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TIME-DEPENDENT CHANGES IN THE EXPRESSION OF TRPV4 AND TRPM8 CHANNELS IN THE COLON OF RATS WITH 6-OHDA-INDUCED PARKINSON'S DISEASE

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Parkinson's disease (PD) is neurodegenerative disease, which is accompanied by degeneration of dopaminergic neurons in subtantia nigra. Non-motor symptoms, in particular, disorders of the gastrointestinal (GI) tract are observed in 20-80% of patients some 15-20 years before clinically diagnosed PD and are not a least important feature of PD pathogenesis. The transient receptor potential (TRP) channels are expressed throughout the GI tract, where they play an important role in taste, thermoregulation, pain, mucosal function and homeostasis, control of interstitial motility etc. The aim of this study was to investigate the contribution of TRPV4 and TRPM8 channels in the GI motor function in the colon of rats with PD, induced by injection of the 12 μg 6-hydroxydopamine (6-OHDA). The studies were performed on the 4th week and the 7th month after PD-induction. The rats were randomly divided into: I group – the sham-lesioned rats, 4 μl 0.9% NaCl, autopsy 4 weeks after injection (n = 5); II group – the 6-OHDA-PD rats, 4 μl 12 μg of 6-OHDA, autopsy 4 weeks after injection (n = 5); III group – the sham-lesioned rats, 4 μl 0.9% NaCl, autopsy 7 months after injection (n = 4); IV group – the 6-OHDA-PD rats, 4 μl 12 μg of 6-OHDA, autopsy 7 months after injection (n = 5). We evaluated the body weight of rats, GI transit time, the cecum weight index and immunohistochemical identification of tyrosine hydroxylase (TH)-positive cells, TRPV4 and TRPM8 expression in rat's colon. We showed that on the 7th month of the experiment, the GI transit time doubles over time; the cecum weight index of 6-OHDA rats increased by 57%; the number of TH-positive cells in colon rats decreased 2-fold, while TRPM8 ion channels were downregulated in PD rats and TRPV4 ion channels were upregulated in the colon of rats with 6-OHDA-PD. It was concluded that TRPV4 and TRPM8 ion channels may be considered pharmacological targets in the progression of PD pathology.

K e y w o r d s: 6-OHDA, transit time, motility, colon, TRPV4 channels, TRPM8 channels.

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic (DA) neurons in substantia nigra pars compacta (SNpc) and depletion of dopamine in striatum. Diagnosis of PD occurs with the onset of motor symptoms (e.g. bradykinesia, muscular rigidity, rest tremor, and postural and gait impairment) which are preceded by 20 years or more with numerous non-motor symptoms [1]. PD is a multi-system disorder involving not only the central nervous system, but also the peripheral and enteric nervous systems [2]. Patients with neurological disorders including PD frequently present with coexistent non-motor bowel diseases such as dysbiosis, constipation, motility dysfunction, immune dysregulation and dysfunction of intestine permeability [3, 4]. The mechanisms of these processes are insufficiently studied, so it is important to find new targets for the study of PD pathology.

Furthermore, PD is most prevalent in older people (>65 y.o.) [5]. But the long-term changes on PD animal models are not given enough attention, while the long-term gastrointestinal (GI) changes in the model of PD have not been studied yet [6-8].

The transient receptor potential (TRP) channels belong to the superfamily of cationic channels. Their activation induces changes of membrane potential (depolarization) and/or intracellular calcium concentration ($[Ca^{2+}]_i$) [9]. TRP channels are expressed throughout the GI tract, where they play an impor-

tant role in taste, thermoregulation, pain, mucosal function and homeostasis, control of interstitial motility etc. [10, 11]. The TRPM channels represent one of the largest and most variable subfamilies of the TRP channels. The chemosensory role of TRPM8 channels is the most probable function in the digestive system, hence a TRPM8-dependent modulation of GI motility is likely. There are findings on the role of these channels in the regulation of the inflammatory responses too [12]. Another large subfamily is transient receptor potential vanilloid-type (TRPV) channels. It was shown that TRPV4 can regulate vascular endothelial permeability during colonic inflammation, gut motility through activation of intestinal muscularis macrophages [7, 9]. The role of these channels in the GI symptoms in PD patients is unclear.

In our previous study [14] we used a rat model of easily reproducible partial lesion of the nigrostriatal system (unilateral injection of 6-hydroxydopamine (6-OHDA) and showed reduced spontaneous and carbachol-stimulated colon motor activity in it. Moreover, pharmacological activation of TRPV4 ion channels by their selective agonist GSK1016790A decreased the contractile activity of colon smooth muscle cells in the PD rats model [15].

The aim of the present study was to investigate the long-term changes in GI motor function in the 6-OHDA model of PD and the possible contribution of TRPM8 and TRPV4 ion channels in it.

Materials and Methods

Animals. Studies were done on male Wistar rats (200-220 g, n = 19). Animals have been bred and housed in a standard temperature condition (21-23°C), lighting (12/12 h light-dark cycle), at humidity (30-35%) in the animal facility of Taras Shevchenko National University of Kyiv. All animals had unlimited access to chow and tap water. The study has been carried out in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and approved by the Bioethical Committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv, Ukraine.

Modelling of 6-OHDA–induced PD in rats. The dopamine neurons were disrupted by unilateral stereotaxic microinjections of 12 μg of selective neurotoxin 6-OHDA (Sigma, USA) in the medial forebrain bundle. The sham-lesioned groups of animals were injected with 4 μl 0.9% NaCl as described in more detail previously [14]. In brief, 6-OHDA was dissolved in 4.0 μl of NaCl with the addition of 0.1% ascorbic acid (as a stabilizer inhibiting oxidation of 6-OHDA). Anesthetized rats (mix of 75 mg/kg ketamine (Sigma, USA) and 2% xylazine (100 μl/rat, Alfasan International BV, the Netherlands), i.p. by total volume 1 ml) were placed on the modified stereotaxic apparatus (SEG-4). The 6-OHDA-model of the PD was modeled by stereotactic coordinates (mm) from the bregma AP = -2.2; ML = 1.5; DV = -8.8.

Study design. The rats were randomly divided into: I group – the sham-lesioned rats, 4 μl 0.9% NaCl, autopsy 4 weeks after injection (n = 5); II group – the 6-OHDA-PD rats, 4 μl 12 μg of 6-OHDA, autopsy 4 weeks after injection (n = 5); III group – the sham-lesioned rats, 4 μl 0.9% NaCl, autopsy 7 months after injection (n = 4); IV group – the 6-OHDA-PD rats, 4 μl 12 μg of 6-OHDA, autopsy 7 months after injection (n = 5). The day of PD-induction was considered as the 1st day of the experiment. The details of these experiments are illustrated in Fig. 1.

The body weight was measured before surgery (the 1st day) (Gr. I-IV), at the 4th week (Gr. I-IV) and the 7th month (Gr. III-IV) of the experiment. At the end of the experiment, rats were euthanized by CO_2 inhalation with further cervical dislocation. A section of colon from the anus to cecum was isolated immediately after euthanasia and processed for immunohistochemistry or mucus biochemical assays.

Gastrointestinal transit assay. Rats were orally gavaged with 0.5 ml aqueous solution of 3% carmine red (ECO Resource, Ukraine) and placed in a new cage with no bedding. The time at which gavage took place was recorded as T_0. Starting at 120 min post-gavage, rats were monitored every 10 min for the production of a red fecal pellet. Total GI transit time was considered as the interval between T_0 and the time of the first observance of carmine red in stool [14].

Immunohistochemistry. Immunostaining was performed using the paraffin-embedded 5 μm sections. The 1 cm of full-thickness colonic tissue samples were fixed in 4% paraformaldehyde (pH 7.4), embedded in paraffin, sectioned (5-μm thick sections). The sections were deparaffinized, unmasked the epitopes with Target retrieval solution (50x) for 15 min (Dako, Envision Flex, DM828, USA), blocked for endogenous peroxidase using Peroxi-

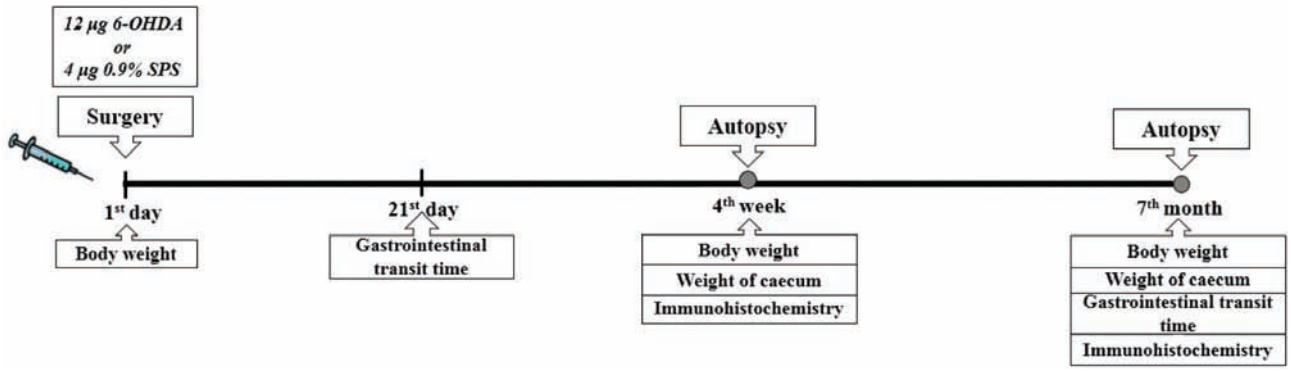

Fig. 1. The scheme of the experiment. Doses and solvents in 6-OHDA-induced PD: 12 μg of selective neurotoxin 6-hydroxydopamine (6-OHDA, Sigma, USA). The sham-lesioned groups of animals were injected with 4 μl 0.9% NaCl

dase blocking reagent for 3 min (Dako, EnVision Flex, DM827, USA), washed in dH_2O, washed in the Wash solution (50x, Artisan), incubated in 4% skimmed milk powder for 20 min. The 30 min incubation at T_{room} with primary antibodies: TH-primary antibodies (1:200, Millipore, AB152, USA), TRPM8 polyclonal primary antibodies (1:200, Invitrogen, OSR00077W) or anti-TRPV4 polyclonal antibody (1:200, Abcam, ab39260), followed by the horseradish peroxidase –linked secondary antibodies (biotinized anti-rabbit, 1:200) for 30 min, T_{room}. Diaminobenzidine (Dako, EnVision Flex, DM827, USA) immunoreactivity detection system was used for 2 min.

Statistical analysis. Data are presented as mean±standard derivation (SD). In cases when the normality hypothesis was not obeyed, the probability index (P) was calculated by the rank nonparametric Mann-Whitney criterion. In other cases the *t*-Student test was applied. The difference was considered statistically significant at $P < 0.05$. GraphPad Prism 8.0.2 was used for making graphs.

Results and Discussion

Body weight is a classic non-motor diagnostic parameter for the detection of PD. Patients affected by PD often show marked changes in body weight: they may gain or lose a lot of weight depending on the stage of the disease [16]. Comorbidity of weight loss and malnutrition may impact PD progression, giving rise to dyskinesia and cognitive decline [17]. The sham-lesioned rats gained 43% of body weight between the 1st day and 4th week of the experiment whereas the 6-OHDA-PD rats only 27% that is 11% less vs. the control group ($P < 0.05$). At the 7th month, we observed increased body weight in the 6-OHDA-PD rats by 16% (216.78 ± 19.98%) vs. age-matched sham-lesioned group (200.62 ± 5.19%) (Fig. 2, *A*).

Despite the fact that the defining symptoms of PD are motor symptoms, patients also often suffer from GI disorders. Gastric and intestinal dysfunction can occur at any stage of PD development, and some of the symptoms may appear many years before the disease is diagnosed [18]. We observed the delay (27%, $P < 0.01$) of GI transit time via whole GI tract in 6-OHDA-PD rats at the 3rd week of the experiment vs. age-matched sham-lesioned group (Fig. 2, *B*). Aging was shown to accelerate the gastric and small intestinal transit significantly [19]. In our study, at the 7th month of the experiment the GI transit time in control rats was 2.5-fold higher vs. control rats on the 3rd week of the experiment ($P < 0.001$). Despite this profound age-related delay in GI tract motility, still 6-OHDA-PD rats had a slower by 9% ($P < 0.001$) GI transit time vs. age-matched control group (Fig. 2, *B*). In our previous study [15], rats with 6-OHDA-PD at the 7th month of the experiment had higher levels of water contents in the feces vs. the 3rd week of the experiment. The water contents in the feces is a marker of colonic inflammation that might be the course of observed less profound GI transit delay on the 7th month of the experiment.

The cecum weight along with a spleen size are macroscopic indicators of colonic inflammation [20]. We did not observe significant changes in the cecum weight index on the 4th week of experiment in 6-OHDA-PD vs. the sham-lesioned rats. While, on the 7th month of the experiment, 6-OHDA-PD rats had a tendency to enlarge the cecum weight index vs. age-matched sham-lesioned group (Fig. 2, *C*). The development of an enlarged cecum is a typical feature in germ-free rodents [21]. It seems that

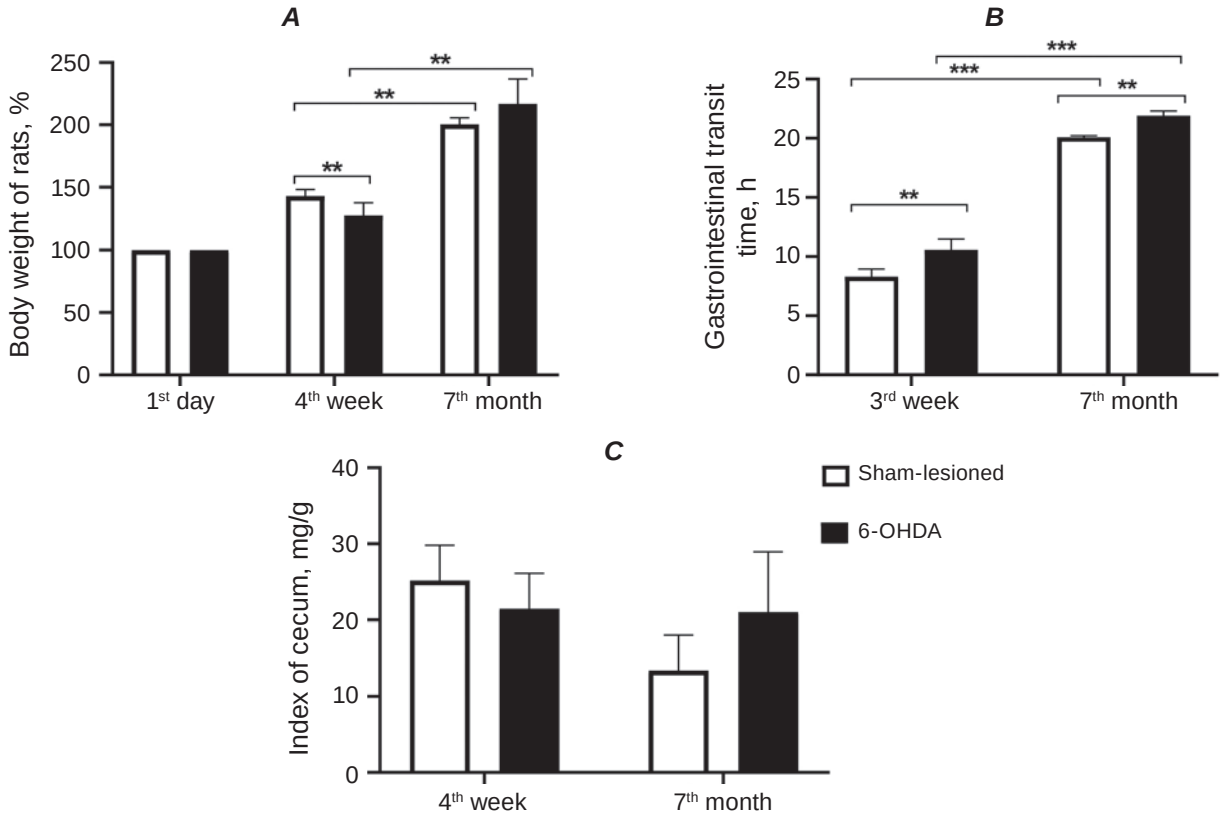

Fig. 2. 6-OHDA-induced PD was associated with a delay in intestinal transit time that became more significant with age. ***A*** *– the changes in dynamics of body weight in rats with PD on 1st day, 4th week and 7th month after surgery, %, The body weight of rats on the 1st day was taken as 100% and each successive was counted in relation to the 1st day;* ***B*** *– the total GI transit time of gut (by 0.5 ml of 3% carmine red) of rats with PD;* ***C*** *– the index of cecum weight of rats with PD, mg/g. *P < 0.05, **P < 0.01, ***P < 0.001 (M ± SD), n = 19*

cecum enlargement depends on the amount of accumulated substances that in the conventional animals should be degraded by microbial fermentation. Moreover, in a recent review [22] has been proposed the contribution of the appendix to PD development while an early childhood appendectomy as the decrease risk for PD. Interestingly, the cecum weight index of the sham-lesioned rats was twice lower on the 7th month of the experiment vs. the 4th week. To our opinion, such a difference can be explained by twice increased rat body weight on the 7th month of the experiment vs. the 4th week (Fig. 2, *A*).

Jeanette M Shultz et al. [23] showed that 6-OHDA-treated monkeys had reduced TH-ir and AADC-ir in proximal colon myenteric plexus 3 months post-neurotoxin. The loss of TH-positive cells in the gut is selectively due to the disappearance of DA neurons within both myenteric and mostly submucosal plexus in the intestine, while no changes are detected in the esophagus and stomach. TH is a key enzyme involved in the synthesis of dopamine. A loss of DA neurons has been described in the myenteric plexus of patients with PD [24]. Immunohistochemically, we observed a 2-fold decrease in the number of TH-positive cells in rat's colon with parkinsonism on the 4th week vs. the sham-lesioned group (Fig. 3). The color intensity of TH-positive cells in the sham-lesioned and PD groups almost disappeared with age. The localization of TH-positive cells is apically in crypt epitheliocytes.

TRPM8 expression has been reported in afferent neurons, myenteric plexus, and distal mouse colon epithelial cells [41] as well as in rat smooth muscles of the stomach and colon [27]. Antonella Amato et al. [12] demonstrated that the exogenously activated TRPM8 receptors were able to reduce the colonic smooth muscle contractility. They suggest that the spasmolytic effects are mediated by a direct action on the muscle cells, involving large-conductance Ca^{2+}-dependent K^+-channels. The TRPM8 re-

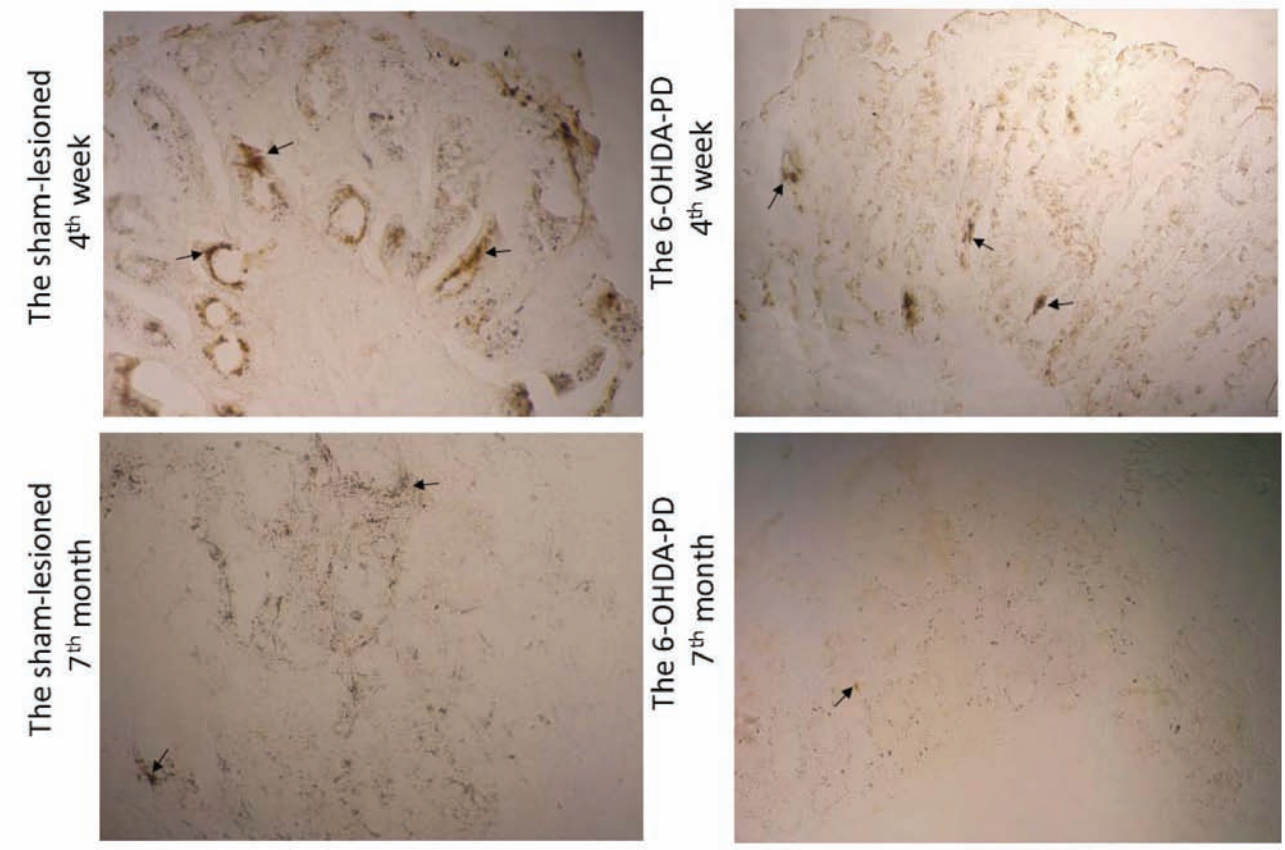

Fig. 3. Photomicrographs of TH-positive immunostaining (→, brown) of dopaminergic neurons in formalin-fixed, paraffin-embedded rat's colon (5 μm) (n = 19) (x20)

ceptors have a preference for Ca^{2+} permeation. The activation of M3 muscarinic receptors in intestine mobilizes Ca^{2+} from intracellular stores to induce mucus secretion [28].

Thus, TRPM8 ion channels may play an important role in GI pathology in PD. We observed complete disappearance of TRPM8 receptors in rat's colon with parkinsonism vs. the sham-lesioned groups (Fig. 4). However, over time, their numbers almost have not been changed in the sham-lesioned group at the 7th month of the experiment. The localization of TRPM8 ionic channels is on epitheliocytes of colons crypts.

TRPV4 channels were detected in thin nerve fibers associated with blood vessels of the submucosa and serous membranes of the stomach, small and large intestine [29], in the mesenteric and pelvic afferent neurons, on the basolateral surface of epithelial cells of the GI tract [30], as well as on the surface of macrophages of the muscular membrane (muscularis macrophages) of the GI tract [11]. When activated, they are able to increase the intracellular level of Ca^{2+}, which can positively affect the motor function of the GI tract. In addition, disruption of calcium homeostasis in neurons leads to the loss of these cells or their functions and the development of neurodegenerative diseases such as PD [31]. Increased expression of TRPV4 ion channels in vascular endothelial cells promotes the development of inflammatory processes in the colon due to increased vascular permeability. The study by Yamawaki et. al. [30]. Showed that TRPV4 ion channels can regulate transepithelial transport of substances in the digestive tract. The use of TRPV4 ion channel agonists causes a significant decrease in transepithelial resistance in IEC-6 cell culture (Intestinal Epithelial Cells) and, as a consequence, causes their hyperpermeability, which is observed in the pathogenesis of PD [32].

We observed increased expression of TRPV4 ionic channels in rat's colon with parkinsonism vs. the sham-lesioned groups (Fig. 5). Over time, their numbers are decreasing in both the sham-lesioned group and the 6-OHDA-PD group. The localization of TRPV4 ionic channels is apically on epitheliocytes of colons crypts.

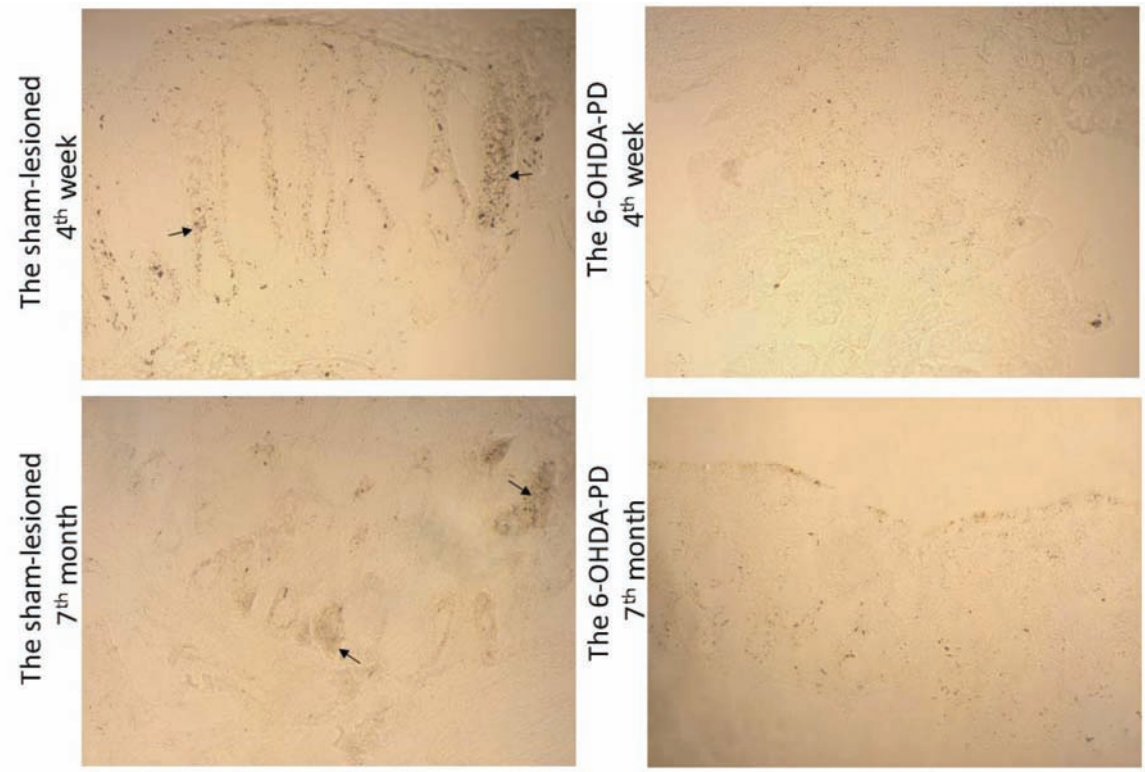

Fig. 4. Photomicrographs of TRPM8 ionic channels – positive immunostaining (→, brown) in formalin-fixed, paraffin-embedded rat's colon (5 μm), (n = 19) (x20)

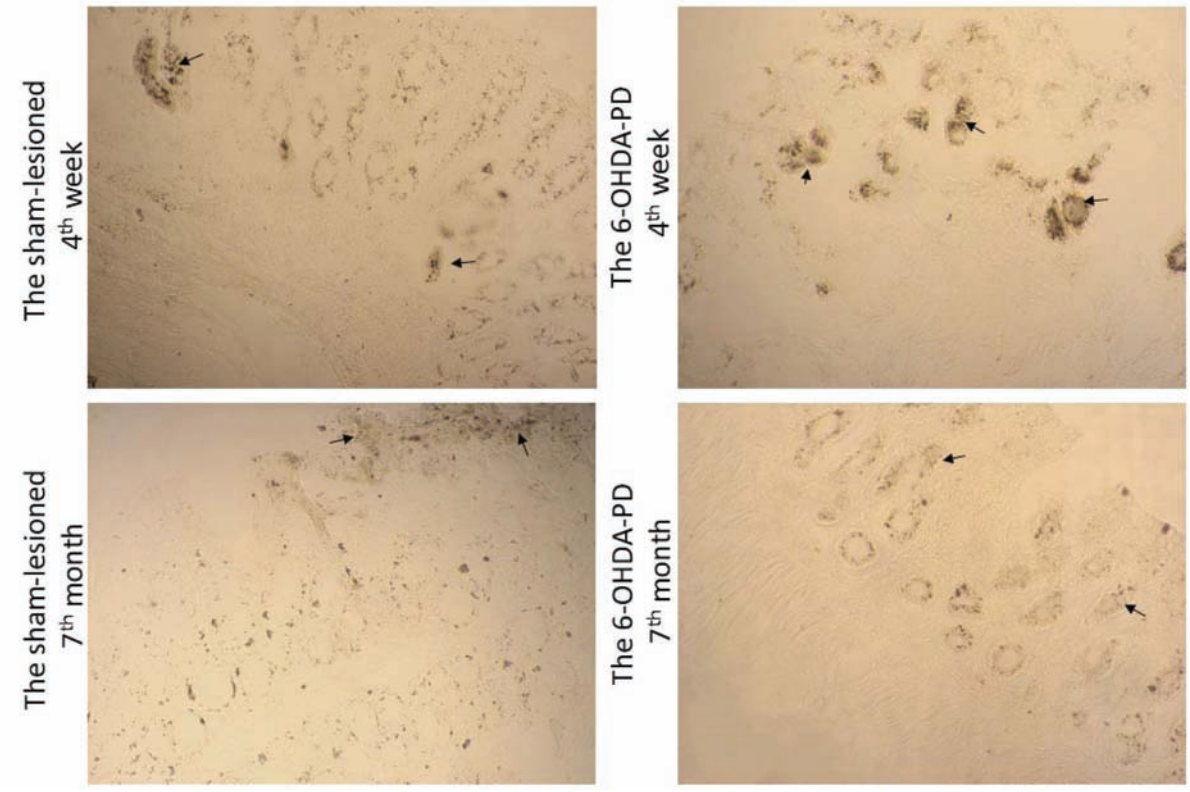

Fig. 5. Photomicrographs of TRPV4 ionic channels - positive immunostaining (→) in formalin-fixed, paraffin-embedded rat's colon (5 μm), (n = 19) (x20)

Conclusions. In the present study, we reported for the first time the long-time changes of intestine in 6-OHDA-induced PD *in vivo* at the 7th month of the experiment. We found that the GI transit time in 7-month-old rats doubles over time. In addition, the changes in GI transit time in rats with PD are greater, the consequence of which is constipation in rats with PD. There is also a 2-fold decrease in TH-positive neurons, disappearance of TRPM8 ion channels and a 2-fold increased expression of TRPV4 ion channels in the colon of rats with 6-OHDA-PD. Thus, it was shown that in the 6-OHDA model of PD there is an increase in non-motor symptoms of PD with age, as well as changes that occur at the level of expression of these ion channels. Thus TRPV4 and TRPM8 ion channels may be a new pharmacological target in PD pathogenesis.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ЗМІНИ В ЕКСПРЕСІЇ TRPV4 ТА TRPM8 КАНАЛІВ У ТОВСТІЙ КИШЦІ ЩУРІВ ІЗ ІНДУКОВАНОЮ 6-OHDA ХВОРОБОЮ ПАРКІНСОНА

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Хвороба Паркінсона (ХП) – це нейродегенеративне захворювання, яке супроводжується дегенерацією дофамінергічних нейронів у чорній субстанції. Немоторні симптоми, зокрема розлади шлунково-кишкового тракту (ШКТ), спостерігаються у 20-80% пацієнтів за 15-20 років до клінічно діагностованої ХП і є не менш важливою ознакою цієї хвороби. Транзієнтний рецептор потенціалу (TRP) каналів експресується у ШКТ, де вони відіграють важливу роль у смаку, терморегуляції, болю, функції слизової оболонки, гомеостазі, контролі інтерстиціальної рухливості тощо. Мета цієї роботи – дослідити внесок TRPV4 та TRPM8 каналів у моторну функцію ШКТ щурів з ХП, яку було індуковано ін'єкцією 12 мкг 6-гідроксидофаміну (6-OHDA). Дослідження проводили через 4 тижні та 7 місяців після моделювання ХП. Щурів було випадковим чином розділено на чотири групи: I група – хибнооперовані щури, ін'єкція 4 мкл 0,9% NaCl, аутопсія через 4 тижні після ін'єкції (n = 5); II група – щури з 6-OHDA індукованою ХП, ін'єкція 4 мкл 12 мкг 6-OHDA, аутопсія через 4 тижні після ін'єкції (n = 5); III група – хибнооперовані щури, ін'єкція 4 мкл 0,9% NaCl, аутопсія через 7 місяців після ін'єкції (n = 4); IV група – щури з 6-OHDA індукованою ХП, ін'єкція 4 мкл 12 мкг 6-OHDA, аутопсія через 7 місяців після ін'єкції (n = 5). Оцінювали масу тіла щурів, час проходження ШКТ, індекс маси цекуму та імуногістохімічну ідентифікацію тирозин гідроксилази (TH)-позитивних клітин, експресію TRPV4, TRPM8 каналів товстої кишки. Показано, що на 7-й місяць експерименту, час проходження ШКТ збільшився вдвічі; індекс маси цекуму в щурів із 6-OHDA збільшився на 57%; у товстій кишці щурів знизилася кількість TH-позитивних клітин в 2 рази, тоді як TRPM8 іонні канали були знижені у щурів із ХП, а TRPV4 іонні канали були підвищені в товстій кишці щурів із 6-OHDA-ХП. Зроблено висновок, TRPV4 та TRPM8 іонні канали можуть бути фармакологічною мішенню у терапії патогенезу ХП.

Ключові слова: 6-OHDA, кишковий транзит, моторика, товста кишка, TRPV4 та TRPM8 канали.

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