EFFECT OF INTRACRANIAL CATHETER PLACEMENT ON MICROGLIA METABOLIC PROFILE IN RATS

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The aim of the work was to investigate the effect of the intracranial catheter placement on the metabolic profile of rat microglia. Microglial cells were isolated by the centrifugation in Percoll gradient. Oxidative metabolism and phagocytic activity were investigated by flow cytometry. Arginase activity was examined by colorimetric method. Nitrite level was assayed in Griess reaction. It was found that intracranial catheter placement causes down-regulation of nitrite synthesis by 3 times, augmentation of the reactive oxygen species generation by 1.5 times, and slightly decreases microglia phagocytic activity. Thus, intracranial catheter placement was associated with anti-inflammatory shift of microglia metabolism in rats in distant time period after the device implantation.

Key words: phagocytes, microglia, metabolic profile, intracranial catheter.

Effective drug delivery to the brain structures for the treatment of neurodegenerative diseases, oncopathology, etc. is a serious problem for medical and biological science. The most popular approaches include systemic introduction of therapeutic agents orally or intravenously. However, many of the systemically introduced drugs have very limited ability to penetrate the blood-brain barrier, that severely affects their therapeutic efficiency. In addition, the systemic drug administration stimulates increasing their single and total doses to optimize the dose load of the target tissue in the brain. Increasing the dose of therapeutic agents, especially chemotherapeutic drugs, is associated with their significant systemic toxicity and the development of side effects [1–3]. Currently, numerous biotechnological strategies have been developed and investigated to facilitate the delivery of therapeutic agents to the brain [4–6]. However, the use of targeted intravascular methods of delivery of drugs is a rather traumatic procedure and can cause non-specific inflammatory processes in the brain tissues, which, in turn, can exacerbate the course of neurodegenerative diseases and the tumor process, the basis of which pathogenesis is inflammation [7, 8].

Key effector cells of inflammatory processes in the brain are the microglia – resident brain phagocytes. The functions of microglia in the brain tissue are similar to those of other resident tissue phagocytes. These cells are characterized by high plasticity of metabolism. On the one hand, microglial cells can exert cytotoxic (microbicidal, tumoricidal, etc.) effects, and on the other — have an ability to participate in reparative processes and promote the remodelling of brain tissue and stimulate neuro- and angiogenesis. These characteristics underlie the theory of microglia metabolic polarization, inherent for all resident and circulating phagocytes, to classical proinflammatory (M1) and alternative anti-inflammatory (M2) activation states [9–11].

This study was aimed to investigate the effect of the placement of intracranial catheter on the metabolic profile of rat microglia.
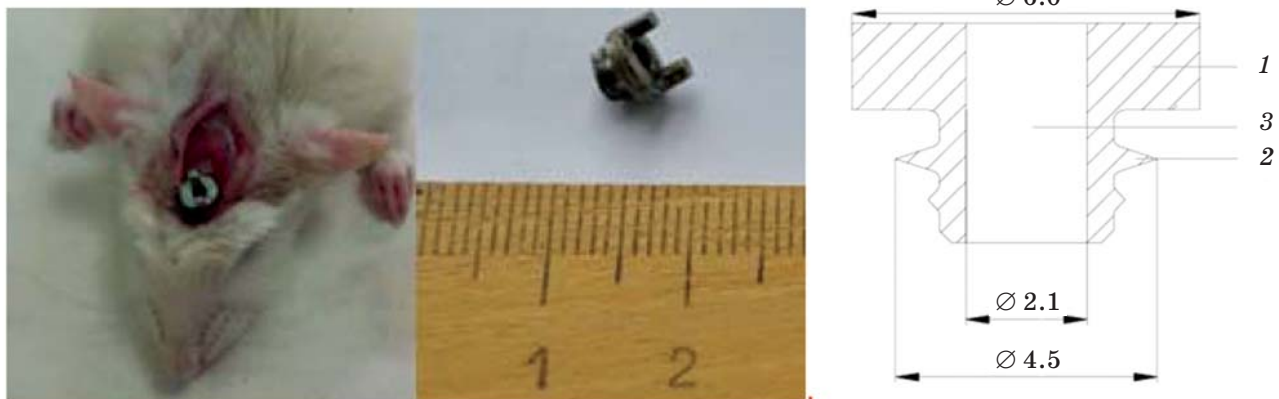
Fig. 1. **Intracranial catheter for multiple intracerebral experimental studies:**
1 — outer sleeve; *2* — self-tapping double-start thread; *3* — inner opening

Materials and Methods

The research was conducted on white outbred adult male rats aged 2–3 months, weighting 200–250 g. Animals were bred at animal facility of Educational and Scientific Center "Institute of biology and medicine" of Taras Shevchenko National University of Kyiv. The animals were kept in the vivarium with free access to water and food. Animal protocol was approved by the Taras Shevchenko National University animal welfare committee according to the Animal Welfare Act guidelines. All animal studies were carried out in accordance with the norms of the Convention on Bioethics of the Council of Europe 'Europe Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes' (1997), the general ethical principles of animal experiments, approved by the First National Congress on Bioethics in Ukraine (September 2001), and national law No. 3447-IV "On the Protection of Animals from Cruel Treatment" [12]. Animals were randomized by weight and divided in two groups ($n = 8$) : group 1 — intact animals, group 2 — animals with intracranial catheter.

For these experiments, we developed the device for multiple intracerebral experimental studies (Fig. 1), containing an intracranial catheter (patent of Ukraine for invention No. 114580 dated 26/06/2017 [13].

Developed intracranial catheter is a boss with an outer sleeve with a diameter of 6 mm and a self-tapping double-start thread with a diameter of 4.5 mm, which allows the boss to be screwed and fastened to the scull. The inner diameter of the catheter, through which all diagnostic and therapeutic manipulations are carried out, is 2.4 mm. The inner opening of the catheter is closed with a cap. Trephine opening in the given coordinates is performed by a drill in the form of a trident. The working diameter of the drill is 3.4 mm. It corresponds to the diameter of the trephine opening, and ensures reliable fixation of the threaded part of the boss in the skull.

1–2 days before the device was used, head skin was depilated. Immediately before the surgery, 0.25% of Marcaine ("Recipharm Monts", France) was administered at a dose of 0.04 ml/g to relieve pain for 6–8 hours. Before the device was placed, animals were anesthetized with a Calipsovetum ("Brovafarta", Ukraine). After this, the scalp incision was performed in the projection of the device. Scalp incision was then expanded, washed with hydrogen peroxide, and connective tissue was removed from the skull. Skull surface was then degreased and dried, and trephine opening with the diameter of 3.4 mm was made using a drill, that is completed with the device. The catheter was placed and fastened in the trephine opening with a special screwdriver. The skin was sutured up in such a way that the catheter remained outside. The control animals were treated similarly, but the catheter was not inserted.

The animals were observed for 21 days. The animal behavior, weight, and survival was analyzed. On the twenty second day, their microglia was isolated and the cell functional characteristics were examined.

For the microglial cell isolation, rats were euthanatized by cervical dislocation, the brain was rapidly extracted on ice in a Petri dish, hippocampus was dissected and perfused using 0.9% NaCl with 0.2% glucose ("Darnytsia", Ukraine), and then was homogenized in Potter homogenizer for 15 min at room temperature. The obtained homogenate was filtered through a 40 nm cell strainer (BD Biosciences Discovery) to remove cell conglomerates. The homogenate was transferred into a test tube and centrifuged at 350 g for 10 min at room temperature". The sediment was suspended in 1 ml 70% isotonic percoll solution ("GE Healthcare", USA) and transferred into another test tube. Two ml of 50% isotonic percoll solution were carefully layered on the top of 70% percoll solution. Over the 50% percoll layer, 1 ml phosphate buffer was gently added. Then, density gradient was centrifuged at 1200 g for 40 min. After centrifugation, the layer at the interface between the 70% and 50% Percoll phases contained highly enriched microglia was aspirated. Microglia cells were washed in PBS by centrifugation for 5 min at room temperature and re-suspended in the RPMI-1640 medium (Sigma-Aldrich, USA) for further examination of functional characteristics. The purity of isolated microglia population was estimated by flow cytometry with the use of anti-CD11b antibodies (BD Pharmingen™). The proportion of CD11b+ cells was ≥ 92%. Cell viability, determined by Trypan blue exclusion test, was 96%.

The phagocytic activity was examined as described by Cantinieaux B. et al. with small modifications [14]. FITC-labeled (Sigma Aldrich, USA) thermally inactivated cells of a one-day culture of *S. aureus* Cowan I (collection of the Dept. of Microbiology and Immunology of ESC "Institute of Biology and Medicine") at a concentration of 1×10^7 cells/ml were used as a phagocytosis object. 2×10^5 microglial cells at the volume of 70 µl were placed in the cytometric test tubes, and 30 µl suspension of FITC-labeled *S. aureus* were added and mixed with pipettes. To obtain FITC-labeled *S. aureus,* we dissolved FITC in DMSO (Sigma Aldrich, USA) and mixed with bacteria at the ratio of 0.05 g FITC per 10^8 bacteria. Then the mixture was incubated for an hour in a dark place at room temperature and labelled bacteria were washed thrice by centrifugation. The negative control sample contained 30 µl PBS instead of labeled microorganisms. The cells mixture was incubated for 30 min in CO_2-incubator at 37 °C. After that, the cells were washed twice by centrifugation in PBS and re-suspended in 400 µl of 0.4% formalin solution ("Enamine", Ukraine). The samples were analyzed by flow cytometry. The results were registered as phagocytosis index that represents the mean fluorescence per one phagocytic cell (that is proportional to the number of ingested bacteria).

Reactive oxygen species (ROS) generation was determined using dichlorodihydro-fluorescein diacetate (DCF-DA) (Sigma Aldrich, USA) [15]. The cells were incubated in a phosphate buffer with 10 µM DCF-DA for 30 min at 37 °C. Fluorescence of the DCF-DA-labeled cells was studied by flow cytometry.

The arginase activity of the microglia was evaluated in colorimetric assay described by Classen et al. [16]. 100 µl 0.1% Triton X-100 (Sigma-Aldrich, USA), 100 µl 50 mmol Tris-HCl (pH 7.5; Sigma Aldrich, USA) with 10 mmol $MnCl_2$ were sequentially added to the cell suspension. The mixture was kept at 56 °C for 7 min to activate arginase activity. 100 µl L-arginine (0.5 mol; pH 9.7; Sigma-Aldrich, USA) was then added to the cells with pre-activated arginase for 2 hours. To stop the reaction of L-arginine hydrolysis, 800 µl of acid mixture (H_2SO_4:H_3PO_4:H_2O = 1:3:7) was prepared immediately before the experiment and were added to the samples. For colorymetric evaluation of urea, α-isonitrosopropiophenon (40 µl, 6 % in ethanol, Sigma-Aldrich, USA) was added to the samples, and the mixture was incubed at 95 °C for 30 min, and than at 4 °C for 30 min. Urea concentration was measured spectrophometrically at $\lambda = 545$ nm. The urea µg was calculated using a calibration curve, that was created using standard urea solutions of known concentration. The data were analyzed using the next formula: µg urea / 60 (MM urea) × 50 (dilution factor)/t (min incubation with arginine) = arginase units per 1×10^6 cells; 1 unit = amount of enzyme necessary to hydrolyze 1 µM arginine per min.

Nitrite production level was measured in cell supernatant by the Griess reaction [16]. To prepare Griess reagent equal volumes of 2% sulfanilamide in 10% phosphate acid and 0.2% naphthylethylenediamine hydrochloride (Sigma-Aldrich, USA) were mixed. 100 µl Griess reagent were added to 100 µl suspension of microglial cells. The mixture was incubated for 30 min at room temperature in the dark. Results were measured spectrophotometrically on a plate photometer Ascent ("Labsystems", Finland) at 540 nm excitation wave length.

Nitrite level was determined using a calibration curve that was created using standard solutions of sodium nitrite. The extinction value was divided by the number of living cells in the sample. Nitrite concentration was presented for 10^6 cells. Each sample was assayed in triplicate, and results are presented as mean ± SD. Statistical significance of the results was determined by Student's *t*-test [17]. For all analyses, $P < 0.05$ was accepted as a significant probability level.

Results and Discussion

Placement of intracranial catheter did not influence significantly animal behavior and did not cause statistically significant changes in their survival or weight throughout the observation period (Fig. 2).

However, on the twenty second day of observation significant alterations in the microglia metabolic profile of catheterized rats were registered. There are several criteria to evaluate metabolic activation profile of phagocytes, including microglia. Arginine metabolism is key characteristics of phagocyte (including microglia) functional polarization. Classic (M1) and alternative (M2) activation of phagocytes is accompanied by different direction of arginine metabolism. Under classic activation, iNOS metabolizes arginine and cytotoxic reactive nitrogen species are formed, while under the alternative activation arginase Arg-1 is induced and metabolizes arginine to urea and ornithine, precursor for polyamines and proline. Biogenic amines (putrescine, spermine and spermidine) are involved in cell growth and can inhibit T-cell immune responses. Increased proline synthesis by phagocytes is characteristic for tumor microenvironment [18, 19]. Proline is a key component of collagen synthesis. Its synthesis is increased during reparation tissue remodelling [20]. Reactive nitrogen species, especially NO, exerts cytotoxic effects and are important signal molecules. In particular, NO activates a number of transcription factors, including NFκB, involved in the synthesis of pro-inflammatory cytokines and several other inflammatory mediators [21, 22]. According to our results, intracranial catheter placement did not cause statistically significant changes in arginase activity of microglia cells (Fig. 3, *A*) while causing statistically significant (almost by three times) decrease in NO synthesis at day 21 of observation period (Fig. 3, *B*).

Arginine metabolism shift to decreased activity of NO-synthase is a sign of alternative or anti-inflammatory metabolic activation of phagocytes, including microglia. In the case of catheter placement, such microglia metabolic skew can result from the activation of prolonged reparation processes.
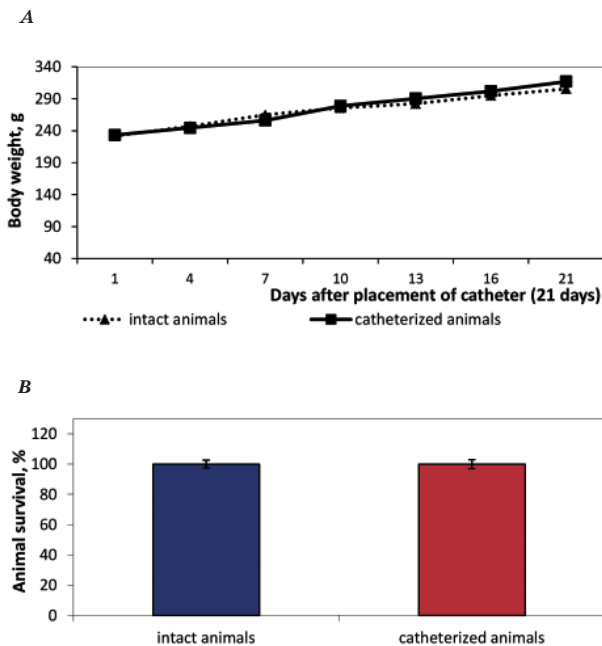

Fig. 2. **The effect of intracranial catheter placement on survival (*B*) and weight of rats (*A*)** (*n* = 8 in all groups)
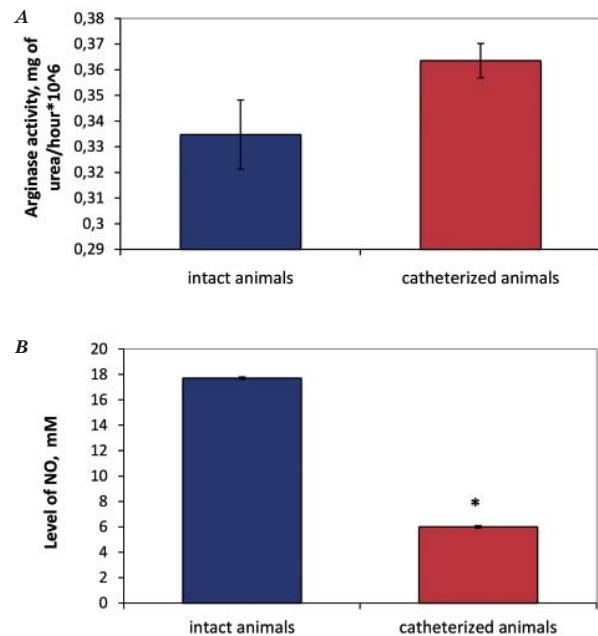

Fig. 3. **The effect of intracranial catheter placement on the arginase activity (*A*) and NO synthesis (*B*) inmicroglia cells** (*n* = 8 in all groups)
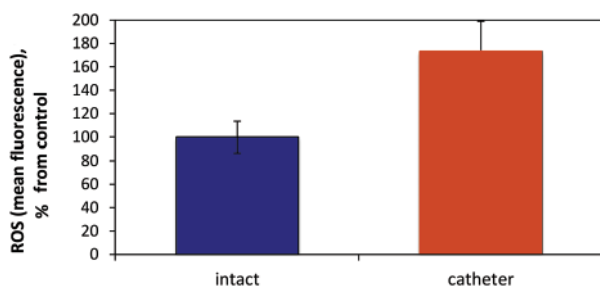* — $P < 0.05$ as compared to intact animal value

Fig. 4. The effect of intracranial catheter placement on reactive oxygen species generation by microglia cells ($n = 8$ in all groups)
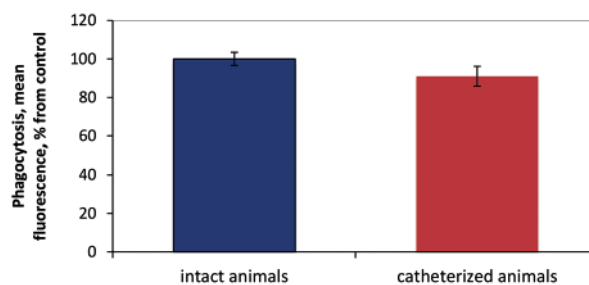* — $P < 0.05$ as compared to intact animal value

Fig. 5. The effect of intracranial catheter placement on phagocytic activity of microglia cells
($n = 8$ in all groups)

ROS synthesis in characteristic for both pro- and in anti-inflammatory metabolic activation of phagocytes, since it is necessary for the regenerative and destructive inflammatory processes [23–25]. Intracranial catheter placement caused 1.5-fold increase in ROS production (Fig. 4). To characterize the functional polarization of phagocytes, the change in their oxidative metabolism is commonly considered in the context of arginine metabolism shift. In our case, increased ROS production along with decrease in reactive nitrogen species synthesis might indicate anti-inflammatory polarization of microglia metabolism.

Phagocytic activity is associated with increased expression of a number of receptors, including scavenger receptors. Generally, up-regulation of this function is considered as a sign of the alternative (M2) cell polarization [26]. In our experiments, phagocytic activity in microglia of experimental animals did not differ from that in control animals (Fig. 5).

Pro-inflammatory phagocyte activation is presumably followed by the decrease of their endocytosis function. Unchanged phagocytic activity along with up-regulated ROS generation and skewed arginine metabolism towards increased arginase activity indicate anti-inflammatory metabolic shift in microglial cells.

Therefore, intracranial catheter placement was associated with anti-inflammatory shift of microglia metabolism in rats in a distant time period after the device implantation. Catheters are devices used for intracerebral drug delivery as well as for modeling of a number of brain diseases such as neurodegenerative disease or tumors. The ability of the device to cause anti-inflammatory metabolic activation of microglia might obscure true results of disease modeling or treatment, and should be taken into account in such experiments.

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ВПЛИВ ІМПЛАНТАЦІЇ ІНТРАКРАНІАЛЬНОГО КАТЕТЕРА НА МЕТАБОЛІЧНИЙ ПРОФІЛЬ МІКРОГЛІЇ ЩУРІВ

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Метою роботи було дослідити впливу встановлення інтракраніального катетера на метаболічний профіль мікроглії щурів. Клітини мікроглії виділяли методом центрифугування в градієнті щільності перколу. Оксидативний метаболізм і фагоцитарну активність досліджували методом проточної цитометрії. Аргіназну активність вивчали колориметричним методом. Синтез нітритів визначали в реакції Грісса. Було виявлено, що встановлення інтракраніального катетера спричинювало зниження синтезу нітритів клітинами мікроглії в 3 рази, посилення синтезу реактивних форм кисню в 1,5 раза і неістотно знижувало фагоцитарну активність. Імплантація катетера не впливала на масу і життєздатність тварин. Таким чином, встановлення інтракраніального катетера було асоційовано з протизапальною метаболічною активацією мікроглії щурів у віддалені терміни після імплантації пристрою.

Ключові слова: фагоцити, мікроглія, метаболічний профіль, інтракраніальний катетер.

ВЛИЯНИЕ ИМПЛАНТАЦИИ ИНТРАКРАНИАЛЬНОГО КАТЕТЕРА НА МЕТАБОЛИЧЕСКИЙ ПРОФИЛЬ МИКРОГЛИИ КРЫС

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Целью работы было исследование влияния установки интракраниального катетера на метаболический профиль микроглии крыс. Клетки микроглии выделяли методом центрифугирования в градиенте плотности перколла. Оксидативный метаболизм и фагоцитарную активность исследовали методом проточной цитометрии. Аргиназную активность изучали колориметрическим методом. Синтез нитритов определяли в реакции Грисса. Было выявлено, что установка интракраниального катетера вызывала снижение синтеза нитритов клетками микроглии в 3 раза, усиление синтеза реактивных форм кислорода в 1,5 раза и незначительно снижала фагоцитарную активность. Имплантация катетера не влияла на массу и жизнеспособность животных. Таким образом, установка интракраниального катетера была ассоциирована с противовоспалительной метаболической активацией микроглии в отдаленные сроки после имплантации устройства.

Ключевые слова: фагоциты, микроглия, метаболический профиль, интракраниальный катетер.