

EFFECTS OF *CAMELLIA SINENSIS* EXTRACT ON PASSIVE AVOIDANCE LEARNING AND HIPPOCAMPAL NEUROGENESIS IN RATS

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We investigated the effects of a concentrate of green tea (extract of *Camellia sinensis*) on passive avoidance learning and hippocampal neurogenesis in male Wistar rats. Twenty-one male rats were divided into control, sham, and experimental groups. Rats of the control group were left intact, sham rats received infusions of 1.0 ml of distilled water for 8 weeks, while experimental rats received 1.0 ml of brewed green tea with a concentration of 0.04 g/ml. After eight weeks and behavioral testing, brain samples were taken, and 6- μ m-thick sections were prepared from the hippocampus and examined microscopically using hematoxylin+eosin staining. Results of the passive avoidance tests confirmed improved learning after green tea concentrate administration ($P < 0.01$). Treatment of the experimental group noticeably the density (number per test area) of neurons (intensity of neurogenesis) in the CA3 area and dentate gyrus of the hippocampus ($P < 0.001$).

Keywords: green tea, *Camellia sinensis*, hippocampus, neurogenesis, passive avoidance, shuttle box.

INTRODUCTION

Learning and memory provide the ability of living organisms to adapt to the environment. Synaptic processes, biochemical activities involved in the formation of memory traces, increased survival of neurons, and long-term changes in gene expression are the factors involved in memory formation [1]. If learning leads to changes in the synaptic strength in neuronal circuits, these changes will provide storage of memories [2, 3].

Memory loss, impaired learning ability, and impaired mental concentration occur at any age but are more common in older individuals. Adult hippocampal neurogenesis is one of the brain plasticity characteristics; it seemingly plays important roles in brain functions, as well as in neurological and neurodegenerative diseases [4]. Certain medicinal plants demonstrated efficacy in maintaining health and treating many diseases. The constituents of these plants are able to prevent cognitive functions from

disorders, such as premature dementia, or to slow down their progressing. Due to the negative side effects of many “standard” chemical drugs on different body systems, their limited effects, inherent risks, and high costs, medicinal plants and natural remedies are receiving more and more attention [5, 6]. Among such remedies, there are some routine food plant products. Antioxidants and anti-inflammatory drugs used for the treatment of neuronal degeneration exert positive effects on learning and memory [7]. These agents also decelerate brain aging and improve quality of life [8, 9].

Such a widespread product as green tea is obtained from the leaves of *Camellia sinensis* [10]. It contains a number of phytochemicals; their amounts depend on the conditions of cultivation, environment, and climate [11]. Green tea is consumed worldwide; 20% of its consumption is in Asia [12]. Regular consumption of green tea was demonstrated to lower blood cholesterol and risk of cardiovascular and liver diseases. It also plays a positive role in weight loss, reduces the risk of arthritis and hypersensitivity of the respiratory system, and protects skin cells against damage by free radicals. Due to its antioxidant effects, it functions as an anti-aging agent. Nonetheless, some effects of green tea and its constituents are far from final elucidation.

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Neurogenesis in the dentate gyrus of the hippocampus can be a unique solution to computational problems in the brain and, hence, a mechanism to regulate the mood, learning processes, memory formation, and many higher cognitive functions in humans [13]. Learning activates the maturation of new neurons and increases their survival and incorporation into neural circuits. There are strong reasons to believe that learning is significantly dependent upon the hippocampal neurogenesis.

In this study, we investigated the effects of a green tea concentrate (*Camellia sinensis* extract) on passive avoidance learning and hippocampal neurogenesis in male Wistar rats.

METHODS

Twenty-one 2-month-old male Wistar rats weighing 230–280 g were purchased from the Pasteur Institute (Tehran, Iran) and used in this study. The rats were kept in standard metal cages in groups (seven animals in each). They were kept free of pathogens in a bed of wood chips and provided *ad libitum* with pellet food and tap water in calibrated glass bottles. The room temperature was $22 \pm 2^\circ\text{C}$, light cycle was 12/12 h, and relative humidity was set at 40–60%. Green tea was brewed according to mostly cited contemporary methods [14]. Briefly, 100 ml stock solution (green tea concentrate, 0.04 g/ml) was prepared and maintained at 80°C for 10 minutes in the brewing process. This extract was used for infusion during eight weeks.

Animals were divided into the control (C), sham (Sh), and experimental (Ex) groups. Group C received regular and adequate food and water and no additional interventions; group Sh received 1 ml of distilled water infusion each day, while group Ex received 1 ml of the green tea concentrate at a dose of 0.04 g/ml during eight weeks.

Passive avoidance learning was tested in an apparatus with two (light, Plexiglas, and dark, black) compartments of the same size (20×20×30 cm each) separated by a guillotine door (7×9 cm). The floor of the dark compartment was equipped with stainless-steel rods (2.5 mm in diameter) separated by a distance of 1 cm. Intermittent electric shocks (50 Hz, 1 mA, and 5 sec) were delivered to the grid floor of the dark compartment from an insulated stimulator.

Training. The rats were allowed to habituate to the laboratory environment and apparatus for 1 hour before

the training and testing experiments were done. All tests were conducted between 08:00 and 14:00. Each animal was gently placed in the light compartment for 5 sec, after which the guillotine door was lifted, and the latency with which the animal crossed a border of the dark (shock) compartment was timed. If an animal waited more than 300 sec to cross the other side, it was eliminated from the experiment. Once the animal crossed the border with all four paws, the door was closed, and the rat was taken into the home cage. The habituation trial was repeated after 30 min and followed after the same interval by the acquisition trial during which the guillotine door was closed and a foot shock (50 Hz, 1 mA, 5 sec) was delivered immediately after the rat had entered the dark compartment. Shaking and trembling of the animals combined with a short scream confirmed an effective shock. After 20 sec, the rat was removed from the apparatus and placed temporarily into the home cage. Two minutes later, the rat was retested in the same way as before. If the rat did not enter the dark compartment in 120 sec, successful acquisition of a passive avoidance response was recorded. Otherwise, when the rat entered the dark compartment, the door was closed again, and the rat received the same shock as above.

Twenty-four hours after training, a retention test was performed to evaluate long-term memory. Each animal was placed in the light compartment for 5 sec, the door was opened, and the step-through latency (STL) for entering the dark compartment was measured. The test session was ended when the animal did not enter the dark compartment after 300 sec (criterion for retention). During these sessions, no electric shock was applied. The increase or decrease in the STL value indicated an increase or decrease in memory retention, respectively.

Microscopy. After behavioral testing, brain samples were taken and placed in 10% formalin for microscopic studies. After sample preparation, dewatering was performed using ascending alcohol concentrations followed by clearing and impregnation with paraffin.

Sections of the hippocampal region (thickness 6 μm) were prepared. Hematoxylin+eosin-stained specimens of the hippocampal regions were examined microscopically. The density of neurons (their number per test area) was measured.

Analysis of variance (ANOVA) followed by Tukey's constant test was used to evaluate the intergroup differences. The criterion for significance of such differences was $P < 0.05$.

RESULTS

Avoidance learning. The results of passive avoidance testing showed that the learning performance in the Ex group compared to the C group was mildly but significantly better ($P < 0.01$) (Fig. 1). This effect was also confirmed by a much lesser time in the dark compartment shown by rats of the Ex group as compared to the C and Sh groups; the difference was about fourfold (Fig. 2).

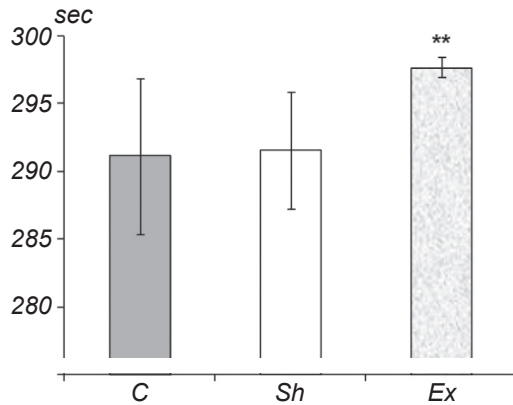


Fig. 1. Diagram of the means of the step-through latencies, sec, in the examined animal groups. C, Sh, and Ex are the control, sham, and experimental (treated with the green tea extract) groups. ** $P < 0.01$ in comparison of the Sh and C groups.

Р и с. 1. Діаграма середніх значень латентних періодів (с) заходу шурів у відсік з електрифікованою підлогою.

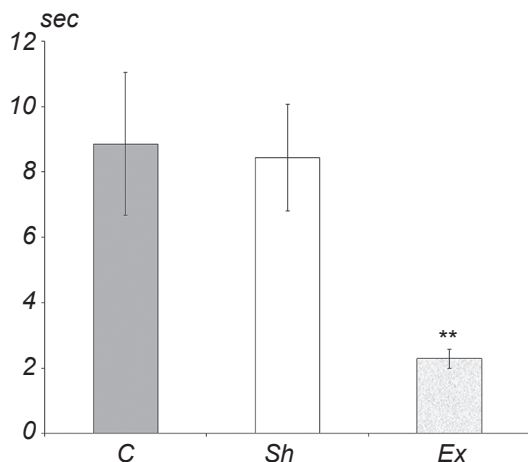


Fig. 2. Diagram of the means of the time, sec, spent in the dark (punished) chamber in the experimental groups. Designations are similar to those in Fig. 1.

Р и с. 2. Діаграма середніх значень часу (с), проведеного щурами в темному відсіку з електрифікованою підлогою.

Microscopic studies. Histological study of the density of neurons in the hippocampal CA3 area (Fig. 3) and dentate gyrus, DG (Fig. 4) in the rat brain revealed significant differences between the Ex group, on the one hand, and groups C and Sh, on the other hand ($P < 0.001$).

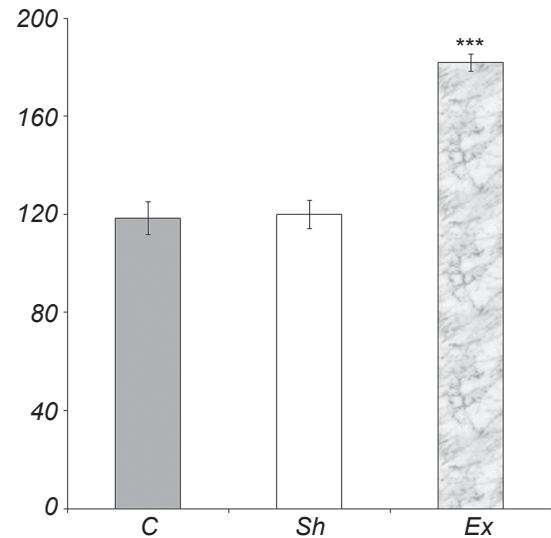


Fig. 3. Diagram of the means of the densities of neurons (their numbers within test zones) in the CA3 area of the hippocampus. *** $P < 0.001$. Designations are similar to those in Figs. 1 and 2.

Р и с. 3. Діаграма середніх значень щільності нейронів у полі CA3 гіпокампа.

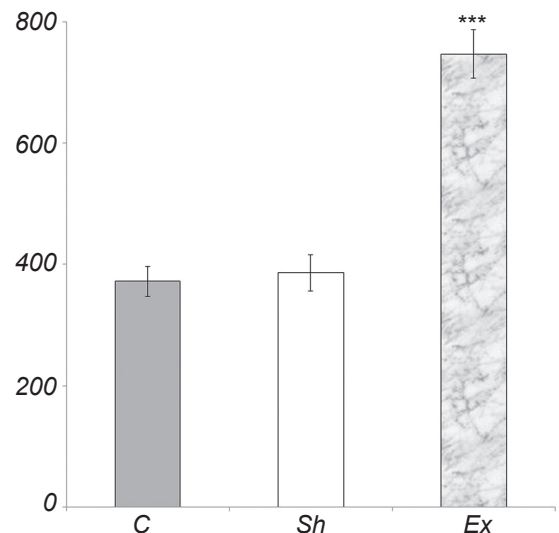


Fig. 4. Diagram of the means of the densities of neurons in the dentate gyrus of the hippocampus. Designations are similar to those in Figs. 1–3.

Р и с. 4. Діаграма середніх значень щільності нейронів у зубчастій звивині гіпокампа.

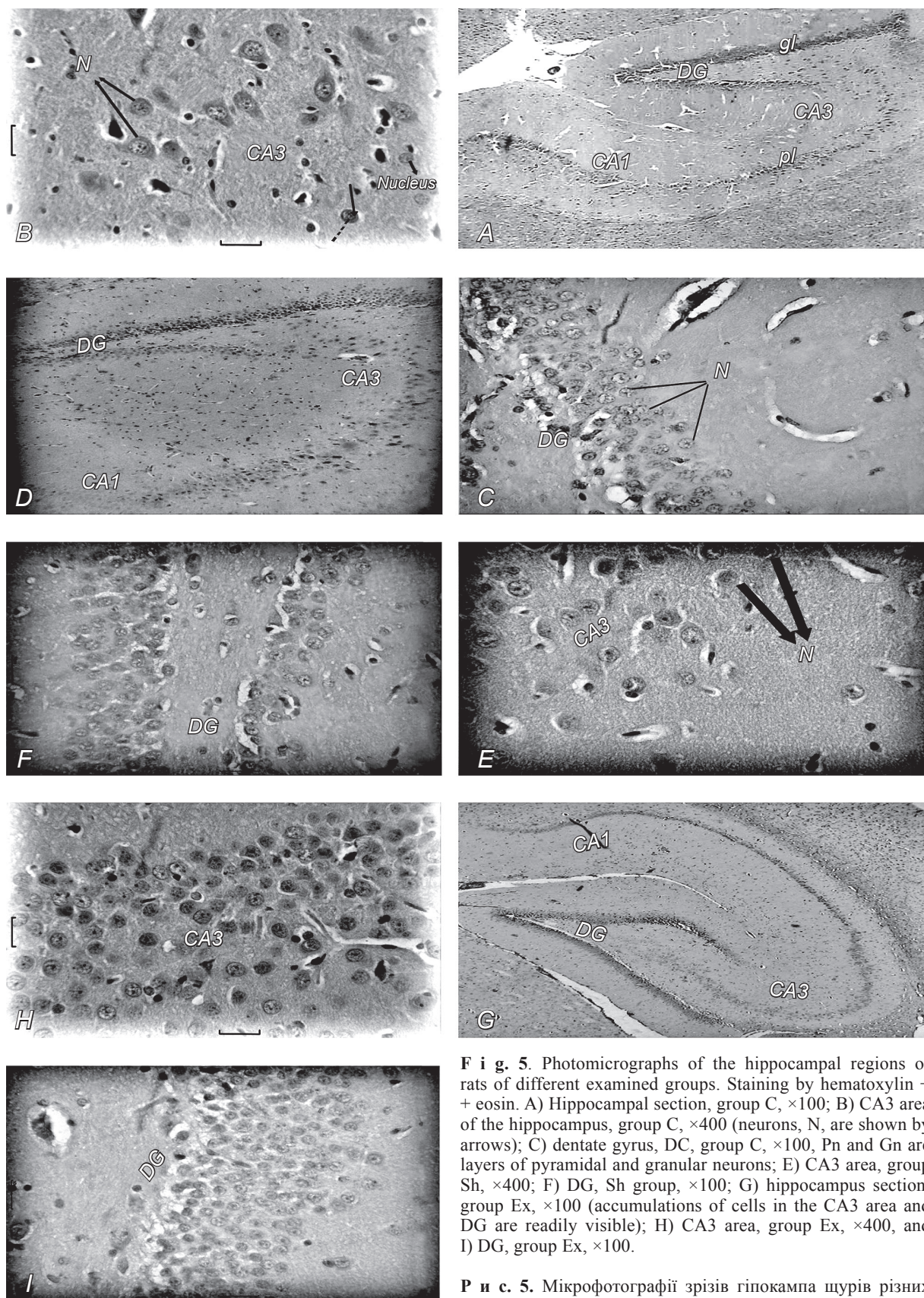


Fig. 5. Photomicrographs of the hippocampal regions of rats of different examined groups. Staining by hematoxylin + eosin. A) Hippocampal section, group C, $\times 100$; B) CA3 area of the hippocampus, group C, $\times 400$ (neurons, N, are shown by arrows); C) dentate gyrus, DC, group C, $\times 100$, Pn and Gn are layers of pyramidal and granular neurons; E) CA3 area, group Sh, $\times 400$; F) DG, Sh group, $\times 100$; G) hippocampus section, group Ex, $\times 100$ (accumulations of cells in the CA3 area and DG are readily visible); H) CA3 area, group Ex, $\times 400$, and I) DG, group Ex, $\times 100$.

Р и с. 5. Мікрофотографії зрізів гіпокампа щурів різних досліджених груп.

Histological examination revealed the healthy (normal) state of the hippocampus in the C and Sh groups. All studies were performed using a conventional optical binocular microscope interfaced with a digital camera (Fig. 5A and D). In the hippocampal regions of the brains of the C group, the Ammon's horn consisted of a thin layer of large C-shaped pyramidal neurons (Pns). The DG region was V-shaped and consisted of a thick layer of cells of relatively small granular neurons (Gns).

There was a significant difference between the number (density) of neurons in the CA3 region of the hippocampus of the brain of rats in the Ex group, as compared to the respective indices in the C and Sh groups ($P < 0.001$). At the same time, the densities of neurons in the CA3 region of rats of the latter groups were quite identical. The measured morphological index (neuronal density) in the Ex group was, on average, 54% greater than that in groups C and Sh (Fig. 3).

A nearly analogous situation was observed in the DG. The densities of neurons in the C and Sh groups were quite similar to each other. At the same time, this index in the Ex group was twofold greater (203%, if the density in the C group was taken as 100%) (Fig. 4).

Significantly increased numbers of cells in the examined hippocampal structures of Ex animals were clearly visible at stained samples of the CA3 area and DG even without accurate calculation (Fig. 5 H and I). Significantly intensified neurogenesis in the above regions of the hippocampus in rats treated for a long time with the green tea concentrate, as compared with that in the control groups (C and Sh), was the reason for such dramatic morphometric differences.

DISCUSSION

In agreement with the earlier known effects of green tea on learning and memory, the main focus of our study was to investigate the role of the constituents of green tea (mostly antioxidants, such as flavonoids and polyphenols) in modulation of the learning ability and morphological characteristics of the hippocampus.

Epigallocatechin gallate (EGCG) and anthocyanins constitute about 35% of the dry weight of the green tea concentrate. The latter also contains flavonols, flavanones, and flavan-3-ols. Flavanols are oligomers or polymers (condensed tannins or proanthocyanidins), and their concentration in green tea is rather high. Anthocyanins intensify the ability of neurons to

respond to chemical intermediates. They also prevent the formation of blood clots and, thereby, prevent thrombosis, maintain the elasticity of blood vessels, and also improve transmission of nerve impulses [15, 16]. From the chemical aspect, catechins are polyphenols and the most abundant catechin in green tea is EGCG, an agent with clear antioxidant properties. Catechins and caffeine inhibit catechol-O-methyltransferase (COMT). Thompson and Kim [17] suggested that flavonoids decrease cAMP degradation and synthesis of IP₃, increase neuronal activity, and strengthen long-term potentiation due to competitive inhibition of phosphodiesterase and COMT. Flavonoids presumably associate with the important cell molecular systems and, hence, have the potential to induce renewed protein synthesis in neurons. Such promoted synthesis causes morphological changes with a drastic impact on memory formation. These effects were readily evident in our microscopic observations (Figs. 3–5). The intensified neurogenesis in the hippocampus might well justify the observed enhanced learning abilities in the Ex rats manifested in terms of reduced latencies in learning (Fig. 1).

EGCG in green tea affects AMP kinase. This process moderates the death of brain cells by stress, and it also prevents the formation of amyloid protein structures containing copper, iron, and zinc. It also interferes with the connection of these sediments to prions (infectious proteins) [18]. On the other hand, EGCG modulates protein kinase C and contributes to gene modulation, cell survival, and the cell cycle [19–23]. Our results also convincingly showed that green tea constituents increase neurogenesis in the DG and CA3 hippocampal regions (Figs. 3 and 4); in total, this is in accordance with previous reports. Therefore, green tea is able to mediate cell survival because of its antioxidant properties in combination with the effects of EGCG, and hence exerts a protective effect on cognitive functions. It was proposed [4, 24] that EGCG may be effective in the prevention and treatment of neurodegenerative diseases. It is likely that green tea-borne agents are effective in strengthening memory through modulation of signaling pathways involved in learning and molecular mechanisms responsible for modulation of gene expression and transcriptional regulation of proteins.

Compounds found in green tea were shown to reduce oxidative damage to DNA; probably, this is a significant factor responsible for the improvement of impaired brain functions in aged rats [25]. In general, the green tea extract exerts moderating effects on

working memory by increasing communication between the parietal lobes and DG and between the frontal brain areas. As a result, this improves memory processing, facilitates the flow of information from one cerebral region to the other, improves cognitive functions, and augments memory [26].

Some other compounds present in the green tea extract deserve significant attention. L-theanine is an L-amino acid found in green tea. It increases the levels of GABA and dopamine in the brain and improves the functioning of the dopamine and serotonin cerebral systems [27–29]. It was found that L-theanine and caffeine also present in green tea can exert synergistic effects in the improvement of brain functions [30]. Catechins function as ligands for GABA_A receptors in the CNS. An increase in the number of GABA_A receptors in the brain exerts modulatory effects on cognitive performance, in particular, via the influence on cholinergic synapses controlled by the GABAergic system [30, 31].

Caffeine strengthens the power stabilization and alertness of the brain acting via several specific pathways [32]. It inhibits the phosphodiesterase enzyme and enhances the effect of norepinephrine [33], influences inhibitory neurotransmitters (in particular, adenosine), and, thus, facilitates concentration of such neurotransmitters as dopamine and norepinephrine, and intensifies firing of neurons [30,34–36]. Flavonoids may impact memory formation, in particular, by induction of new protein synthesis. The respective morphological changes may directly influence memory consolidation and storage [37]. It should be taken into account that flavonoids can cross the blood-brain barrier and reach the CNS, exerting significant effects on gene expression and regulation of protein synthesis. All these shifts may protect the brain against damages from neurotoxins, enhance the ability to suppress neural inflammation, facilitate proliferation of neuronal cells in the hippocampus, and, thus, positively affect memory formation [38].

The results of our behavioral tests (Figs. 1 and 2) are in agreement with the existing literature data and complement the latter. These results demonstrated significant facilitation of the learning process in animals treated with green tea concentrate. The effect of the latter can be attributed to the synergistic action of flavonoids and other compounds present in it, to the positive vascular effects that increase the cerebral

blood flow, and to intensification of neuronal cell proliferation in the hippocampus (Figs. 3–5).

In general, such a widespread “routine” food product as green tea should be qualified as an easy to use but clearly effective neuroprotective mean [39].

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All procedures were performed in accordance with the international guidelines for the care and use of laboratory animals and were approved by the Animal Ethics Committee (Islamic Azad University, Science and Research Branch, Tehran, Iran).

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ВПЛИВИ ЕКСТРАКТУ *CAMELLIA SINENSIS* НА НАВЧАННЯ ПАСИВНОМУ УНИКАННЮ ТА НЕЙРОГЕНЕЗ У ГІПОКАМПИ ЩУРИВ

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Резюме

Ми досліджували впливи концентрату зеленого чаю (екстракту з листя *Camellia sinensis*) на навчання реакції пасивного уникання та процес нейрогенезу в гіпокампі самців щурів лінії Вістар. Щури ($n = 21$) були розподілені на три рівні групи – контрольну, «псевдо» та експериментальну. Щури контрольної групи залишались інтактними, особини другої групи щоденно протягом восьми тижнів отримували інфузію дистильованої води (1.0 мл), а експериментальні щури отримували по 1.0 мл концентрату зеленого чаю (0.04 г/мл). Після згаданої експозиції (вісім тижнів) та тестування поведінки вилучали зразки мозку, робили зрізи гіпокампа завтовшки 6 мкм і після забарвлення гематоксиліном та еозинном піддавали їх мікроскопічному дослідженню. Результати тестування реакції пасивного уникання свідчили про те, що навчання такій реакції під дією концентрату зеленого чаю вірогідно покращувалося ($P < 0.01$). В експериментальній групі спостерігалася збільшена щільність нейронів (їх кіль-

кість у межах тест-зон) у ділянці CA3 та зубчастій звинині гіпокампа ($P < 0.001$), тобто в цих структурах інтенсифікувався нейрогенез.

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