EXPERIMENTAL ARTICLES

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LONG-TERM EFFECTS OF SHAM SURGERY ON PHAGOCYTE FUNCTIONS IN RATS

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Animal models of inflammatory disorders, including those of the nervous system are commonly used to explore the pathophysiological role of immune cell response in disease triggering and course and to develop biotechnology products for therapeutic use. Modeling some of these disorders, particularly neurodegenerative diseases, implies surgical manipulations for the intracerebral introduction of disease-initiating substances (toxins, amyloids etc.). Design of these experiments involves the use of sham-operated animals as a control of non-specific intrinsic side-effects elicited by surgical manipulations *per se*, including local and systemic inflammation, where phagocytic cells are key participants. Short-term post-surgical immunomodulatory effects are widely reported. However, no study thus far has examined the long term effects of sham-surgery on phagocyte functions.

The *purpose* of this study was to evaluate the effect of sham-surgery, commonly used for modeling neurodegenerative diseases, on phagocyte functions in the far terms after the surgical manipulations.

Materials and Methods. Adult male Wistar rats were used in the study. Sham surgery consisted of stereotactic unilateral injection of saline solution into the median forebrain bundle (sham-operated 1, SO1) or directly into the substantia nigra (sham-operated 2, SO2). Before the placebo surgery, animals were anaesthetized using nembutal and ketamine/xylazine correspondingly. Functional characteristics (phagocytic activity, oxidative metabolism, CD80/86 and CD206 expression) of phagocytes (microglia, peritoneal macrophages, circulating monocytes and granulocytes) were examined by flow cytometry. Differential leukocyte count was conducted using hematological analyzer.

Results. Phagocytes from animals underwent of different protocols of placebo surgery, demonstrated various patterns of functional changes on day 29 after the manipulations. In animals from SO1 group, we observed signs of residual neuroinflammation (pro-inflammatory shift of microglia functional profile) along with ongoing resolution of systemic inflammation (anti-inflammatory metabolic shift of circulating phagocytes and peritoneal macrophages). In rats from SO2 group, pro-inflammatory polarized activation of peritoneal phagocytes was registered along with anti-inflammatory shift in microglia and circulating phagocytes.

Conclusions. Sham surgery influences functions of phagocytic cells of different locations even in the far terms after the manipulations. These effects can be considered as combined long-term consequences of surgical brain injury and the use of anesthetics. Our observations evidences that sham associated non-specific immunomodulatory effects should always be taken into consideration in animal models of inflammatory central nervous system diseases.

Key words: sham surgery; phagocytes; neuroinflammation; systemic inflammation.

Animal experiment in biomedical research is a subject of debates in view of the growing awareness of ethical questions, and as a result of a gradually changing place of animals in our society. Nevertheless, the use of animal models currently stays a mandatory

practice in medicine and veterinary, as well as in pharmaceutical biotechnology [1–4]. In particular, animal models of inflammatory disorders of the nervous system are commonly used to explore the pathophysiological role of immune cell response in disease triggering and course and to develop biotechnology products for therapeutic use. The regulations concerning the use of animals for scientific purposes require making it under restrictive conditions, taking into account 3Rs — replacement, reduction, and refinement — expressed in 1959 by Russel and Burch [5]. One of the aspects of controversy regarding these regulations is the use of sham surgery (placebo surgery) in animal models of diseases. Sham surgery is a false surgical intervention that leaves out the step thought to be therapeutically or experimentally necessary. Historically, researchers have used sham-operated animals in numerous models of human diseases (cardiovascular, orthopedics, central nervous system diseases etc.). In particular, modeling of neurodegenerative diseases including Parkinson's and Alzheimer's diseases implies surgical manipulations for the intracerebral introduction of disease-initiating substances (toxins, amyloids etc.) [6, 7]. Ethical reasons demand consideration of unmanipulated (unoperated, intact) control animals to minimize animal suffering and unnecessary procedures [8]. However, surgical manipulation *per se* triggers intrinsic side effects, including local tissue damage, inflammation, and wound healing. In addition, non-physical factors can also influence inflammatory response to surgical stress [9, 10]. Therefore, sham surgery control groups are essential to eliminate the influence of these effects on the study results evaluation [11, 12].

Phagocytes — circulating neutrophils and monocytes, as well as tissue-resident macrophages — are key participants of the onset, progression and resolution of inflammation involved inter alia in tissue damage and wound healing [13]. Resident macrophages drive local tissue inflammation and are to a large extent responsible for recruiting of circulating phagocytes into the inflamed area [14, 15]. Literature data and our own results amply evidence, that metabolic features of circulating phagocytes mirrors the course of systemic inflammatory response [16-18]. According to current concept concerning phagocyte plasticity, these cells respond to different environmental stimuli by polarized activation. In this process, phagocytes can acquire two polar functional states: M1 (pro-inflammatory) and M2 (anti-inflammatory), as well as numerous intermediate functional states. M1 polarized activation is associated with increased microbicidal capacity and enhanced secretion of pro-inflammatory cytokines to ulterior initiation and enhancement the cell-mediated adaptive immunity. M2 polarized activation endows phagocytes with ability to participate in antiparasitic immune responses, as well as in allergic reactions, wound healing, resolution of inflammation, and tissue remodeling [19, 20].

Surgical manipulations elicit local and systemic response to injury that leads to alterations in the immune and circulatory systems, including phagocyte function changing. These alterations can manifest in the form of temporary postoperative immunosuppression or post-surgery inflammatory response [21, 22]. All reports concerning postoperative immunomodulation describe fluctuations of immune system cells including phagocytes over short-term period after the surgical manipulations including sham-surgery. However, no study thus far has explored the potential long-term impact of sham surgical procedures on phagocyte functions. The purpose of this study was to evaluate the effect of sham-surgery, commonly used for modeling neurodegenerative diseases, on phagocyte functions in the far terms after the surgical manipulations.

Materials and Methods

Animals and study design. The study was conducted on adult male Wistar rats (220-250 g) bred in the vivarium of the Educational and Scientific Centre "Institute of Biology" of Taras Shevchenko National University of Kyiv, Ukraine. The animals were kept in standard conditions with *ad libitum* access to water and standard diet. Animal protocol was approved by the University Ethics Commitee according to Animal Welfare Act guidelines. All procedures with animals were performed in conformity with the principles of humanity as it was written in "General principles of animal experimentation" approved by the National Congress on bioethics (Kyiv, 2001–2007) and in conformity with Council directive of November 24, 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). Forty rats were used in this study. Before the experiment, animals were randomized by

weight and divided into three groups: I- intact (n=20), II — sham-operated 1 (n=10), and sham-operated 2 (n=10). At the day of surgery, rats from groups II were anesthetized with nembutal (50 mg/kg, i.p., "Sigma", USA), placed in a stereotaxic instrument (SEJ-4, Ukraine), and injected unilaterally with 4 µl of 0.1% ascorbic acid in isotonic saline solution into the left lateral ascending bundle (2.2 mm caudal and 1.5 mm lateral from the bregma in accordance with the atlas coordinates) [23]. Solution was injected into the brain tissue at a rate of 1 µl/min (every 15 s). The injector was left in place for 5 min before slowly withdrawing it to allow for liquid diffusion and prevent reflux. Rats from groups III were anesthetized with a mixture of ketamine (75 mg/kg diluted in sterile water for injection, Sigma, USA) and 2% Xylazine (400 µl/kg, Alfasan International BV, Netherlands) i.p., and injected unilaterally with 10 µg of 0.9% sodium chloride in the volume of 2 µl into the substantia nigra (AP = −5.3; ML = ± 2.0; DV = −7.2) according to Hoban et al., 2013 [24]. Solution was also injected into the brain tissue at a rate of 1 µl/min (every 15s). The injector was left in place for 5 min before slowly withdrawing it to allow for liquid diffusion and prevent reflux.

Hematological parameters, as well as phagocyte metabolic and functional characteristics were investigated on day 29 after the surgery.

Hemogram analysis. Hematological indicators were determined using an analyzer "Particle counter model PCE 210" (ERMA, Japan), adapted for the study of blood cells in rats and mice. In addition to differential leukocyte count, systemic inflammation markers were calculated. Neutrophil to lymphocyte ratio (NLR) was defined as the absolute neutrophil count/absolute lymphocyte count. Derived NLR (dNLR) was calculated as absolute neutrophil count/ white blood cells total count. Lymphocyte to monocyte ratio (LMR) was defined as the absolute lymphocyte count/absolute monocyte count. Systemic immune-inflammation index (SII) was defined as platelet count × NLR. [25].

Microglia cell isolation

Microglia cells were isolated from hippocampus homogenates as described previously [26]. For this purpose, brain was rapidly extracted on ice. Hippocampus was dissected and perfused using a phosphate buffered saline. Isolated tissue was softly dissociated in ice cold phosphate buffered saline supplemented with 0.2% glucose using Potter homogenizer. Tissue homogenate was filtered through a 40 nm cell strainer (BD Biosciences Discovery) and sedimented at room temperature. After this, microglia cells were isolated in Percoll density gradient. Purity of isolated microglia fraction was evaluated by flow cytometry using fluorescein isothiocyanate (FITC) mouse anti-rat CD11b (BD PharmingenTM) and phycoerythrin (PE) mouse anti-rat CD45 (BD PharmingenTM). The proportion of CD11b+CD45+ cells was 88.9 ± 3.7%. Cell viability was determined by Trypan blue exclusion test. The proportion of viable cells was ≥ 93%.

Peritoneal macrophage isolation

Peritoneal macrophages (PM) were isolated without preliminary sensitization as described previously [27]. Rats were sacrificed and PM were harvested using phosphate buffered saline containing 100 U/mL of heparin. Cells were sedimented at 300 g for 5 min at 4 °C, washed twice with serum−free DMEM, and resuspended in DMEM containing 10% FCS and 40 µg/mL gentamycin.

Phagocyte metabolic and phenotypic characteristics assessment

Phagocytic activity was detected as described earlier [17]. FITC-labeled thermally inactivated cells of Staphylococcus aureus Cowan I (collection of the Department of Microbiology and Immunology, ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv) were used as an phagocytosis object. Blood/microglia/ PM samples were incubated at 37 °C for 30 min with bacterial cells. Phagocytosis was arrested by adding a 'stop' solution (phosphate buffered saline with 0.02% EDTA and 0.04% paraformaldehyde). Data are presented as the percentage of phagocytizing cells (PP) and phagocytosis index (PhI) (the average fluorescence per phagocytic cell). The oxidative metabolism (reactive oxygen species (ROS) generation) of phagocytes was explored using 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) as described previously [17].

For phagocyte phenotyping, FITC-labeled anti-CD80/86, and phycoerythrin (PE)-labeled anti-CD206 antibodies (Becton Dickinson, Pharmingen, USA) were used. Results were assessed using FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, USA). Granulocytes and monocytes were gated according to forward and side scatter.

Statistical analysis

All data are presented as mean ± SD. Statistical differences were calculated

using ANOVA with Tukey's post-hoc test. Differences were considered significant at $P \leq 0.05$.

Results and Discussion

Sham-surgery as a control for intracerebral drug introduction was associated with alterations in functional state of phagocytes of different locations in the far terms after the manipulations. Probably, one can regards these alterations as combined long-term consequences of surgical brain injury and the use of anesthetics. Encephalon belongs to immunologically privileged sites, and is isolated form the immune system by the blood-brain barrier (BBB). Instead, brain is equipped with autonomous immune system represented by 80% by microglial cells — resident brain phagocytes [28]. These cells are responsible for immune patrolling and immune surveillance in the brain tissue. In addition, these cells are key drivers of the neuroinflammation. However, recently it has become obviously, that immune-privileged nature of the brain is not absolute, and there is afferent transportation of brain antigens to the peripheral lymphoid tissues, which normally induces immune tolerance to these antigens. BBB weakening is accompanied by the increased exposure peripheral immune cells with brain antigens, and can be associated with the development of local and systemic inflammatory and autoimmune responses [29, 30].

To assess whether sham surgery initiated long-standing inflammatory response, we compared metabolic and phenotypic characteristics of microglia population in intact and lesioned animals. In our experiments, microglia from rats in different sham-groups demonstrated distinct patterns of functional alterations. Proportion phagocytizing cells in microglia population was increased in both sham-groups as compared with intact animals (Fig. 1, *A*), indicating activated state of these cells [31]. At the same time, phagocytic activity in sham-operated group 1 was lower than that in other two groups (Fig. 1, *B*). Microglia from these animals was also characterized by increased ROS generation. Taken together, such results indicate residual

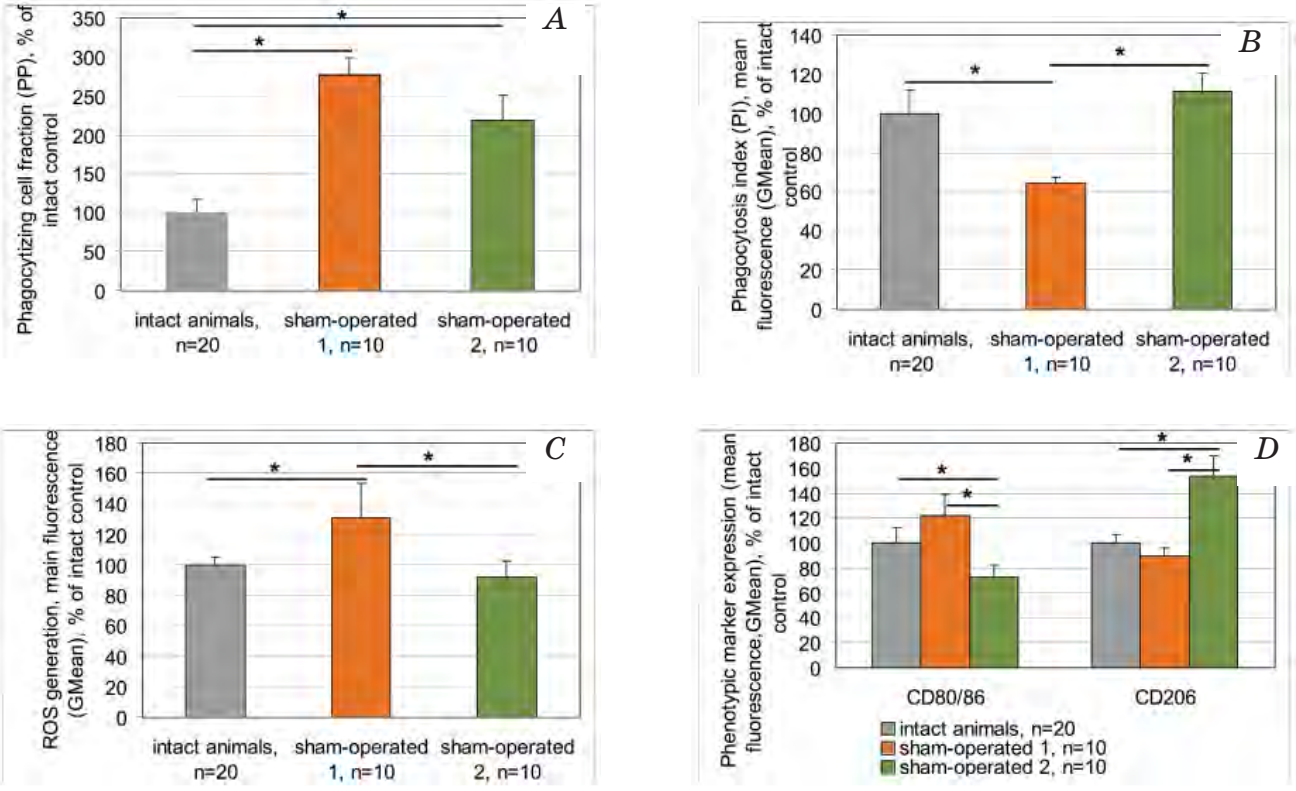

Fig. 1. **Functional and phenotypic characteristics of microglial cells of rats in the far terms after the sham-surgical manipulations:**

A, B — phagocytic activity; *C* — oxidative metabolism; *D* — phenotypic marker expression. Data are presented as Mean ± SD. * indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test)

pro-inflammatory shift of these cells [32]. One of the reasons of this effect can be the ability of sodium pentobarbital to induce local and systemic inflammation after the i.p. injection [33]. In microglial cell from rats in sham-operated group 2, down-regulated CD80/86 expression along with overexpression of CD206 was registered. It indicates moderate anti-inflammatory functional skew of these cells, probably, associated with the resolution of neuroinflammation caused by surgical brain injury.

Both the relative and absolute counts of circulating leukocytes, including myeloid cells, are key indexes for quantifying the magnitude of a systemic inflammation [25]. In our experiments, monocytosis was revealed in rats from sham-operated group 2 (Fig. 2, *A* and *B*). Our observations are in agreement with data reported by Spencer et al., 2022 concerning the ability of ketamine (used in this group) to exert sex-specific anti-inflammatory effect. This effect manifested as a decrease in the plasma level of interleukin 6, of which the principal source is monocytes [34]. Monocytosis entailed the decrease of LMR value in rats from sham-operated group 2 (Table 1). Values of remaining indicators in sham-operated animals didn't differ significantly from those in intact rats.

Minor changes in quantitative indices of circulating phagocytes in sham-operated animals were associated with rather substantial alterations of their functions. Minor changes in quantitative indices of circulating phagocytes in sham-operated animals were associated with rather substantial alterations of their functions. In expanded monocyte fraction in rats from sham-operated group 2, increased phagocytizing cell fraction was detected (Fig. 3, *A*) with low phagocytic intensity (Fig. 3, *B*). It can indicate the recruitment of bone marrow derived monocytes, which might be released into the peripheral blood under stress conditions, and are characterized by lowered phagocytic activity [35]. ROS generation of circulating phagocytes in sham-operated animals from both groups was decreased as compared to intact animals (Fig. 3, *C*), whereas both CD80/86 and CD206 were overexpressed (Fig. 3, *D*). CD206 up-regulation is a phenotypic marker of phagocyte anti-inflammatory metabolic shift, whereas CD80/86 overexpression is a marker of pro-inflammatory metabolic shift [36]. Nevertheless, it is necessary to note that CD80 is also up-regulated in highly phagocytizing, CD206-overexpressing circulating myeloid-derived suppressor cells (MDSC) [37].

We inclined to suggest, that taken together metabolic and phenotypic characteristics of circulating phagocytes in rats in the far terms after the sham-surgical manipulations indicate ongoing resolution of systemic inflammation.

Peritoneal macrophages (PM) belong to the omentum-associated lymphoid tissue (Omentum-Associated Lymphoid Tissue, OALT), which has many features in common with MALT and is actively involved in initiating and controlling local and systemic inflammatory processes. OALT is closely related to the systemic vascular network and interacts with the central nervous system and the hypothalamic-pituitary-adrenal axis [38]. The biological characteristics of this tissue are not yet fully understood. However, there is ample evidence of the integrative role of PM and other OALT cells in the pathophysiology of inflammatory diseases. This circumstance as well as the fact that anesthetics in our experiments were introduced i.p. raised our

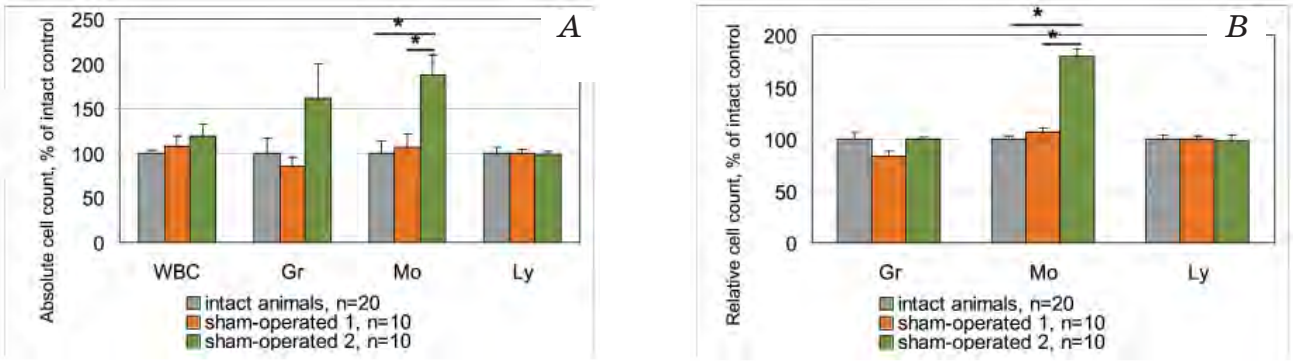

Fig. 2. **Differential leukocyte count in rats in the far terms after the sham-surgical manipulations. Data are presented as Mean ± SD. * indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test)**

Table 1. **Leukocyte ratios in rats in the far terms after the sham-surgical manipulations**

Animal group	Coefficients			
	NLR	dNLR	LMR	SII
Intact (***n*** = 20)	0.32 ± 0.03	0.32 ± 0.07	11.0 ± 2.44	33.73 ± 9.09
Sham-operated 1 (*n* = 10)	0.25 ± 0.05	0.34 ± 0.12	8.45 ± 2.28	32.85 ± 8.04
Sham-operated 2 (*n* = 10)	0.25 ± 0.06	0.25 ± 0.07	5.52 ± 2.28*	41.91 ± 7.83

Note: * indicates significant ($P \leq 0.05$) differences in comparison with intact animals (ANOVA with Tukey post-hoc test), Mean ± SD. NLR — neutrophil to lymphocyte ratio, dNLR — derived neutrophil to lymphocyte ratio, LMR — lymphocyte to monocyte ratio, SII — systemic immune inflammation index.

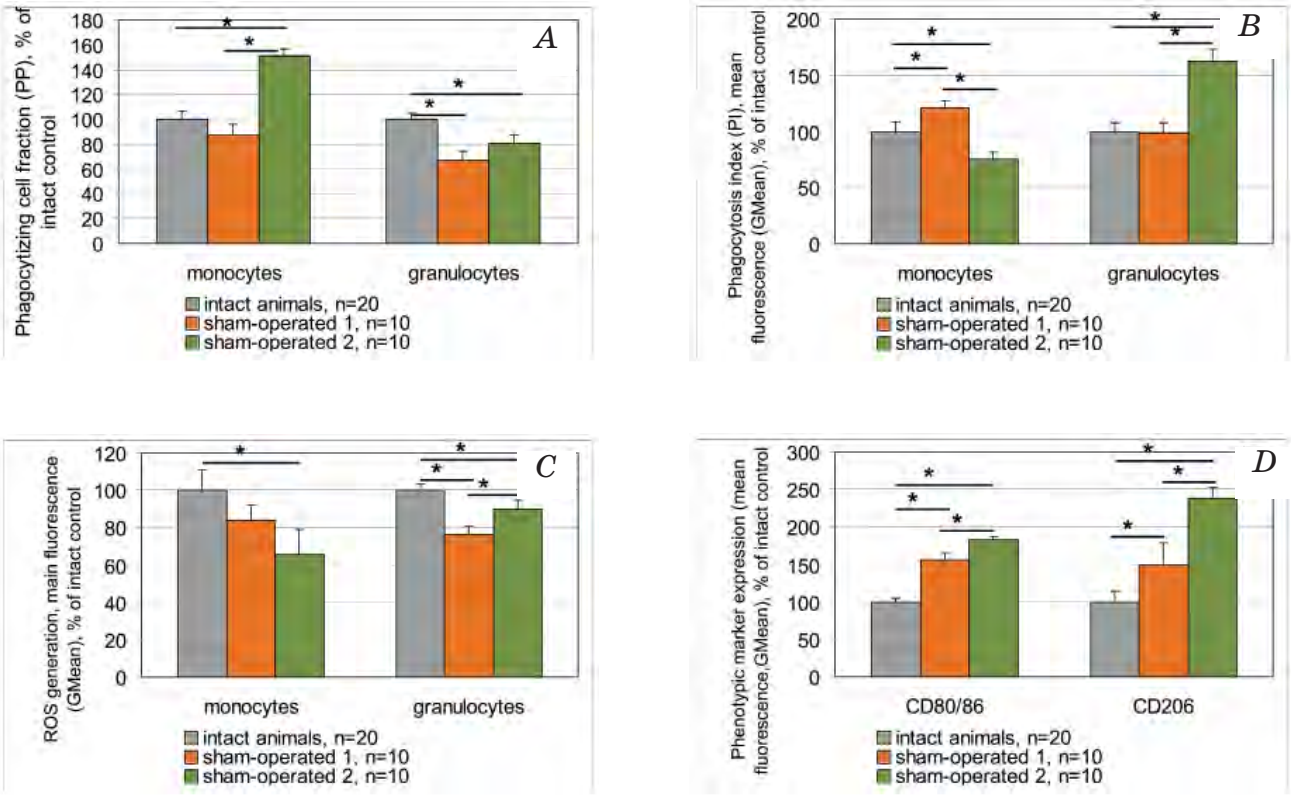

Fig. 3. **Functional and phenotypic characteristics of circulating phagocytes of rats in the far terms after the sham-surgical manipulations:**

A, B — phagocytic activity; *C* — oxidative metabolism; *D* — phenotypic marker expression. Data are presented as Mean ± SD.

* indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test).

interest to examining these cells in sham-operated animals. Metabolic and phenotypic characteristics of PM in sham-operated rats differed from those in intact animals in the far terms after the sham-surgical manipulations, and varied slightly between placebo-surgery groups. It might be stipulated by the difference in the effects of ketamine/xylazine and barbiturates on phagocytic cells, reported by Hristovska et al. [39]. In rats from sham-operated group 2, which received i.p. injection of ketamine/xylazine, we observed highly increased proportion of phagocytizing PM (Fig. 4, *A*).

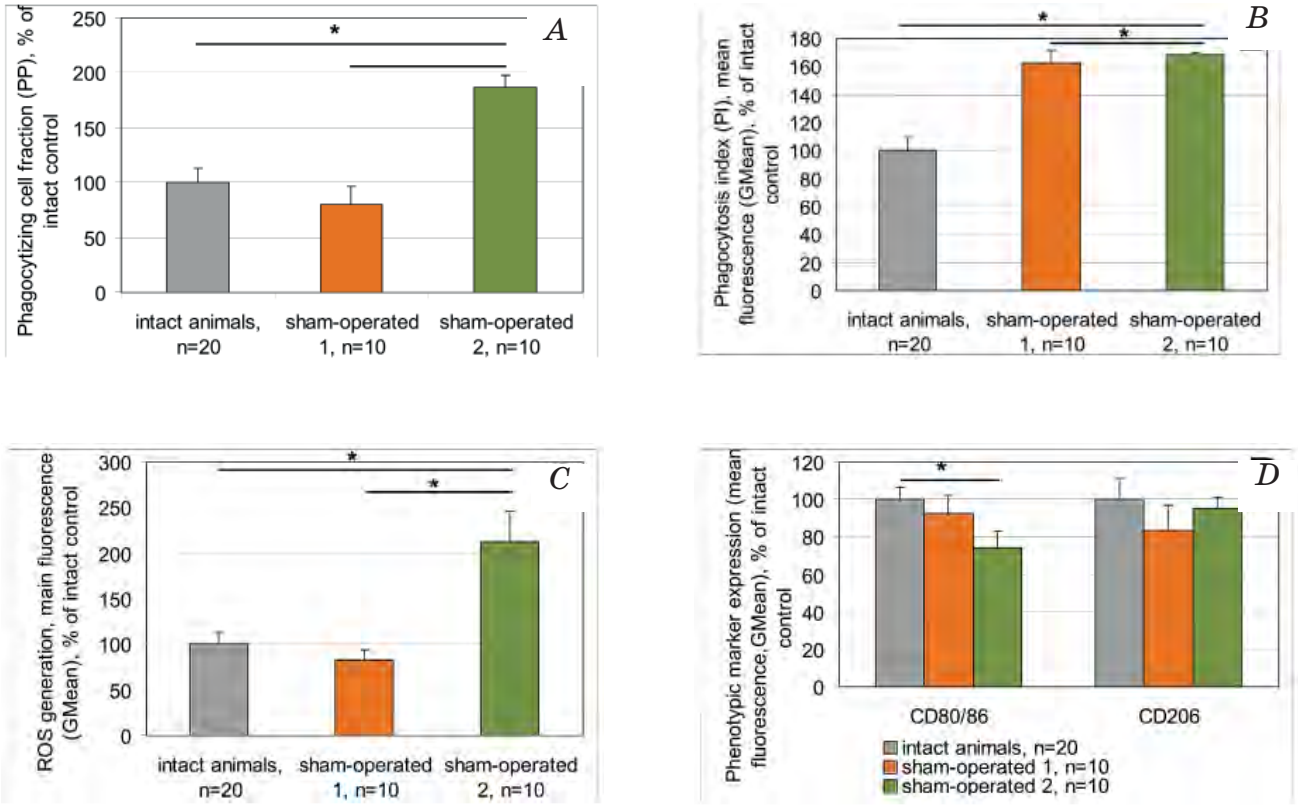

Fig. 4. **Functional and phenotypic characteristics of peritoneal macrophages of rats in the far terms after the sham-surgical manipulations:**
A, B — phagocytic activity; *C* — oxidative metabolism; *D* — phenotypic marker expression. Data are presented as Mean ± SD. * indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test)

These cells were characterized by up-regulated phagocytic intensity (Fig. 4, *B*) and augmented ROS generation (Fig. 4, *C*). CD80/86 membrane expression was decreased in these cells, the level of CD206 membrane expression didn't differ significantly as compared to intact animals. Although CD80/86 up-regulation indicates pro-inflammatory phagocyte metabolic shift, whereas down-regulation is a marker of anti-inflammatory polarized activation of phagocyte, overexpression of these co-stimulatory molecules is associated with acquiring antigen-presenting capacity by activated phagocytes, which is characteristic for terminal phases of inflammation. Phagocytes during earlier phases of inflammation are featured by decreased co-stimulatory molecules expression along with increased phagocytic activity and high production of reactive oxygen and nitrogen species [40]. Thus, metabolic and phenotypic characteristics of PM in rats from sham-operated group 2 evidence ongoing inflammation in peritoneal cavity 28 days after the sham-surgery with the use of i.p. ketamine/xylazine as anesthetics. Surprisingly, PM in rats from sham-operated group 1, which received i.p. injection of nembutal during placebo-surgery, demonstrated no substantial alterations in their functional and phenotypic characteristics excluding increased phagocytic activity, which can be considered as a sign of residual resolution of inflammation in peritoneal cavity.

Thus, our results exposed a hitherto underappreciated impact of sham-surgery on phagocytes of different subsets and location even in the far terms after the sham-surgical manipulations. Based on our results, we conclude that sham associated confounding effects should always be taken into consideration when examining phagocyte subsets in animal models of inflammatory central nervous system diseases.

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Conflicts of Interest

Authors declare no conflict of interest.

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ДОВГОСТРОКОВІ ЕФЕКТИ ПЛАЦЕБО-ХІРУРГІЇ НА ФУНКЦІЇ ФАГОЦИТІВ У ЩУРІВ

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Експериментальне моделювання запальних захворювань, у тому числі нервової системи, зазвичай використовується для вивчення патофізіологічної ролі клітин імунної системи у виникненні та перебігу захворювання, а також для розробки біотехнологічних продуктів для терапевтичного застосування. Моделювання деяких запальних захворювань, зокрема нейродегенеративних, передбачає проведення хірургічних маніпуляцій із внутрішньомозкового введення тригерних субстанцій (токсинів, амілоїдів та ін.), що спричиняють розвиток захворювання. Схема цих експериментів включає використання хибнооперованих тварин, як контроль неспецифічних побічних ефектів, викликаних хірургічними маніпуляціями, як такими, у т.ч. місцевого та системного запалення, в якому ключовими учасниками є фагоцитарні клітини. Широко відомі короткострокові післяопераційні імуномодуляторні ефекти. Однак досі не вивчено довгострокових ефектів плацебо-хірургії на функції фагоцитів.

Метою дослідження було оцінити вплив плацебо-хірургії, що зазвичай використовується для моделювання нейродегенеративних захворювань, на функції фагоцитів у віддалені терміни після хірургічних маніпуляцій.

Матеріали та методи. У дослідженні використовували дорослих щурів-самців лінії Вістар. Плацебо-операція полягала в односторонньому стереотаксичному введенні фізіологічного розчину в серединний пучок переднього мозку (група 1) або безпосередньо в чорну субстанцію (група 2). Перед плацебо-операцією тварин анестезували нембуталом та кетаміном/ксилазином, відповідно. Функціональні характеристики (фагоцитарну активність, оксидативний метаболізм, експресію CD80/86 та CD206) фагоцитів (мікроглії, перитонеальних макрофагів, моноцитів та гранулоцитів, що циркулюють) досліджували методом проточної цитометрії. Диференціальний підрахунок лейкоцитів проводили за допомогою гематологічного аналізатора.

Результати. Фагоцити тварин, що зазнали різних протоколів плацебо-операцій, демонстрували різний характер функціональних змін на 29 добу після маніпуляцій. У тварин із групи 1 ми спостерігали ознаки залишкового нейрозапалення (прозапальний зсув функціонального профілю мікроглії) поряд з триваючим завершенням системного запалення (протизапальний метаболічний зсув циркулювальних фагоцитів і перитонеальних макрофагів). У щурів з групи 2 реєстрували прозапальну поляризовану активацію перитонеальних фагоцитів поряд з протизапальним зсувом мікроглії та циркулювальних фагоцитів.

Висновки. Плацебо-хірургія впливає на функції фагоцитуючих клітин різної локалізації навіть у віддалені терміни після маніпуляцій. Ці ефекти можна розглядати як комбіновані віддалені наслідки хірургічної травми головного мозку та застосування анестетиків. Наші спостереження свідчать про те, що неспецифічні імуномодуляторні ефекти плацебо-хірургії завжди слід брати до уваги при експериментальному моделюванні запальних захворювань центральної нервової системи.

Ключові слова: плацебо хірургія; фагоцити; нейрозапалення; системне запалення.