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Flavonoid quercetin reduces gliosis after repetitive mild traumatic brain injury in mice

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The effect of water-soluble form of quercetin on the structural changes of glial cells in hippocampus was investigated in mice after repetitive mild traumatic brain injury. Reactive astro- and microgliosis in hippocampus were observed after brain injury. Iba-1 and GFAP immunohistochemistry was used to visualize astrocytes and microglia cells. Immunopositive cells were counted in hippocampal CA1-area at 5th, 10th and 30th days since the first injury and at 5th, 10th and 30th days since the first quercetin injection. Administration of quercetin leaded to the decrease in number of activated glial cells. Thus, our study demonstrates the following: repetitive mild traumatic brain injury in mice is associated with reactive gliosis; quercetin showed neuroprotective effects by reducing this gliosis. In view of the described, use of quercetin is appropriate for pharmacological correction of cerebrovascular disorders after traumatic brain injury.
Key words: repetitive mild traumatic brain injury; hippocampus; microgliosis; astrogliosis; quercetin.

INTRODUCTION

According to Centers for Disease Control and Prevention [1], traumatic brain injury (TBI) is caused by a bump, blow, or jolt to the head or a penetrating head injury that disrupts the normal function of the brain. The severity of a brain trauma is defined depending on the following characteristics [2, 3]: 1) whether or not a person had a loss of consciousness; 2) how long the loss of consciousness was; 3) the length of amnesia; 4) the resulting cognitive, behavioral and physical problems; 5) the recovery.

TBI is considered to be mild, if the loss of consciousness lasted 030 minutes and the length of amnesia wasn't longer than 24 hours [4]. Among other common symptoms of concussion, there are dizziness, nausea, reduced attention and concentration, memory problems and headache [5]. However, the signs of initial mild trauma are not always obvious [6], while the recurrence of a brain injury may call more dramatic consequences.

The main activities associated with an increased risk of repeated mild trauma are contact sports (boxing, American football, ice hockey, soccer, rugby, etc) and participation in military events [7]. Specifically, the evidence of long-term effects of repetitive mild traumatic brain injury (rmTBI) was found in the autopsied brains of retired NFL players which contained accumulations of abnormal tau protein [8]. Unfortunately, standard diagnostic methods (MRI, CT, EEG) [9] still lack sensitivity for immediate tissue changes in mild TBI, while the postmortem data on late neurodegenerative disorders (chronic traumatic encephalopathy, Alzheimer's disease, Parkinson's disease) due to repeated trauma is growing [10]. Respectively, animal models are still necessary for studying pathophysiology of rmTBI.

Each case of TBI is not a single event, but a whole cascade, starting from an immediate mechanical injury (primary injury) and continuing with metabolic, cellular and molecular events (secondary injury) that may evolve from minutes to months [11, 12]. Mild TBI, being a closed head trauma, is defined by the secondary processes which nevertheless

may lead to consequences not considered as mild [13].

With help of animal models, it was shown that concussion induces such consecutive changes as ion flux (increased potassium and calcium and decreased magnesium), metabolic alterations (activation of glycolysis and increased energy demands for cell membrane pumps to restore cellular ionic homeostasis) and disruption to cerebral blood flow (impaired autoregulation) [14]. As a result, TBI is followed by membrane degradation of vascular and cellular structures and ultimately necrotic or programmed cell death (apoptosis) [15].

One of the main phenomena that cause cell death at brain trauma is oxidative stress related to the generation of reactive oxygen species (superoxides, hydrogen peroxide, nitric oxide and peroxinitrite). Thus, oxidative stress plays a role of a specific target for the treatment of mild TBI [16]. Antioxidants derived from natural products are considered to be potentially effective scavengers of reactive oxygen species [17]. Particularly, flavonoids are substances of such kind and are already used for treating many important common diseases [18].

Flavonoids form a subclass of polyphenols, which are characterized as containing two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge, and are widely distributed in nature and in foods (fruit, vegetables, grains, bark, roots, stems, flowers, tea and wine) [19]. Many studies suggest evidence of neuroprotective, cardioprotective and chemopreventive features of flavonoids [20].

Quercetin is the most studied flavonoid known for inhibiting production of pro-inflammation producing enzymes (cyclooxygenase and lipoxygenase) as well as TNF-α, nitric oxide production and nitric oxide synthase expression [21]. Anti-inflammatory effect of quercetin was shown both in *in vitro* and *in vivo* animal experiments [22–24]. Given the data on positive effect of quercetin at cerebrovascular disorders, we decided to test its water-soluble form corvitin as a treatment for rmTBI to prevent long-term neurodegenerative changes.

METHODS

All stages of the experiment were performed in accordance to the law of Ukraine "On the Protection of Animals from Cruelty" (Article 26; No 3447 – IV) and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

Animals. Subjects were 66 mice from 8 to 12 weeks old males of wild-type mouse (weighing 24–40 g). Animals were housed in cages on a 12-hour light–dark cycle, with free access to food and water. The groups were formed as follows: (1) controls – not anesthetized, not injured (n=11); (2) sham-injected mice – injured (n=28); (3) mice injected with quercetin – anesthetized, injured (n=27).

The model of repetitive mild traumatic brain injury. We induced rmTBI by using a model proposed earlier [25]. Its design represents a modification of Marmarou's weight-drop model [26] which reproduced single diffuse TBI from mild to severe in rats. Both in original scheme and in modified, the skull of a mouse is exposed to an impact with a weight guided by a vertical tube. The tube is pre-fixed to a stationary object like wall or rack. In both schemes, the severity of injury depends on the height from which the load falls and the weight of the latter. Respectively, in our study the 95 g weight fell from the 1 m height. The weight of the load was revised from 450 g to 95 g in accordance with the ratio between the weight of the brain of rats and mice. In our study, an experimental animal, lightly anaesthetized with diethyl ether, was not fixed in the apparatus, and after the weight fell, it fell too (through a piece of aluminum foil pre-attached on top of the H-shaped box), landing on a damper support. We avoided rebounding of the weight and, consequently, additional blows to the skull by using a nylon fishing line that stopped the load from falling after the impact

due to its length. According to this model, neither scalp incision nor attachment of helmet to the mouse skull were done. Mice were subjected to 5 head impacts (1/day for 5 successive days).

Drug administration. Injured mice were intraperitoneally injected with corvitin (PJSC SIC "Borshchahivskiy CPP", Ukraine) which is a complex of quercetin with povidone. Corvitin dissolved in saline was injected to animals i. p. in a dose of 100 mg/kg (quercetin amount in a dose – 10 mg/kg) according to the following scheme: (1) the first day – the first injection (in an hour after the last injury) and the second injection in 6 hours; (2) the second day – two injections within 6 hours; (3) from the third day to the fifth day – single injection daily.

Immunohistochemical staining. Immunohistochemical staining of glial cells was performed as previously described [27]. At 5th, 10th, and 30th days after rmTBI, mice were deeply anaesthetized with an i. p. injection of calipsol and intracardially perfused with 0.1 M phosphate buffer (PB), followed by 4% phosphate-buffered paraformaldehyde (PFA). After perfusion, the brains were quickly removed, immersed in the same fixative and postfixed overnight at 4°C. Frontal 50-µm-thick brain sections were cut on the vibratome (VT1000A Leica, Wetzlar, Germany). The sections were left overnight at 4°C for incubation with primary antibodies diluted in the solution (0.1 M PB, 1% bovine serum albumin, 0.3% Triton X-100). To identify the glial cells the following primary antibodies were used: polyclonal rabbit anti glial fibrillary acidic protein (GFAP, 1:1500; Dakocytomation, Glostrop, Denmark), polyclonal rabbit anti-Iba-1 (1:1000; Wako, Osaka, Japan). On the next day, sections were incubated with the secondary anti-rabbit antibody Alexa Fluor 594 (1:1000, Molecular Probes Inc., USA) diluted in the solution (0.1 M PB, 0.5% bovine serum albumin, 0.3% Triton X-100). The immunostained sections were mounted with fluorescent mounting medium Immu-Mount (Thermo Scientific, Waltham, MA). Confocal images of glial cells were acquired with a laser scanning microscope (FV1000-BX61WI, Olympus, Tokyo, Japan). For the evaluation of cells, the program "Image J" was used. The number of cells was calculated in 0.1 mm^2. The area of interest (Fig. 1) was located in hippocampal CA1-zone.

Statistical analysis. Statistical analysis was performed in StatSoft Statistica 6.0. The two-tailed Student's t–test was used to assess the significance of differences between samples ($P<0.05$ was considered to indicate statistical significance). All data were shown as mean ± SD.

RESULTS AND DISCUSSION

No signs of skull fractures and hemorrhages were detected after delivering rmTBI. In our previous study, evaluation of water content in brain in controls and injured animals also didn't show edema presence [28]. However, immunohistochemical analysis revealed glial changes following the mechanical trauma. The results of our study showed the development of astro- and microgliosis due to experimental rmTBI, which was presented by gradual increase in number of astrocytes and microglial cells up to 30th day. The injection of quercetin to the injured mice lead to the inhibition of gliosis.

Astrocytes and microglia cells play mixed role in brain injury, both promoting and inhibiting regeneration of neural tissue, depending on

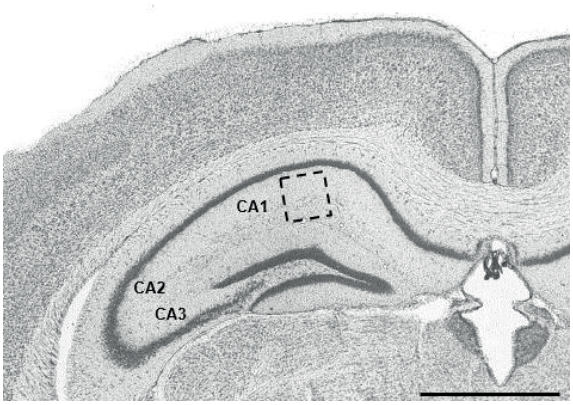

Figure 1. ROI in hippocampal CA1-zone. CA1, CA2, CA3 – fields of hippocampus. The dotted square indicates the location and the size of the ROI. Scale bar = 1 mm

specific conditions [29]. For example, reactive astrocytes secrete molecules of extracellular matrix that form glial scar and thus limit axonal regeneration. On the other hand, this way astroglia isolates the site of damage and prevents its extension. Furthermore, astrocytes express trophic factors necessary for neuronal survival. Microglia also produces neuroprotective factors, additionally clearing cellular debris and orchestrating neurorestorative processes in an injured brain [30]. However, the effect of dysregulated microglia may be detrimental to neurons due to intensive production of proinflammatory and cytotoxic mediators [31]. Astro- and microgliosis are responses of the brain to the development of pathology and are presented as morphological and quantitative changes of astrocytes and microglia cells. The task of our research was the quantitative analysis of glial changes in CA1-zone of hippocampus after delivering rmTBI and subsequent corvitin injection in mice.

Immunohistochemical studies have shown that the number of GFAP-positive astrocytes increased significantly on 5th day (37.55±8.23), 10th day (45.36±9.57) and 30th day (44.54±9.79) since the first injury in comparison with the control (19.44±4.45) (Fig. 2, A). It should be noted that the number of GFAP-positive astrocytes was doubled on 5th day since the injury in comparison with the control (P<0.001). The number of activated astrocytes gradually grew up to 10th day, and such indicators maintained up to a month. Immunohistochemical study showed that the increase of the GFAP-positive astrocytes was observed in layers (*str. pyramidale, str. radiatum, str. lacunosum-moleculare*) of the hippocampal CA1zone. Thus, reactive astrogliosis in hippocampal area CA1 was observed. GFAP-positive astrocytes acquired pronounced hypertrophied structure: their processes were thickened and shortened; volume of soma increased.

Study of the microglial reaction at the traumatic brain injury revealed that the number of Iba1-positive microglia increases and its indicator on 5th day amounted to 48.00±9.97, on 10th day – 45.29±8.70, on 30th day – 40.63±10.62 compared to controls (22.40±3.71), (P<0.001)

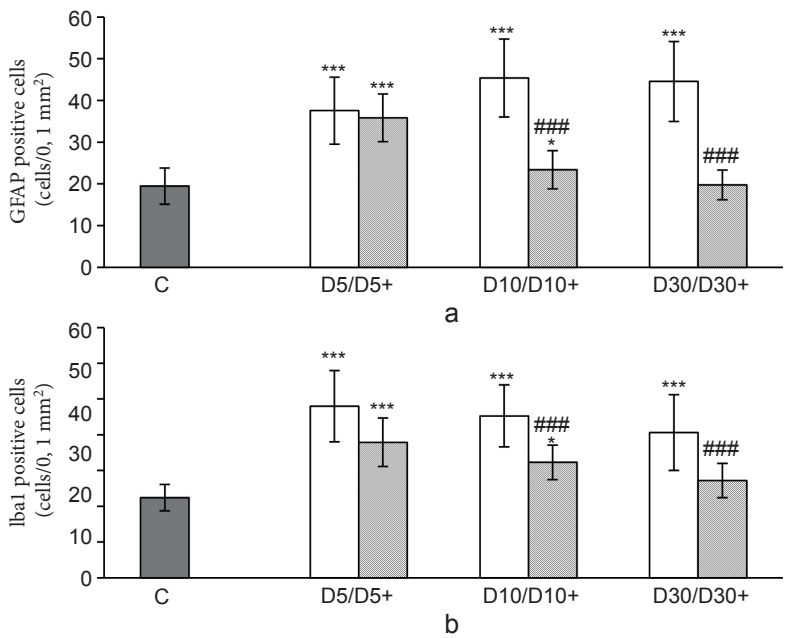

Figure 3. Changes in number of astrocytes (A) and microglial cells (B) of hippocampal area CA1 in control, after delivering brain injury (on day 5, day 10 and day 30 since the first impact) and after quercetin treatment (on day 5+, day 10+ and day 30 + since the first injection). *, ** and *** P<0.05, P<0.01 and P<0.001 in comparison with control, ##, ### P<0.05 and P<0.001 in comparison within each pair of the bars. Results are the mean ± SD

(Fig. 2, B) The number of activated microglial cells gradually reduced to a month after the injury. Iba1-positive microglia cells were observed in all layers of the hippocampal CA1-zone. The morphology of the microglial cells transformed from the resting (ramified) to an activated (amoeboid shape) form with short thick processes and the hypertrophic soma. Thus, after rmTBI, reactive gliosis is observed in CA1-zone of the hippocampus, which indicates the development of inflammatory processes in the brain.

Administration of quercetin resulted in the inhibition of reactive astrogliosis. The calculation of astrocytes after the quercetin administration revealed the decrease of cell numbers, and by 30th day this indicator reached the level of control (Fig. 2, A). The numbers of astrocytes were 35.82±5.91 on 5th day, 23.36±4.70 – on 10th and 19.77±3.68 - on 30th day. Among the samples with rmTBI and those with rmTBI plus the quercetin administration, statistically significant difference was shown between the groups on 10th day and 30th day ($P<0.001$). Thus, the level of activated astrocytes decreased after the quercetin administration.

Microgliosis reduce was less evident than one of astrogliosis (Fig. 2, B). The number of microglia cells on 5th day (37.89±6.80) since the first quercetin injection slightly differed from one on 5th day after the injury (48.00±9.97). On 10th day (32.27±4.80) after the treatment, the number of cells was less than on day 10 (45.29±8.70) after the injury, however, it hasn't decreased much in comparison with the sample five days ago (37.89±6.80). The maximum decrease of Iba1-positive microglial cells was observed on 30th day (27.18±4.78). The difference was statistically significant ($P<0.01$) between the groups of mice on 5th day since the first injury and ones with injury and quercetin injection. The difference was statistically significant ($P<0.001$) between the groups of animals on 10th and 30th days with quercetin injection in comparison with those without drug administration. Despite the fact that the level of activation of microglia following quercetin administration decreases, it remains high enough in comparison with the control. The quercetin application reduces the degree of reactive microgliosis after the rmTBI which allows to make a conclusion about its neuroprotective effects.

It is known that long-term effects of the mild TBI leads to neurodegenerative diseases such as chronic traumatic encephalopathy, Alzheimer's disease, Parkinson's disease, etc [11, 15, 16]. The manifestations of secondary damage are mainly associated with cytotoxic and inflammatory processes that lead to metabolic changes in brain homeostasis due to vascular insufficiency [12, 17, 23, 32]. Oxidative stress as a consequence of a mechanical brain trauma is a powerful damaging factor that in turn leads to alterations in cerebral blood flow. After TBI occurs, the release and activation of free radicals take place. It must be noted that natural flavonoids possess a powerful antioxidant effect, and quercetin is the most studied flavonoid among them. Among diverse properties of phenols, to which quercetin belong, their ability to produce a protective effect against lipoperoxidative damage stands out [33].

Numerous experimental models have been developed to replicate various aspects of the TBI in humans to understand better the mechanisms of damage, their correction and treatment. We used a new model of rmTBI, that simulates the most common form of head injury in humans. Our data indicate the effective neuroprotective impact of water-soluble form of quercetin at the repetitive mild traumatic brain injury.

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ФЛАВОНОЇД КВЕРЦЕТИН ЗМЕНШУЄ ГЛІОЗ ПІСЛЯ ЛЕГКОЇ ЧЕРЕПНО-МОЗКОВОЇ ТРАВМИ У МИШЕЙ

Вплив водорозчинної форми кверцетину на структурні зміни гліальних клітин у гіпокампі досліджували у мишей після повторюваної легкої черепно-мозкової травми. Реактивний астрогліоз і мікрогліоз спостерігали у гіпокампі після механічного пошкодження головного мозку. Астроцити та клітини мікроглії візуалізували за

допомогою імунофлуоресцентного забарвлення анти-GFAP- та анти-Iba1-антитілами. Імунопозитивні клітини підраховували у CA1-зоні гіпокампа на 5, 10 та 30-ту добу після першої травми, а також на 5, 10 та 30-ту добу після першої ін'єкції кверцетину. Введення кверцетину призводило до зниження кількості активованих гліальних клітин. Таким чином, за результатами нашого дослідження, повторювана легка черепно-мозкова травма пов'язана з реактивним гліозом; кверцетин виявив нейропротективні властивості, призвівши до зменшення реактивного гліозу. Є підстави для використання кверцетину у фармакологічній корекції цереброваскулярних порушень після черепно-мозкової травми.
Ключові слова: повторювана легка черепно-мозкова травма; гіпокамп; мікрогліоз; астрогліоз; кверцетин.

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ФЛАВОНОИД КВЕРЦЕТИН УМЕНЬШАЕТ АСТРОГЛИОЗ ПОСЛЕ ЛЁГКОЙ ЧЕРЕПНО-МОЗГОВОЙ ТРАВМЫ У МЫШЕЙ

Влияние водорастворимой формы кверцетина на структурные изменения глиальных клеток в гиппокампе исследовали у мышей после повторяемой лёгкой черепно-мозговой травмы. Реактивный астроглиоз и микроглиоз наблюдали в гиппокампе после механического повреждения головного мозга. Астроциты и клетки микроглии визуализировали с помощью иммунофлуоресцентного окрашивания анти-GFAP- та анти-Iba1-антителами. Иммуноположительные клетки подсчитывали в CA1-зоне гиппокампа на 5, 10 и 30-е сутки после первой травмы, а также на 5, 10 и 30-е сутки после первой инъекции кверцетина. Введение кверцетина приводило к снижению количества активированных глиальных клеток. Таким образом, по результатам нашего исследования, повторяемая лёгкая черепно-мозговая травма связана с реактивным глиозом; кверцетин проявил нейропротективные войства, приведя к уменьшению реактивного глиоза. Существуют основания для использования кверцетина в фармакологической кореекции цереброваскулярных нарушений после черепно-мозговой травмы.
Ключевые слова**:** повторяемая лёгкая черепно-мозговая травма; гиппокамп; микроглиоз; астроглиоз; кверцетин.

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