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Histomorphological changes in the rat cerebral cortex following long-term caffeine consumption

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Investigating the effects of coffee and caffeine on the frontal cortex is essential for understanding how these widely consumed dietary compounds influence higher cognitive functions such as decision-making, behavioral regulation, planning, and social interaction. This study used transmission electron microscopy to assess the ultrastructural changes in the frontal cortex of adult rats following long-term oral administration of either pure caffeine or coffee. While the overall cytoarchitecture remained preserved in both experimental groups, subtle subcellular alterations were observed, with more pronounced structural changes in nerve fibers than in somata. Myelinated axons displayed increased diameter, accumulation of neurofilaments, and elongated mitochondria. Most mesaxon curls remained structurally organized but showed signs of loosening. Neuronal somata largely preserved organelle integrity; however, occasional autophagolysosomes and dilated rough endoplasmic reticulum were detected. Vascular components, particularly endothelial cells, maintained their general structure, though exhibited occasional membrane discontinuity and increased caveolae formation, while arterioles showed elevated smooth muscle tone and a higher density of actin filaments, indicating remodeling. Caffeine exposure resulted in slightly more pronounced mitochondrial and axonal alterations, suggesting higher metabolic stress. Importantly, while the coffee and caffeine groups shared many similarities, caffeine exposure resulted in slightly more pronounced mitochondrial and axonal changes, suggesting higher metabolic demand or stress response. In conclusion, long-term consumption of caffeine or coffee induced mild ultrastructural modifications, particularly in mitochondria, endoplasmic reticulum, and vascular endothelium, without overt neuronal damage. This may reflect early adaptive or stress-related responses. These findings highlight the importance of distinguishing between the effects of caffeine and those of complex coffee mixtures on brain structure and function.
Key words: rats; frontal cortex; coffee; caffeine; electron microscopy.

INTRODUCTION

Caffeine, a component of everyone's daily diet found in items such as coffee, tea, cocoa, cola, chewing gum, and pharmaceuticals, is the most widely used neurostimulant [1, 2]. In addition, it attracts consumers worldwide precisely because of its taste [3, 4]. The main effects of caffeine on human health are associated with the central nervous system, cardiovascular system, inflammatory mechanisms, carbohydrate metabolism and cancer [5, 6]. The role of caffeine and its analogs or derivatives in neuronal plasticity, neuroinflammation, and neurodegenerative pathology has been extensively documented. Our research is focused on studying the cerebral cortex histomorphology in rats. Caffeine belongs to methylxanthines, a class of the most extensively studied phytochemicals, commonly found in coffee, tea, cocoa, and other sources. They influence neural network activity, promote sustained cognitive functioning, and may potentially protect neurons from dysfunction and cell death in animal models of conditions like stroke, Alzheimer's (AD) and Parkinson's diseases [7].

Although caffeine (trimethylxanthine) is the main component of coffee, it also contains

other compounds such as trigonelline, which can affect nerve function (neurite outgrowth) [8]. Coffee is also rich in polyphenols, mainly phenolic acids (e.g., chlorogenic, caffeic, and ferulic acid), quinic acid, quercetin, catechin, N-methylpyridine, 5-hydroxytryptamine, pyrogallol, and others [9, 10]. The effects of caffeine-containing compounds on the body are quite ambiguous and are subject of varying interpretations. Links between coffee consumption and various conditions and diseases has been widely studied [8, 9], often yielding conflicting results.

The primary safety concerns related to coffee arise from its caffeine content. Over the last decade, food regulators have concluded that coffee/caffeine consumption is safe if consumed at 200 mg per serving (about 2 cups of coffee) or 400 mg per day [10]. The toxic dose for adults is estimated at 10 g of caffeine per day.

To date, scientists have proven that caffeine and other coffee compounds affect brain activity and are the central nervous system stimulants [3]. Several main mechanisms of caffeine action in the CNS were described (however, at high non-physiological concentrations of caffeine): antagonism to adenosine receptors, mobilization of intracellular calcium from intracellular stores, the inhibition of specific phosphodiesterases, and antagonism to benzodiazepine receptors [11]. Caffeine increases energy metabolism throughout the brain but simultaneously reduces cerebral blood flow, causing relative cerebral hypoperfusion. Also, it activates norepinephrinergic neurons and probably affects the local release of dopamine.

Methylxanthines effects on humans is often subtle and not easily detectable. The effects of caffeine on learning, memory, performance, and coordination are more closely linked to its impact on arousal, alertness, and fatigue. Caffeine's influence on anxiety and sleep varies depending on individual sensitivity to methylxanthines. These phenomena can be explained by the depletion of the body's energy systems, which, in humans, can become chronic with prolonged caffeine consumption. Moreover, there is an increased risk of stroke and heart attack even without significant atherosclerotic lesions in the cerebral arteries. Most importantly, the long-term traditional use of caffeinated foods and beverages can lead to apoptotic neurodegeneration.

METHODS

The studies were carried out following the existing International Animal Welfare Requirements and the requirements of Directive 86/609 / EEC on the protection of animals. Permission to conduct the study was obtained from the Bioethics Committee of Oles Honchar Dnipro National University (protocol No. 1 dated January 5, 2022). The experiments were performed on nonlinear white male rats (125-140 at the start of the experiments). The animals were kept in conventional sanitary and hygienic conditions with a standard diet [12]. To obtain the heterogeneity of emotional stress reactions in animals of the studied groups, we performed preliminary testing to identify individual behavior patterns [13]. In addition, we preferred to use rats with less homogeneous genotype (from the same parents). The first group included control animals (n = 3), which lived under standard conditions throughout the experiment. The second group animals (n = 4) received "Caffeine-sodium benzoate" ("Darnitsa", Ukraine) in the amount of 150 mg/kg/day [14-16]. Animals of the third group (n = 4) received food mixture of roasted coffee beans in 150 mg/kg/day of caffeine.

At 35 weeks from the start of the experiment, animals were decapitated under light ether anesthesia following the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (Strasbourg, 1986) and following the General Principles on animals", approved by the I National Congress on Bioethics (Kyiv, Ukraine, 2001). We studied the cerebral cortex of rats sections of the precentral gyrus of the frontal cortex. Tissue samples of 1 μm^3 were fixed for 3 h at +2°C in a 2.5% solution of

glutaraldehyde ("SPI," USA) prepared on 0.2 M phosphate buffer (pH 7.4). The material was freed from glutaraldehyde with 0.2 M sucrose and transferred for post-fixation to 1% buffered (pH 7.4) osmium tetroxide solution ("SPI," USA) at +21°C for 1 h. Then, samples were dehydrated with propylene oxide in solutions of increasing concentration. The Epon-Araldite composition was used to make epoxy blocks. Ultrathin sections were produced using an ultramicrotome UMTP-6M ("SELMI," Ukraine) in automatic mode.

The prepared samples were examined according to the scheme, proposed by Mironov et al. [17], on a transmission electron microscope PEM-100-01 ("SELMI", Ukraine) at an acceleration voltage of 75 kV and initial magnifications from 5000 to 40,000 with photo registration of images on a specialized film MACO ™ EM Film Type S ("SPI", USA). Sections of rat frontal cortex were placed on G200 Square Mesh copper mesh ("SPI", USA), contrasted with 2% uranyl acetate solution at +37°C for 15-20 min, followed by Reynolds lead citrate impregnation at room temperature for 30 min.

RESULTS AND DISCUSSION

The control group results of histomorphological examination are presented in Figs. 1 and 2.

The frontal cortex cytoarchitecture remained generally intact but with moderate subcellular alterations. In cortical layers II and III, we observed a moderate number of neurons undergoing apoptosis. In pyramidal neurons, the cytoplasm was densely populated with Golgi complex tubules and cisterns. These neurons exhibited a significant presence of autophagolysosomes containing lipoprotein inclusions. The rough endoplasmic reticulum's tubules showed partial disorganization and dilation. Some mitochondria had blurred profiles and signs of crystallization.

Astrocyte growths that adhered to the hemocapillary stems appeared to have expanded and partially lysed cytoplasm. These astrocytes

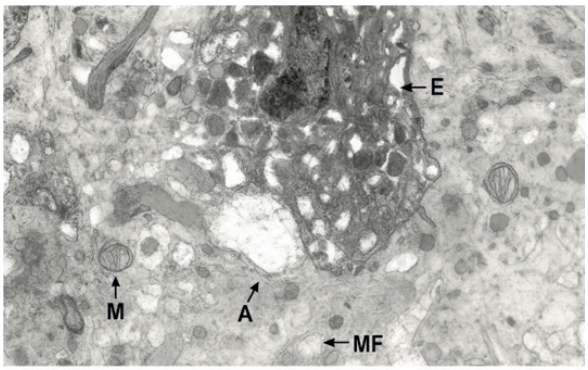

Fig. 1. The frontal cortex of the rat brain (control group). Electronogram, 20,000×. M – mitochondrion with preserved cristae; rER – rough endoplasmic reticulum adjacent to the nucleus; A – autophagolysosome with partially degraded contents; E – endothelial cell forming part of the capillary wall; MF – myelinated fiber with visible compact myelin sheath. The general ultrastructure is preserved, with well-defined organelles and no signs of cellular degeneration

contained autophagolysosomes, lipoprotein inclusions, and fragments of rough endoplasmic reticulum. The plasma membrane, surrounded by astrocytic extensions, appeared uninterrupted.

Microglial cells exhibited an amoeboid form with increased electron-dense cytoplasm, a hypertrophic Golgi complex, and significant development of autophagolysosomes and dense

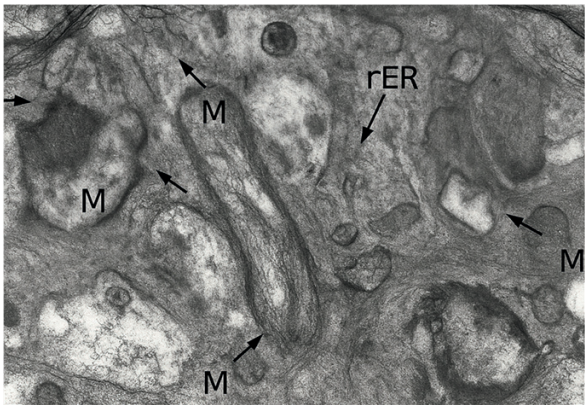

Fig. 2. The frontal cortex of brain of the control group. Electronogram 30,000×. M – mitochondria with moderately elongated profiles and preserved cristae; rER – rough endoplasmic reticulum with attached ribosomes; A – autophagolysosome indicating low-level activation of degradation pathways; MF – myelinated fiber. The overall ultrastructure remains intact, with signs of moderate cellular activity and mitochondrial adaptation

mitochondria. The hyaloplasm contained ribosomes, polysomes, and extensions of agranular endoplasmic reticulum elements.

Oligodendrocytes with partially lysed cytoplasm were typically found away from hemocapillaries. In their nuclei, the peripheral chromatin regions had fuzzy contours and were represented by homogeneous masses, indicating the early stages of karyolysis. Both astrocyte and oligodendrocyte processes contained lipoprotein granules and exhibited loose or stratified plasma membranes.

The lumens of the cortical hemocapillaries displayed narrowing and significant accumulations of flake-like masses with high electron density, along with remnants of disorganized erythrocytes. In most instances, the plasma membranes of erythrocytes were notably damaged and loose. Typically, these loosely surfaced erythrocytes were in close proximity to the disorganized portions of the luminal surface of endothelial cells. In these areas, the endothelial cell cytoplasm appeared thinner and contained osmophilic, homogeneous masses. Pericytes near these areas exhibited increased electron density, and their damaged cortical layers were associated with thickened and loose basement membranes.

The organelle zone of endothelial cell cytoplasm maintained its typical structure, but in most parts, the luminal and basal segments of the plasma membrane were loose. The luminal surface of endothelial cells exhibited a wavy relief pattern with significant invaginations in the form of caveolae, filled with uniformly dense material. In some instances, the luminal surface of the endothelium featured a significant number of microvilli, suggesting circulatory hypoxia. The basal portion of endothelial cell cytoplasm contained indistinct profiles of individual mitochondria and ribosomes. In these areas, the plasma membrane was not observed and, along with the basement membrane, constituted a homogeneous material with high electron density. The nuclei of such endothelial cells appeared enlarged, filled with a substantial mass of heterochromatin, and contained a disorganized nucleolus. The karyolemma membranes surrounding the nucleus had loose regions.

Myelinated fibers were disorganized in the vast majority of cases. Their axoplasm contained coagulates, lysis sites, and mesaxon curls that merged into homogeneous masses of varying shapes and sizes.

In general, the described ultrastructural changes in the frontal cortex of neurons in the brain of rats indicate the development of plasma-dependent coagulation-peptization dystrophy.

Many clinical observations and experimental studies in animals indicate that the effects of adverse environmental factors in specific periods of brain differentiation (so-called critical periods) leave a significant mark, thus creating a basis for developing various CNS pathologies [18]. In our study, caffeine was one of the environmental factors that significantly shifted homeostasis because we did not find significant histomorphological features of the cerebral cortex of animals that took caffeine or coffee. Based on this, we concluded that caffeine has a natural biological effect among all the alkaloids of coffee (Fig. 3).

Upon analyzing the data from the experimental groups of animals, the following indicators were observed. The general cytoarchitecture of the frontal cortex in caffeinated rats remained undisturbed. Neurons featured spherical nuclei filled with nearly homogeneous euchromatin masses and a moderate amount of condensed chromatin. The nuclei displayed no significant pathological signs, were of significant size, and were organized into fibrillar centers, fibrillar and granular components.

The cytoplasm of neurons had a moderate electron density, fine-grained hyaloplasm, several ribosomes, and a reduced number of polysomes. The elements of the rough endoplasmic reticulum showed no significant changes. The effect of caffeine was of an activating nature and directed toward the energy system of the neuronal apparatus. This was supported by the qualitative and quantitative alterations in

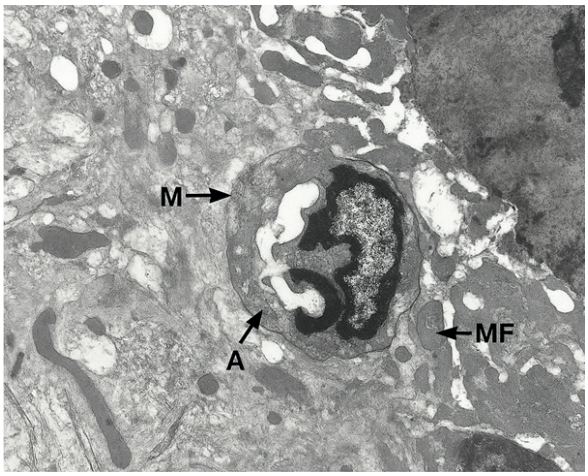

Fig. 3. The frontal cortex of the rat brain after long-term coffee consumption. Electronogram, 15,000×. M – mitochondrion with elongated profile and preserved cristae; A – autophagolysosome near the perinuclear area; MF – myelinated fiber. The endothelial cell is clearly outlined with well-organized nuclear chromatin. No severe structural damage is observed; cellular elements retain functional integrity

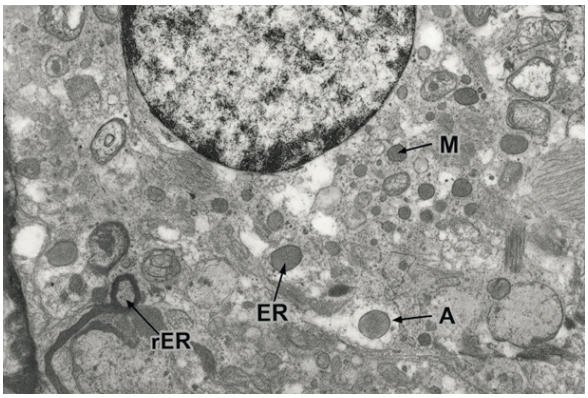

Fig. 4. The frontal cortex of the rat brain after long-term caffeine consumption. Electronogram, 15,000×. M – mitochondrion with moderately reduced cristae and electron-lucent matrix; rER – rough endoplasmic reticulum with preserved ribosomes; ER – agranular endoplasmic reticulum; A – autophagolysosome indicating activation of intracellular degradation pathways. The neuron displays an intact euchromatic nucleus and preserved cytoplasmic architecture, suggesting functional adaptation under caffeine exposure

mitochondria. The mitochondria had a smaller diameter and contained a moderate number of cristae. The mitochondrial matrix had a slightly reduced electron density. There was an increased presence of agranular endoplasmic reticulum tubules and isolated autophagolysis. In most cases, the plasma membrane of neurons displayed a clear outline (Figs. 4; 5).

Oligodendrocytes displayed a high nuclear-cytoplasmic ratio. Their ovoid nuclei were filled with homogeneous masses of euchromatin, which were primarily concentrated near the karyolemma at the periphery. The amount of heterochromatin was less than the decondensed portion. We observed a significant presence of ribosomes, polysomes, and mitochondria in the electron-transparent cytoplasm of these cells. In certain areas, the plasma membrane of these oligodendrocytes appeared loose.

Endothelial cells exhibited a typical cytoplasmic structure, but there were open regions in both the luminal and basal portions of the plasma membrane. The luminal surface featured numerous invaginations with caveolae, filled with a homogeneous substance of high electron density. A moderate number of microvilli were present on the luminal surface of the endothelium. The ribosome count was reduced, and mitochondria had a lightly stained matrix with a limited number of crystals. The nuclei of

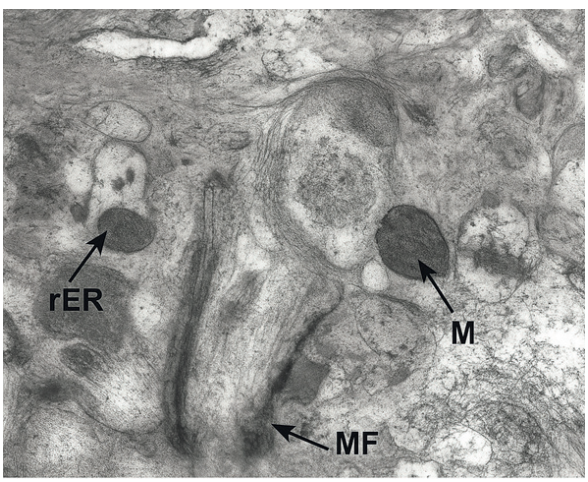

Fig. 5. The The frontal cortex of the rat brain after long-term caffeine consumption. Electronogram, 25,000×. M – mitochondrion with electron-dense matrix and slightly reduced cristae; rER – rough endoplasmic reticulum with associated ribosomes; MF – myelinated fiber. The ultrastructure reveals stable neuronal elements with signs of mild subcellular reorganization and energy-demand adaptation

endothelial cells were enlarged and contained a moderate amount of heterochromatin. The karyolemma membranes surrounding nucleus had loosely arranged loci. Typical nucleolar organizer components were observed within the nucleoli's structure.

Myelinated fibers near neurons exhibited an expanded diameter. Considerable-length mitochondria and neurofilaments were present in their axoplasm. In most areas, the karyotheca membranes of myelinated fibers appeared loose, although they generally maintained their structural integrity and parallel orientation.

Our recent studies have shown that coffee and caffeine influence calcium homeostasis of endoplasmic reticulum (ER) in hippocampal cells [9]. They provoked the increase of intracellular basal calcium level and increased leaking out calcium from ER. It is well known that changes in Ca^{2+} homeostasis, contributing to cell death, can be involved in neurodegenerative disorders [19-21]. "Ca^{2+} hypothesis of neurodegeneration" was also proposed for AD. The intracellular calcium depots which regulate the basal calcium level and signaling are ER, mitochondria and Ca^{2+} channels of the plasma membrane [19, 20, 22]. We previously have shown that hypercalcemia [23] or glutamate excitotoxicity, producing calcium overload, can be involved in proceeding with AD. Besides, the dysfunctions in mitochondria-dependent changes of calcium homeostasis can participate in brain pathologies [24, 25]. However, such changes in the function of mitochondria and ER can be caused by high doses of coffee/caffeine or by their long-term effect. These long-lasting events can lead to apoptotic changes in the brain.

In conclusion, experimental animals exposed to coffee and caffeine exhibited distinctive changes compared to the control group. Our investigations have revealed alterations in the composition of cerebral vessels, with the microvessels of the drainage system remaining unchanged. Nerve fibers appeared to be more susceptible to damage than cell bodies, and we observed adverse changes in intracellular organelles, possibly linked to disturbances in neuronal calcium homeostasis. Notably, the quantitative ratio of neuroglia cells in all groups remained consistent, with astrocytes predominating. These

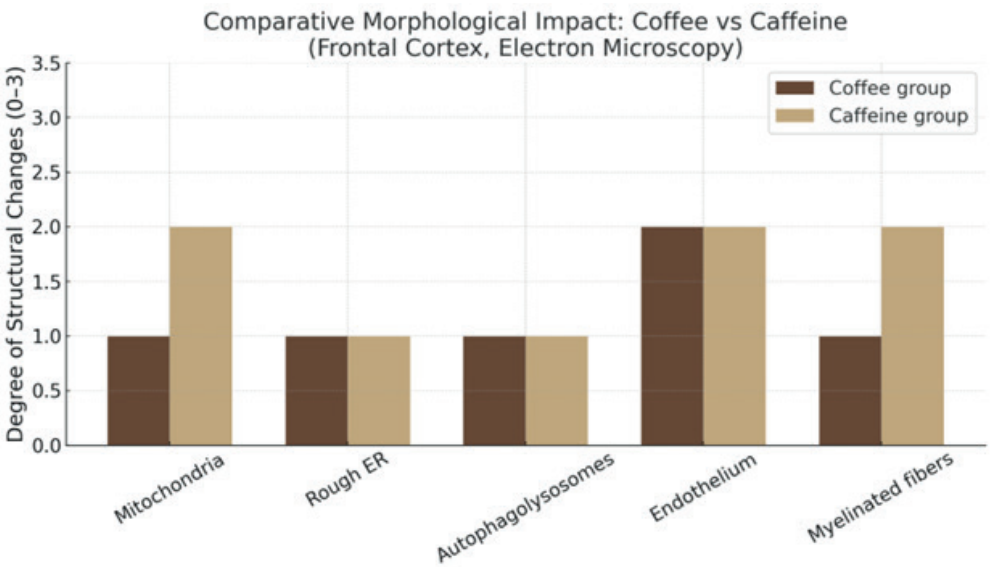

Fig. 6. Semi-quantitative comparative analysis of ultrastructural changes in the frontal cortex of rats after long-term exposure to coffee or caffeine. Each structure was scored from 0 (no change) to 3 (severe change). The diagram summarizes alterations observed in mitochondria, rough endoplasmic reticulum, autophagolysosomes, vascular endothelium, and myelinated fibers based on electron microscopy findings

findings contribute to our understanding of the effects of caffeine and coffee consumption on the neurological and vascular aspects of the brain.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ОСОБЛИВОСТІ ГІСТОМОРФОЛОГІЇ КОРИ ГОЛОВНОГО МОЗКУ ЩУРІВ ЗА УМОВ ДОВГОТРИВАЛОГО СПОЖИВАННЯ КОФЕЇНУ

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Вивчення впливу кави та кофеїну на лобову кору головного мозку є важливим для розуміння того, як вони впливають на вищі когнітивні функції, такі як прийняття рішень, регуляція поведінки, планування та соціальна взаємодія. Метою нашого дослідження було оцінити ультраструктурні зміни у фронтальній корі дорослих щурів після тривалого перорального введення чистого кофеїну або кави за допомогою трансмісійної електронної мікроскопії. Показано, що, незважаючи на збереження загальної цитоархітектоніки фронтальної кори у обох експериментальних групах, були виявлені помірні субклітинні зміни. Зокрема, нервові волокна зазнали більш виражених структурних змін порівняно з сомами нейронів. У мієлінізованих волокнах збільшувався діаметр аксонів,спостерігалося накопичення нейрофіламентів і подовження мітохондрій. Хоча більшість мезаксонів зберігали структурну організацію, виявлялися ознаки їхнього послаблення. Органели нейронів, включаючи ядра та гранулярну ендоплазматичну сітку, переважно залишались інтактними; однак були виявлені поодинокі ознаки утворення аутофаголізосом і дилатації ендоплазматичної сітки. Судинні компоненти, зокрема ендотеліальні клітини, загалом зберігали свою структуру, хоча спостерігалися окремі ділянки з розривами плазмолеми та збільшенням кількості кавеол. У стінках артеріол підвищувався тонус гладких м'язів і щільність актинових філаментів, що вказує на судинну перебудову. Важливо зазначити, що хоча обидві експериментальні групи мали подібні зміни, при дії кофеїну спостерігалися дещо більш виражені зміни в мітохондріях та аксонах, що може свідчити про більшу метаболічну напругу або стрес–відповідь. Таким чином, тривале споживання кофеїну або кави не призводить до вираженого ураження нейронів, але викликає помірні ультраструктурні зміни – насамперед у мітохондріях, ендоплазматичній сітці та ендотелії судин – що можуть відображати ранні адаптивні або стресасоційовані реакції нервової тканини. Отримані результати підкреслюють важливість диференційованого підходу до оцінки впливу ізольованого кофеїну та комплексних кавових сумішей на структуру та функцію головного мозку.

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