial markers could be also useful as indicators of endothelial dysfunction. In the study of Cobbs et al., an induction of the endothelial nitric oxide synthase (eNOS) isoform was found in microvessels surrounding the cortical contusion by 24 h post-injury in the rats after moderate TBI. It was concluded that such altered microvascular state may contribute to barrier breakdown and hyperemia which characterize traumatic brain injury [28].

The aim of this work was to study the effect of metformin on the formation of conditional skills, markers of neurogenesis and oxidative stress in the brain of rats with acute intracerebral hemorrhage in the setting of streptozotocin-nicotinamide-induced diabetes.

MATERIALS AND METHODS OF RESEARCH

Drugs. Antidiabetic drug Metformin hydrochloride (Siofor® tablets, Berlin Chemie, Germany, Met, 250 mg/kg/day) was used in the current study.

Experimental animals and groups. The study was carried out on 31 male Wistar rats weighing 200-250 g. The study design was approved by the Biomedical Ethics Committee of the Dnipro State Medical University (protocol No. 8 dated 17.12.2019). Experiments were performed in compliance with the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. The animals were kept in standard vivarium conditions (air temperature 22-24°C, relative humidity 50%, 12-hour day/night cycle) of the Dnipro State Medical University (Ukraine) with free access to water and food. According to the result of the oral glucose tolerance test, all rats with a similar degree of glycemia were randomized into four groups: group 1 – intact control/naive [saline, 5 ml/kg/day] (n=8); group 2 – pathology control 1 (T2DM+saline, n=9); group 3 – pathology control 2 (T2DM+ICH+saline, n=7); group 4 – animals that received metformin (T2DM+ICH+Met, n=7).

Metformin tablets were suspended in water with the addition of Polysorbate-80. The studied drugs were administered intragastrically for 20 days, starting from the 50th day after the induction of diabetes. Rats of the intact control and pathology control groups 1 and 2 received saline intragastrically (5 ml/kg/day).

Type 2 diabetes mellitus was simulated with a single intraperitoneal injection of nicotinamide (NA, 230 mg/kg) and streptozotocin (STZ, 65 mg/kg) in citrate buffer (pH=4.5, 0.1 M) to overnight fasted rats [29, 30]. Blood glucose level was measured 72 hours after NA/STZ injection. Animals with values less than 8.3 mmol/L were excluded from the study. The results that confirm the presence of diabetes mellitus in these experimental groups of animals are described in previous publications [31].

Intracerebral hemorrhage (ICH) in rats was induced by microinjection of 1 μL of bacterial collagenase 0.2 IU/μL (Type IV-S) [32]. On the 60th day after NA/STZ injection, a Hamilton microsyringe was inserted into the striatum of anesthetized rats by the following stereotactic coordinates: 0.2 mm anterior, 2.8-3,0 mm lateral, and 5.5 mm ventral to the bregma.

Fig. 1. Schematic representation of the method for inducing ICH (a). Representative images of coronal section (straight-cut at injection site) in vehicle-treated T2DM+ICH group and metformin-treated T2DM+ICH group at 24 h and on the 10th day after ICH induction (b)

Behavioral analysis. The *passive avoidance test* represents a fast and a reliable method that can be utilized to evaluate the impact of pharmacological agents on emotional learning. Rats have an innate preference for dark and enclosed environment. Testing was divided into 2 phases: acquisition (training) phase and test phase. During the first training phase on the 69th day of the experiment, animals were placed in the light compartment and allowed them a maximum of 5 min to cross the gate and enter into the dark compartment (latency time 1). After entering the dark box, the guillotine door was closed and the animal was given 5 shocks with a current of 1 mA through the electrified floor with an interval of 5 s and a duration of each pulse of 1 s. Rats were removed from the dark compartment 20 s later to their home cages until testing 24 h later. On the second day (the testing phase, on the 70th day of the experiment), the rats were placed into the starting light compartment and their latencies to enter the dark compartment with all four paws (maximum latency of 180 s) were measured (latency time 2). Shock was not administered on the test. Immediately after the training or testing of every animal, the chamber was wiped with a 10% ethanol solution [33].

To evaluate the effectiveness of reproducing the skills of *passive avoidance test*, the antiamnestic activity coefficient of a drug (Aa) was calculated according to the formula [34]:

$$\text{Aa} = \frac{\Delta Latency\ (group\ 4) - \Delta Latency\ (group\ 3)}{\Delta Latency\ (group\ 1) - \Delta Latency\ (group\ 3)} \times 100\%,$$

where ΔLatency = Latency time 2 $_{\text{(testing phase)}}$ – Latency time 1 $_{\text{(training phase)}}$

Preparation of brain. On the 70th day of the study the rats were anesthesized, sacrificed, and their brains were quickly removed with following perfusion on ice-cold PBS, and then washed, dried, and dissected to separate the cortex. Specimens from every brain were separated, weighed, and stored at -45°C for later analyses. Finally, brain samples from ICH-affected hemisphere in each group were collected and kept in neutral buffered formalin 10% for histopathological examination.

The first half of cortical tissues was homogenized in 5% w/v 20 mM phosphate buffer (pH=7.4) and centrifuged at 10000 g for 10 min at 4°C. Obtained supernatants were used to measure the levels of AGEs, AOPPs and lactate. Another half of cortical tissues was homogenized in a buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride and 0.01% merthiolate in a ratio of 1:10 (tissue:buffer). The homogenate was centrifuged at 10000 g for 10 min at 4°C and the supernatant was collected for measurement of 8-OHdG by ELISA.

Biochemical analysis. The level of *advanced glycated end products* (AGEs) was measured by fluorescence method [35], using Hoefer DQ 2000 Fluorometer (USA) with fixed wavelengths (excitation/emission = 365 nm/460 nm). The results were expressed as arbitrary units (AU) per mg of protein.

The level of *advanced oxidation protein products* (AOPPs) was determined by measuring the absorbance at 340 nm, according to the modified method described by Witko-Sarsat [36, 37]. Briefly, the reactant mixture used for the AOPPs assay contained 30 µl of 50% acetic acid and 15 µl of 1.16 M potassium iodide. Then, 1.9 mL of this mixture was added to 100 µL of the test sample (plasma, HSA, modified has, or PBS-blank), and the absorbance was recorded immediately. The results were expressed as chloramine T equivalents per mg of protein (µM/mg of protein).

Lactate levels were measured spectrophotometrically using a standard test kit "Lactate, PZ Cormay S.A., Poland" according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA). The assay of brain 8-OHdG (Rat 8-hydroxy-2-deoxyguanosine (8-OHdG) ELISA Kit, MyBioSourse, Inc., San Diego, CA, USA) was performed according the manufacturer's protocols.

Histopathological Examination. The material was fixed in a 10% neutral buffered formalin solution (pH 7.4) for 24 hours at room temperature [38]. Samples' infiltration was performed using a Microm STP-120 histoprocessor (Thermo Fisher Scientific, Germany). Samples were dehydrated in ascending concentrations of isopropanol (70%, 80%, 95%, and in three changes of 100% for 90 min per shift), cleared in xylene, and infiltrated in two changes of molten paraffin for 120 min per change. After that, the paraffin-embedded tissues were embedded into paraffin blocks using a HistoStar embedding station (Thermo Fisher Scientific, USA). Serial sections of maximum 4 µm thickness were obtained using a Thermo HM 355S microtome (Thermo Fisher Scientific, Germany). Sections of each tissue sample were used for general histological staining of tissues using hematoxylin and eosin [39], luxol fast blue for myelin with cresyl violet counterstaining, silver impregnation

according to the Bilshovsky-Kahal method [40] and for further immunohistochemical staining.

Microscopy of histological sections was performed using an Axio Imager 2 microscope (Zeiss, Germany) at ×100, ×200, and ×400 magnifications.

Immunohistochemical methods. Sections of 4 μm thickness were mounted on Superfrost adhesive slides (Thermo, Germany), then deparaffinised with xylene and rehydrated. The activity of endogenous peroxidase was blocked with a 3% hydrogen peroxide solution in 70% methanol for 20 minutes at room temperature. Thereafter the sections were washed in three changes of phosphate buffer saline (PBS) followed by heat-induced antigen retrieval (HIAR) by heating in a water bath in citrate buffer with pH 6.0 (20 minutes after reaching a temperature of 98°C) with a symmetrical arrangement of the slides in a jar with the addition of 2 ml of Triton-X100 detergent (Sigma, Germany) per 200 ml of buffer [41].

After washing in three changes of PBS, the slides were placed in a humid chamber and incubated with a 1% blocking serum (normal goat serum) for 20 minutes. For the current study the following primary antibodies were used: anti-endothelial NO synthase, eNOS (mouse monoclonal, clone M221, dilution 1:400, Abcam, United Kingdom); anti-GAP43 (rabbit polyclonal antibody, 1:1000, Thermo Fisher Scientific, USA); anti-HIF-1α (mouse monoclonal, clone ESEE122, 1:5000, Thermo Fisher Scientific, USA); anti-N-cadherin (rabbit polyclonal antibody, 1:1000, Thermo Fisher Scientific, USA); anti-VE-cadherin (rabbit polyclonal antibody, 1:500, Abcam, United Kingdom).

Sections were incubated with primary antibodies in a humid chamber at 4°C overnight. Visualisation was performed using the Master Polymer Plus Detection System reagent kit (Master Diagnostica, Spain) and was completed by the reaction of DAB chromogen with hydrogen peroxide in the presence of horseradish peroxidase to form a brown colour at the sites of binding of the primary antibody [41]. Thereafter, sections were additionally counterstained with Gill's haematoxylin for 30 seconds, dehydrated in ascending concentrations of alcohol, cleared in xylene, and mounted with coverslips.

Statistical analysis. The data obtained in the study were processed by statistical methods using the licensed program GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA, GPS-2169913-THSG-DF1FF). Before applying the statistical criteria, the hypothesis of the normal law of distribution of random variables was tested (according to the Shapiro-Wilk test). All results are expressed as mean±S.D. The reliability of intergroup differences was established using the parametric Student's t-test or one-way analysis of variance ANOVA and the Mann-Whitney or Kruskal-Wallis test for abnormal distribution [42]. The level of statistical significance of the obtained results was $p<0.05$.

RESULTS AND DISCUSSION

The analysis of latency time shows that the induction of T2DM (group 2) led to a deterioration in learning process, as evidenced by 33.2% lower values of this indicator ($p<0.05$) compared to the group 1 (intact animals). Modelling of ICH (group 3) contributed to a further decrease in this indicator, and its values were 52.5% ($p<0.05$) lower than those of group 1 (Fig. 2a). Metformin, although it has some anti-amnestic activity, did not change significantly the latency time as compared to the group 3 (Fig. 2a; Fig. 2b).

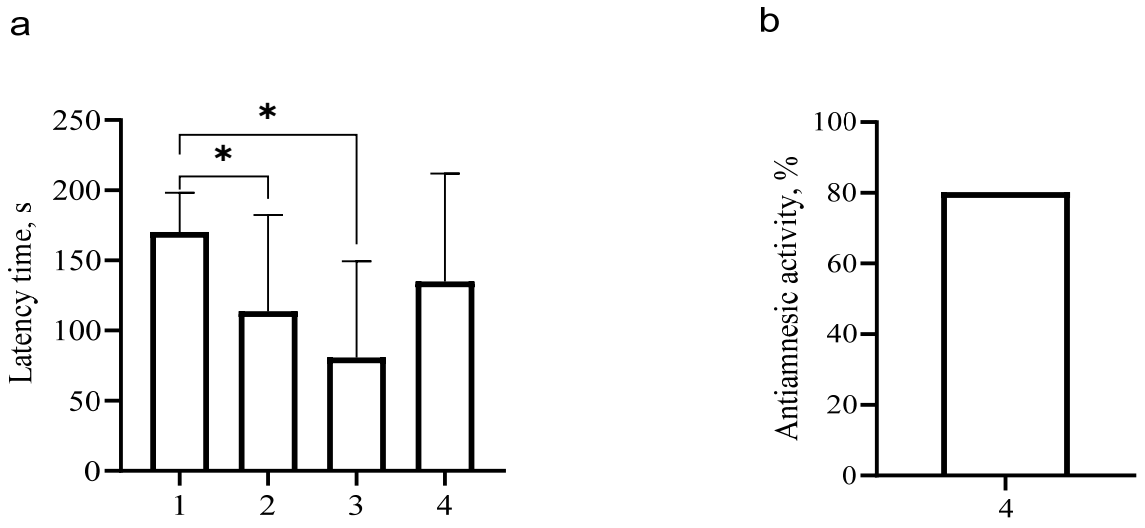

Fig. 2. Latency time (a) and antiamnesic activity in T2DM+ICH group treated with metformin (b). Data are presented as mean±S.D

Acute ICH in rats with T2DM led to a statistically significant rise in the AGEs level in brain homogenates by 18.5% ($p<0.05$) in comparison with the group 1 but not with the group 2 **(**Fig. 3a). At the same time, there was an increase in the content of the AOPPs by 53.1% ($p<0.001$) in comparison with the group 1 and by 27.2% ($p<0.01$) in comparison with the group 2 (Fig. 3b). The content of 8-OHdG was also higher in both group 2 by 48.6% ($p<0.05$) and group 3 by 90.9% ($p<0.001$) as compared to intact animals (Fig. 3c). Moreover, statistical significance was observed between groups 2 and 3 ($p<0.05$) (Fig. 3c). In addition, rats with T2DM and acute ICH were characterized by a statistically significant increase in lactate levels both in comparison with the group 1 by 51% ($p<0.01$) and with the group 2 by 31% ($p<0.05$) (Fig. 3d).

It should be noted that metformin reduced AGEs levels in brain cortex homogenates of rats with T2DM and acute ICH by 29.1% ($p<0.001$) (Fig. 3a). Metformin did not affect the lactate content but reduced the AOPPs and 8-OHdG contents by 24.9% ($p<0.015$) and 29.3% respectively (Fig. 3b, Fig. 3c, Fig. 3d).

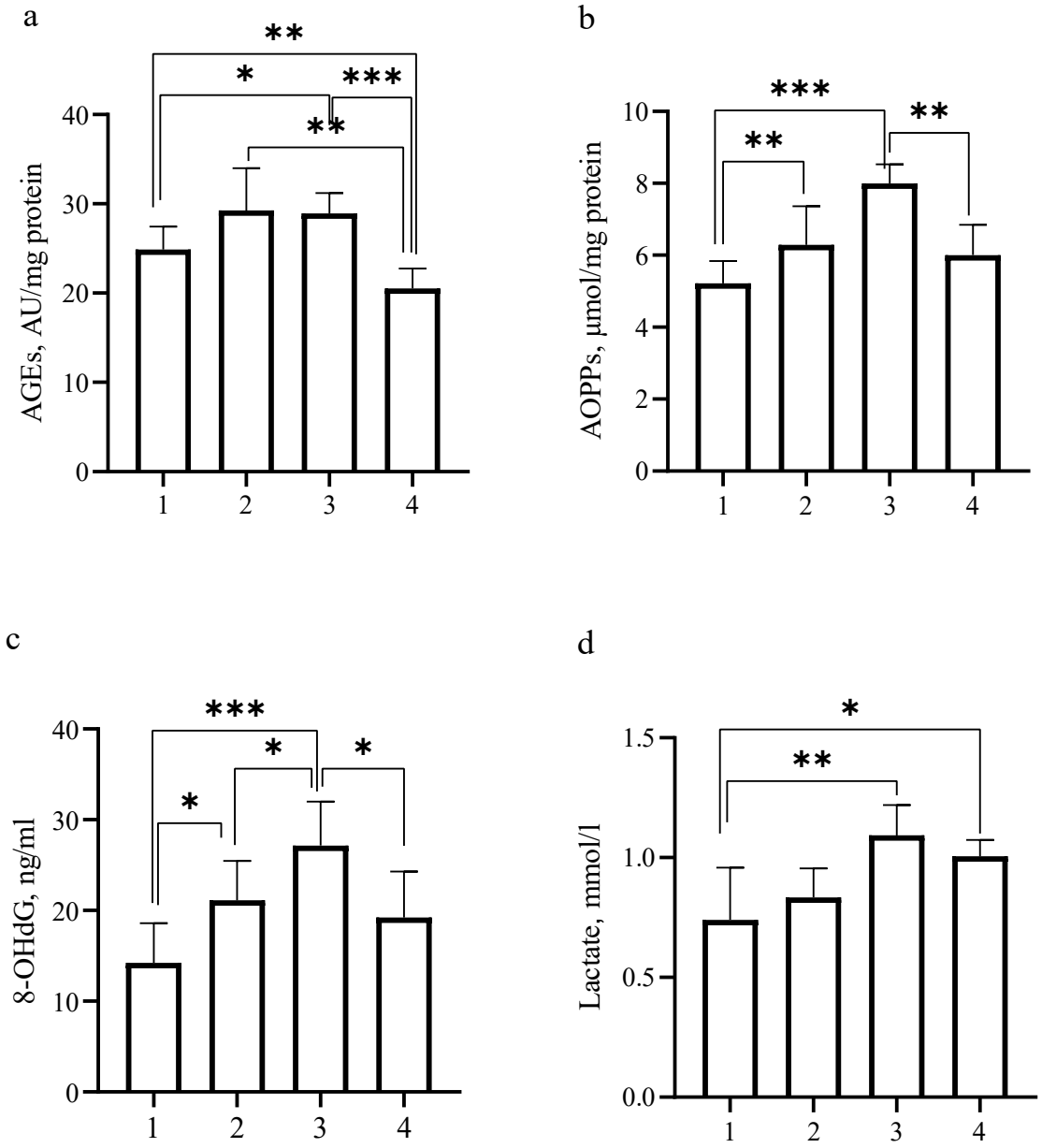

Fig. 3. Level of AGEs (a), AOPPs (b), 8-OHdG (c) and Lactate (d) in the cortical homogenates. Data are presented as mean±S.D

The histology of the brain tissue of intact animals corresponded to the normal histoarchitecture in rats, however in the negative control group a slight edema and satelliteosis of individual neurons were found. Histological examination of the material from both positive control and experimental group animals revealed stereotypical changes in the white matter of the cerebral hemisphere: a destroyed area of nervous tissue with a microglial reaction, edema, areas of haemosiderin deposition and single red blood cells infiltrating the neuropil. Some observed neurons from the areas adjacent to the lesion also revealed satellitosis. There were also clusters of foamy macrophages and haemosiderophages at the border between healthy tissue and the area of injury (Fig. 4, first row). Luxol fast blue staining demonstrated an abrupt loss of myelin distribution integrity at the border of damaged and intact brain tissue just prior to area of macrophage infiltration (Fig. 4, second row). The similar pattern was observed after silver impregnation of the brain tissue in the lesion area, the interruption of argyrophilic fibres at the border of damaged and intact tissue.

Immunohistochemical staining of HIF-1α demonstrated the accumulation of this factor mainly in the neuropil, cytoplasm of glia and neuronal cells, and in endothelial cells. The intensity of neuropil staining for this marker in the intact group was considered as the lowest. The most intense and uniform staining for HIF-1α was demonstrated in both positive and negative control groups, with the most intensely stained areas up to 20 μm thick being localized along the brain tissue defect at the site of injury. Individual glial cells in this area also demonstrated nuclear localisation of this marker. The same nuclear expression of HIF-1α was observed in individual neurons. The expression of HIF-1α in the samples of the study groups did not differ between the groups, but was characterised by a lower intensity than in the control groups, although higher than in the intact groups, with a characteristic intensification of staining in the tissue adjacent to the defect. Also, individual cells (both neurons and gliocytes) with nuclear-positive staining were observed (Fig. 4, sixth row).

Microscopic examination of eNOS immunolabeling demonstrated the presence of immunopositive endothelial cells in all control and test samples, but it should be noted that the staining for this marker was more pronounced in the endothelial cells of cerebral vessels in the control groups. In addition, in the groups with simulated haemorrhage, eNOS staining was observed in the nuclei of some ipsilateral cortical neurons to the injury side (Fig.4, third row).

Labeling of GAP43 was only remarkable in terms of its increased intensity around the injury in investigated groups. Noticeable, that this marker revealed the expression only in neuropil (Fig.4, seventh row).

Immunoexpression of N-cadherin was observed in neuronal bodies and their processes and almost did not differ between the study and control groups either quantitatively or qualitatively, although it was slightly lower in the intact group. In general, the staining of neuropil and neurons in the areas around the brain injury was significantly more intense than in other areas (Fig.4, fifth row).

The most prominent difference in the levels of immunohistochemical staining was observed in the expression of VE-cadherin. In the group of intact animals, the expression of VE-cadherin was focal, mainly around small vessels. In the control groups, the staining patterns for this marker concerned both the vascular component and the bodies of individual neurons likewise the glial cells. In contrast, in the groups treated with metformin, the most intense neuropil staining was revealed, and it was observed in the hemisphere ipsilateral to the lesion. There was also a remarkable staining of neuronal bodies for this marker (Fig.4, fourth row).

According to the data obtained, the severity of cognitive deficits in rats with diabetes mellitus in the acute phase of intracerebral hematoma was higher, but we did not find any statistical difference between the control groups, although many experimental studies have demonstrated the negative impact of DM on memory and learning domains after ischemic stroke [4]. Probably, the differences in the results obtained in humans and animals can be largely related to the size and location of the hematoma, as well as the severity of cerebral edema, which requires further research. This may be especially true for the involvement of the hippocampus in the pathological process, as it is known that both types of diabetes are an independent factor in the development of cognitive impairment, which is probably associated with intense hippocampal neuronal apoptosis and impaired synaptic plasticity [4]. It has been experimentally confirmed that animals which exhibit longer latencies and avoidance of the dark compartment are more likely to have formed hippocampus-dependent contextual memory [43]. Moreover, in preclinical ICH models, it was found that hyperglycemia initiates the hematoma expansion by activating the plasma kallikrein, which inhibits the platelet aggregation [44].

High levels of AOPPs, AGEs, and 8-OHdG indicate oxidative stress in the brain tissue in T2DM and confirm its importance in the pathogenesis of cognitive dysfunction in this pathology [45]. Furthermore, we found that ICH significantly increases the severity of oxidative stress in the brain tissue of diabetic rats,

which can have a negative effect on both neurons and endothelial cells of the brain microvasculature.

It is known that elevated blood sugar levels worsen the breakdown of the BBB, increase brain water content, and lead to cellular apoptosis after ICH [4, 46]. DM further complicates the outlook for ICH by disrupting various mechanistic pathways involved in the secondary brain injury, including neuroinflammatory processes, oxidative stress, vascular dysfunction, and systemic inflammation [8, 47, 48].

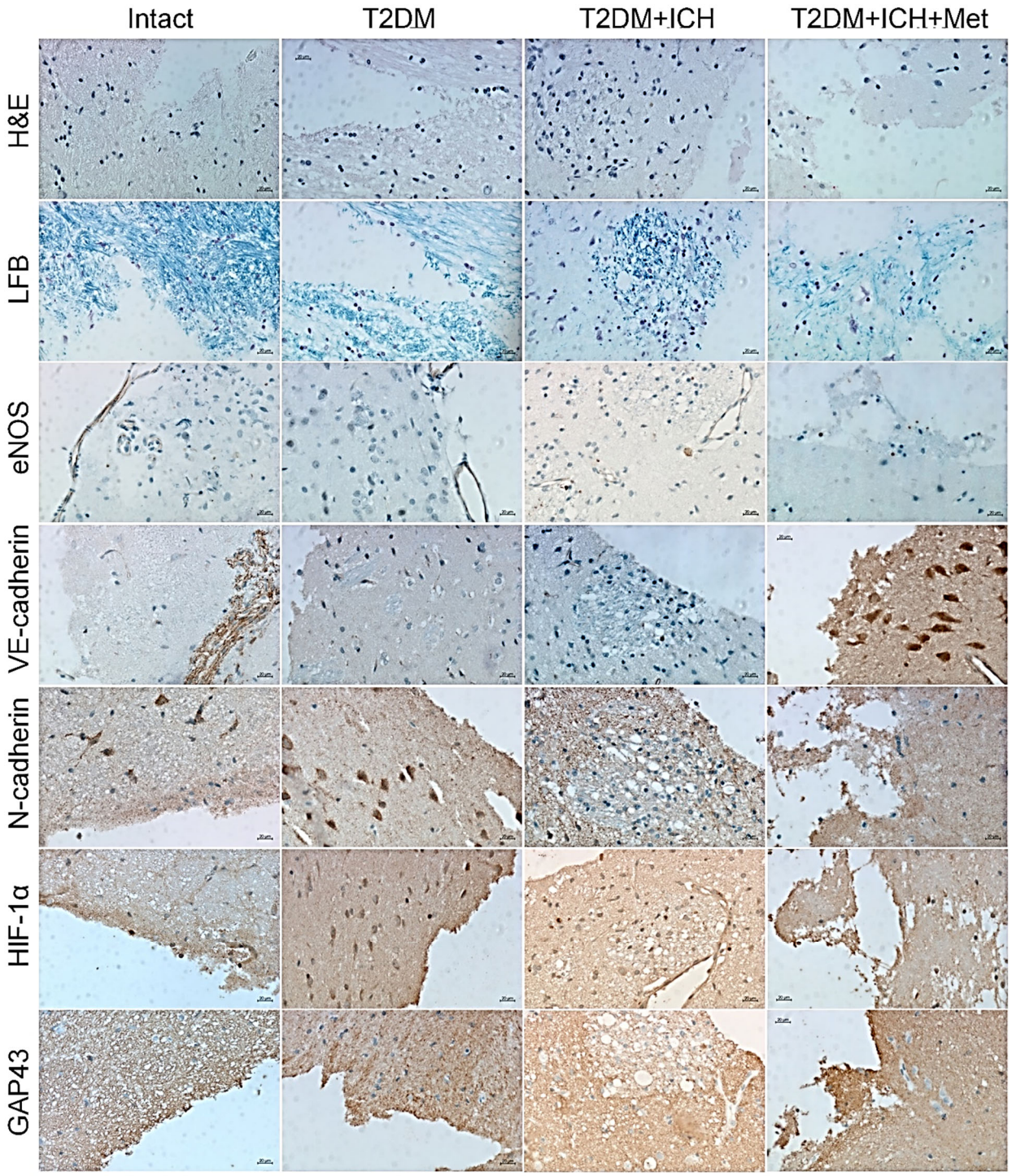

Fig. 4. Histological and immunohistochemical staining in brain tissue samples of experimental groups. Different staining techniques are arranged in rows, groups are arranged in columns. See description in text

Increased oxidative stress and neuroinflammation are closely correlated and lead to microvascular endothelial dysfunction in diabetes. Moreover, endothelial dysfunction may be a key causative factor

in the development of ICH in diabetes [44]. The results of our previous studies have shown that the development of intracerebral hemorrhage in rats with T2DM can intensify the manifestations of oxidative stress, worsen lipid profile, and aggravate endothelial dysfunction. In this case, the pathological process may have the character of a "vicious circle" [48]. We established that both rats with T2DM and rats with T2DM+ICH had a significantly elevated glycemic profile as compared to intact animals. But combined pathology was additionally characterized by an impairment of lipid profile (increased triglyceride level and decreased total cholesterol and high-density lipoprotein) resulting in a rise in the atherogenic index of plasma. A significant increase in the content of the markers of oxidative modification of proteins was observed in both experimental groups. But the rats with T2DM+ICH had higher levels of AGEs in comparison with intact animals. The highest levels of endothelin-1, as a biomarker of endothelial dysfunction, were observed in animals with T2DM+ICH. Such effect was not accompanied by a marked increase of asymmetric dimethylarginine level in blood serum, although there was a clear trend [49]. These changes were associated with oxidative modification of proteins (OMP), such as, neutral and basic carbonyls (PC370 and PC430), advanced glycation end products (AGEs), ischemia modified albumin (IMA), as well as the activity of matrix metalloproteinases MMP2/MMP9 (gelatinases) in rats with experimental T2DM+ICH. These data indicate that ICH in diabetic rats is accompanied by an increased level of AOPPs, AGEs, neutral and basic carbonyls (PC370), and activity of matrix metalloproteinases MMP2/MMP9 (gelatinases) [50]. It is known that high *in vitro* glucose exposure on human brain endothelial cells induces apoptotic cell death mediated by caspase-3 through activation of MMP-9 [51].

In our study, metformin had both nootropic activity and an effect on oxidative stress markers (AGEs, AOPPs and 8-OHdG), which indicates its positive effect on the course of free radical oxidation reactions intensified by both diabetes and ICH. It is known that metformin decreases the synthesis of AGEs and hyperglycaemic-induced ROS production in the endothelium, hence improving the cardiovascular prognosis [52, 53]. These findings are in line with previous studies which found that metformin in conditions of diabetes mellitus complicated by acute intracerebral hemorrhage had advantages over perindopril in relation to endothelial dysfunction. The action of metformin was characterized by a significant decrease in AGEs, ET-1 and vWF levels. But endotheliotropic properties of the studied drug were not associated with an effect on homocysteine levels [54]. It is likely that its protective properties on vascular endothelium play a key role in ensuring BBB functioning and thus exerting neuroprotective properties. However, the ability of metformin to penetrate the BBB may also provide its direct neuroprotective effect, which may be associated with different mechanisms. In particular, metformin is known to improve the expression of brain-derived neurotrophic factor (BDNF) [55]. Elevated levels of lactate in rats with ICH require further study because they can indicate both compensatory activation of astroglia and manifestations of lactic acidosis.

CONCLUSIONS

1. Metformin is highly active against oxidative stress biomarkers in diabetic rats with intracerebral hemorrhage, which further emphasizes its neuroprotective properties and the possibility of using metformin in diabetic patients at risk of hemorrhagic stroke. This study may serve as a reference for the feasibility of studying the clinical efficacy of the drug in these conditions.
2. Considering the increase in VE-cadherin expression in the brain by metformin, it is possible to predict its positive effect on the function of BBB.
3. While these findings provide valuable insights into the potential therapeutic effects of metformin in patients with diabetes mellitus and intracerebral hemorrhage, further research is needed to elucidate the underlying mechanisms of this drug. Long-term studies assessing functional outcomes, histopathological changes, and neuroprotective effects of metformin treatment are warranted to validate its therapeutic efficacy.

Contributors:

Holubiev V.L. – methodology, investigation, data curation, formal analysis, resources, visualization;

Oberemok M.H. – investigation, data curation;

Tkachenko V.A. – investigation, data curation, formal analysis;

Kharchenko Yu.V. – investigation, methodology, data curation;

Bondarenko O.O. – writing – original draft, formal analysis, visualization;

Lievykh A.E. – investigation, methodology, formal analysis, writing – review & editing;

Zhyliuk V.I. – project administration, conceptualization, methodology, visualization, writing – original draft, review & editing.

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