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Atrophy of the testes endocrine apparatus under cerebral hypoperfusion in rats and its correction

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Brain damage (stroke, trauma, hypoperfusion) is accompanied not only by neurological disorders but also by endocrine deficiency. It may manifest in different forms, ranging from barely noticeable to those that radically alter life, affecting the regulation of metabolism, sexual health, psychological well-being, and rehabilitation potential. To assess the atrophy of the endocrine apparatus of the testes under cerebral hypoperfusion in rats and the possibility of its pharmacological correction. Cerebral hypoperfusion in rats was modeled by bilateral common carotid artery occlusion (BCCAO). Atrophic changes in the testicular interstitium and the effects of clomiphene and metformin on their course were studied. Clomiphene, as an activator of the hypothalamic–pituitary–testicular axis, exhibited these properties also under severe chronic cerebral hypoperfusion in rats and prevented/restored the morphofunctional state of the endocrine apparatus of the testes. Metformin showed a tendency toward depression of the testicular endocrine apparatus in intact animals. At later stages of cerebral hypoperfusion in rats, it may have improved the condition of Leydig cells by compensating for progressively accumulating metabolic disturbances. Combined administration of clomiphene and metformin under cerebral hypoperfusion demonstrated lower effectiveness in restoring the morphofunctional state of the testicular endocrine apparatus compared to clomiphene alone. Considering the latter, it is necessary to correlate the results of testicular changes with the reduction of neurological symptoms that develop under cerebral hypoperfusion during the action of clomiphene and metformin, to conclude the expediency of correcting the observed alterations. Cerebral hypoperfusion leads to atrophic changes in the endocrine apparatus of the testes. By causing a decrease in testosterone production, this may reduce the effectiveness of compensatory and recovery processes. Clomiphene was shown to be a promising drug for correcting atrophic changes in the testicular endocrine apparatus under chronic cerebral hypoperfusion. In contrast, metformin demonstrated lower/negative effectiveness in this direction, and its combined administration was less effective than clomiphene alone.

Key words: cerebral hypoperfusion; bilateral occlusion of common carotid arteries (BCCAO); testes; interstitium; Leydig cells; clomiphene; metformin.

INTRODUCTION

Cerebral hypoperfusion is a condition in which the blood supply to the brain is insufficient, resulting in a decrease in the delivery of oxygen and nutrients to brain cells. It can be caused by various factors such as atherosclerosis, hypertension, etc. Also, phenomena of decreased cerebral blood flow occur in brain edema, strokes, and injuries. As a rule, chronic phenomena of hypoperfusion cause various progressive neurological problems, and first, neurocognitive disorders [1].

Brain lesions (stroke, trauma, hypoperfusion) are accompanied not only by neurological disorders but also by endocrine deficiency [2, 3]. The levels of hormones undergo significant changes after a stroke or brain injury, which is reflected in the regulation of neuronal plasticity, reduction of neurotrophic factor formation, prevention of cell death, enhancement of apoptosis and neuroinflammation, increased excitotoxicity, oxidative and nitrosative stress, and brain edema [4]. Recently, numerous studies have been conducted on the roles of thyroid hormones, growth hormone, testosterone, prolactin, oxytocin, glucocorticoids, parathyroid hormone, and dopamine in ischemic stroke, demonstrating their importance in the occurrence of compensatory and reparative processes.

The hypothalamus is a part of the brain that, simultaneously, functions as an endocrine organ. It produces regulatory factors, influences the cells of the anterior pituitary, and stimulates the secretion of hormones by endocrine glands under its control [5]. Moreover, brain damage, even in regions distant from the hippocampus, leads to a decrease in the functional activity of its neurosecretion cells, and accordingly, of the hypothalamic-pituitary axis and its dependent peripheral endocrine organs. Neuroendocrine dysfunction after brain injury may manifest in different forms – from barely noticeable to those that radically change life, affect the regulation of metabolism, sexual health, psychological well-being, and rehabilitation potential [2].

Special attention is drawn to the hypothalamic-pituitary-gonadal (testicular) axis, as its decreased activity results in reduced testosterone production, leading not only to impairment of sexual function but also to a decrease in the intensity of anabolic processes [2]. Rapid nongenomic mechanisms mediate them through cascades of membrane-associated signaling receptors, while slow genomic mechanisms mediate them through classical sex steroid receptors [6]. At the same time, a significant number of authors share the opinion that estrogens and testosterone exert a neuroprotective effect [6, 7]. However, their effects depend on age, sex, dose, and treatment protocol [8]. It should be noted that the neuroprotective properties of testosterone rely not only on its level, but also on the expression of androgen receptors, which decreased after cerebral ischemia. An increase in their expression reduced infarct size after ischemic stroke [9].

This study aimed to evaluate testicular endocrine apparatus atrophy under conditions of cerebral hypoperfusion in rats and to investigate the possibility of its pharmacological correction.

METHODS

Experimental studies were conducted on 119 male Wistar rats aged 4 months and weighing 360-380 g in the vivarium of Bogomolets National Medical University, Ukraine. The experiment was carried out according to the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the rules of the International Committee of Medical Journal Editors (ICMJE), as well as the recommendations "Bioethical expertise of preclinical and other scientific research carried out on animals" (Kyiv, 2006).

All animals were divided into 17 groups (7 rats each). The group of conditionally intact rats (Int) was not subjected to any interventions; PO – sham-operated animals, in which access and mobilization of the carotid arteries were performed without ligation. Clo – animals that received clomiphene orally for 42 days; Met – animals that received metformin orally for 42 days; Clo+Met – animals that received clomiphene and metformin orally for 42 days. BCCAO (bilateral occlusion of common carotid arteries) – rats that underwent two-stage ligation of the left and right common carotid arteries with material collected after 28 (BCCAO28), 42 (BCCAO42), and 56 (BCCAO56) days after ligation of the second carotid artery. Groups BCCAO+Clo – animals that, after BCCAO received clomiphene, and material was collected after 28 (BCCAO28+Clo), 42 (BCCAO42+Clo), and 56 (BCCAO56+Clo) days after ligation of the second carotid artery. Groups BCCAO+Met – animals that, after BCCAO received metformin, material was collected after 28 (BCCAO28+Met), 42 (BCCAO42+Met), and 56 (BCCAO56+Met), respectively. Groups BCCAO+C+M – animals that, after ACL received clomiphene and metformin, and material was collected after 28 (BCCAO28+C+M), 42 (BCCAO42+C+M), and 56 (BCCAO56+C+M) days of reproduction of the experimental model.

Surgical interventions were performed under intraperitoneal thiopental anesthesia (50 mg/kg). After removal of the fur and treatment of the skin with 5% iodine solution, the rats

were subjected to an incision of the skin and fascia along the left lateral surface of the neck. The muscles were bluntly separated, and the left carotid artery was mobilized and ligated. The wound was sutured layer by layer, and the operative field was treated with 5% iodine solution. A week later, a similar intervention was performed on the right side. Fourteen days after reproduction of the experimental model (after ligation of the second carotid artery), the rats of the experimental groups were administered orally: clomiphene citrate (Clostilbegyt®, Egis Pharmaceutical Ltd, Hungary) at a dose of 1.5 mg/kg; metformin (Metformin-TEVA, TEVA, Hungary) at a dose of 20 mg/kg; simultaneously clomiphene and metformin in the specified doses. Research material was collected after 28, 42, and 56 days of reproduction of the experimental model. At these times, material was collected from the animals of the ACL groups. In the animals of the control groups (which did not undergo ligation of the carotid arteries), which were administered clomiphene, metformin, and these drugs simultaneously in synchrony with the experimental groups, as well as in the PO group, the research material was collected synchronously with the experimental groups at the last stage of the experiment.

Euthanasia of the experimental animals was performed using an overdose of sodium thiopental (200 mg/kg), which was administered intraperitoneally. After removal, the material was fixed in 10% buffered formalin (pH 7.4; 4°C) for 48 h, embedded in paraffin, and histological sections 4 μm thick were made, which were stained with hematoxylin and eosin.

The obtained preparations were studied and photographed using an Olympus BX53 microscope with an Olympus SC digital camera and Olympus cellSens Entry 2.3 software. On digital images (×1000; 240×180 μm) of the testes, the area of interstitial islets between seminiferous tubules was determined, excluding blood vessels if present in them.

The obtained numerical data were processed using standard statistical methods, including calculation of the arithmetic mean, standard deviation, and standard error of the mean. The exact Kolmogorov–Smirnov test showed that all experimental measurement data did not contradict the normal distribution. To assess the significance of intergroup differences, a Student's t-test was used; differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The conducted observations revealed that in intact rats, the testicular interstitium was predominantly represented by islets of triangular or occasionally quadrangular shape, located in the spaces between seminiferous tubules. Where the latter were closely adjacent to each other, only single peritubular myoid cells with strongly elongated (rod-shaped) nuclei were found.

In the interstitial islets, small arteries or veins were sometimes present. More often, blood capillaries were found in them. In a significant part of such islets, blood vessels were not detected in the histological section. In the composition of the islets, groups of relatively large cells of irregular shape with moderate eosinophilia of the cytoplasm and a large, round nucleus were observed (Fig. 1). The latter often contained a nucleolus, and chromatin formed clumps unevenly distributed in the nucleus. Such morphology most closely corresponded to Leydig cells.

In the composition of the islets, cells of irregular, and sometimes process-bearing shape, were also found. They had lighter cytoplasm than Leydig cells, an oval-shaped nucleus with peripheral localization of heterochromatin under the karyolemma, often in the form of a continuous rim. Such cells were identified by us as testicular macrophages. In addition, small cells with weakly basophilic or weakly oxyphilic cytoplasm and a small, round, relatively dense nucleus were encountered. They can be located between Leydig cells, but are more often found perivascularly. These cells, which we identified as mesenchymal, should also have included precursors of Leydig cells.

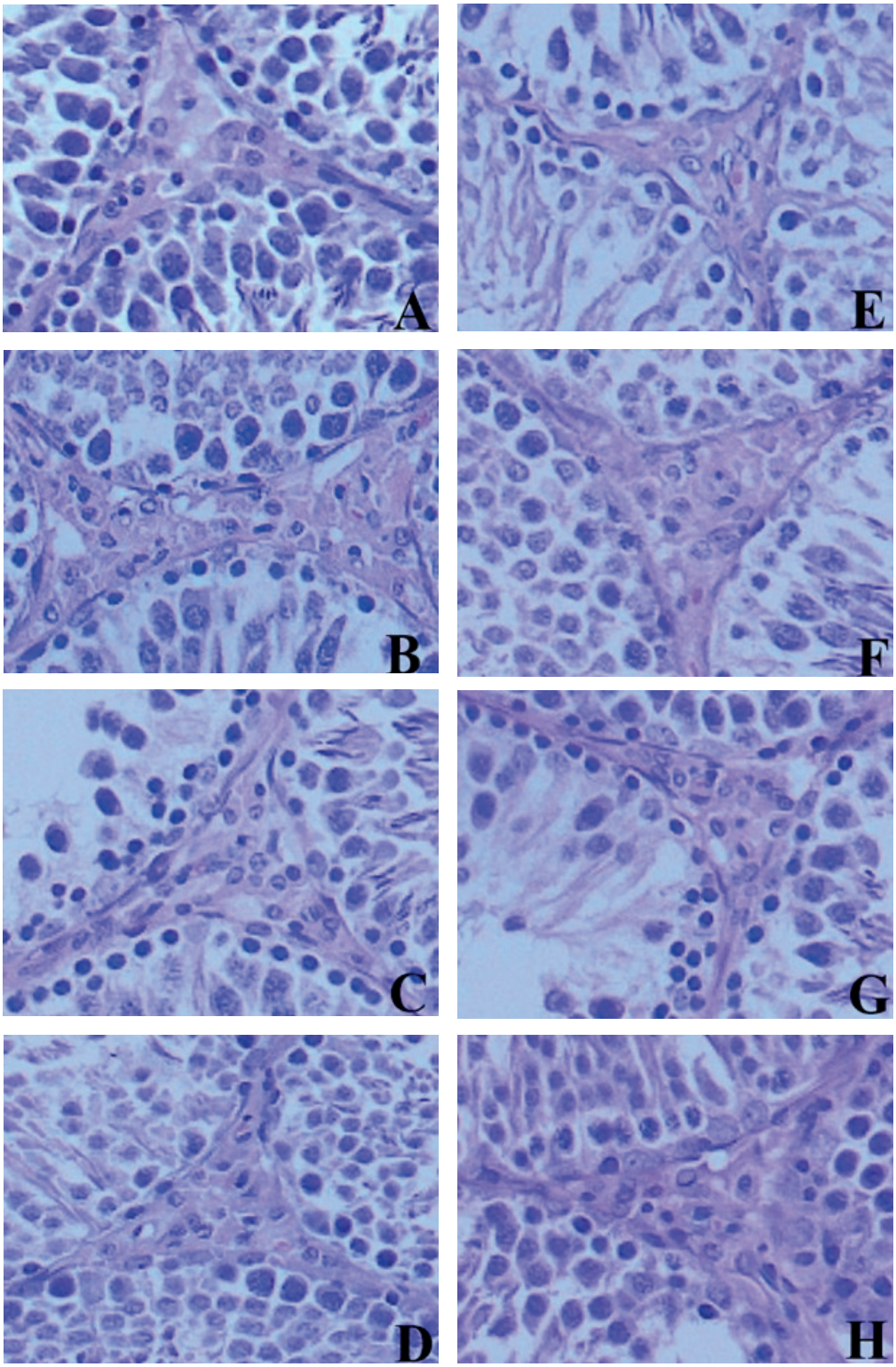

Fig. 1. Condition of the interstitial islets of rat testes in intact animals, under modeling of cerebral hypoperfusion, and under the action of clomiphene and/or metformin (A – Int; B – Clo; C – Met; D – Clo+Met; E – ACL; F – BCCAO+Clo; G –BCCAO+Met; H – BCCAO+Clo+Met). Stained with hematoxylin and eosin, 1000×

The average area of interstitial islets in conditionally intact rats was 626±26 μm². In the testes of rats that did not undergo surgical interventions and received clomiphene (Clo) for 42 days, a certain increase in the size of interstitial islets was observed. Quantitative evaluation showed that compared to intact animals, their average area increased by almost 20% to 748.7 ± 29.8 μm² (Fig. 2). At the same time, an increase in the size of Leydig cells, as well as an enhancement of the intensity of their staining, was noted (Fig. 1B). Under the influence of metformin (Met), there was a tendency toward reduction of the size of interstitial islets (Fig. 2), mainly due to a decrease in the size of Leydig cells (Fig. 1C). With the combined action of clomiphene and metformin (Clo+Met), a moderate increase (Fig. 2) in the size of islets (12%, 704.52 ± 27.5 μm²) was observed. At the same time, Leydig cells, in most cases, stained more intensively than in intact animals (Fig. 1D).

The state of the testicular interstitium in rats of the PO group did not visually differ from that of intact ones. Morphometric evaluation showed a decrease in the area of interstitial islets by almost 6%, but this difference from the indicators in animals of the intact group was statistically insignificant (Fig. 2).

In BCCAO rats, during the second month after reproduction of the experimental model, a significant reduction in the size of interstitial islets was observed (Fig. 1E). Concurrently, both the number of Leydig cells, as verified visually, and their size decreased. Relatively, the number of cells that could be identified as testicular macrophages and mesenchymal cells increased. Morphometry revealed a decrease in the average area of islets after 28 days of model reproduction by approximately 13%, and after 56 days, up to 22% of the control values (Fig. 3).

Rats that, after BCCAO, received clomiphene for 28 days (BCCAO28+Clo) had an average area of interstitial islets 9% less than intact animals. At the same time, Leydig cells were often smaller and had lighter cytoplasm. Gradually, an increase in the area of islets was noted, which after 56 days of observation became somewhat larger (not significantly) compared to Int and

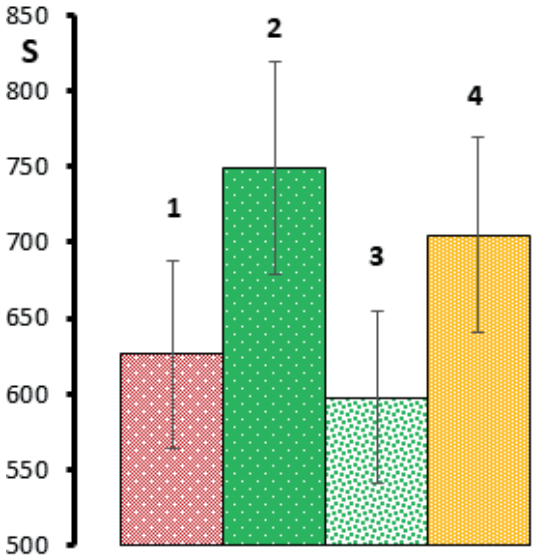

Fig. 2. Area (S, μm²) of testicular interstitial islets in conditionally intact animals (1) and those receiving clomiphene (2), metformin (3), and clomiphene and metformin together (4) for 42 days

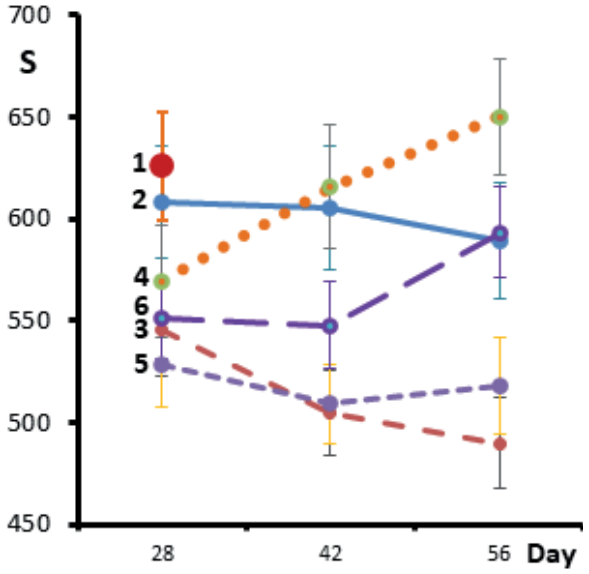

Fig. 3. Area (S, μm²) of testicular interstitial islets in conditionally intact animals (1), sham-operated animals (2), animals with BCCAO (3), and those receiving clomiphene (4), those receiving metformin (5), and those receiving metformin and clomiphene (6)

PO rats (Fig. 3). At the same time, the number, size, and intensity of staining of Leydig cells practically did not differ from the control. Testicular macrophages and mesenchymal cells were rarely encountered (Fig. 1F).

Metformin (BCCAO+Met) practically did not change the state of interstitial islets after 28 days of hypoperfusion reproduction compared to ACL animals, and their area did not differ statistically (Fig. 3). Further, by the 56th day, a tendency toward an increase in the area of islets relative to BCCAO was observed. At the same time, comparatively small Leydig cells with light cytoplasm were found in the islets. Also, more cells identified as testicular macrophages and mesenchymal cells were detected (Fig. 1G).

In BCCAO animals that received clomiphene and metformin (BCCAO+Clo+Met), after 28 and 42 days of hypoperfusion modeling, the interstitial islets were smaller than the Intact Ones by 12%, and their area increased only by the 56th day. Although it remained smaller than Int, these differences were not significant. In BCCAO+Clo+Met rats, the cellular composition of the islets differed by relative heterogeneity. Leydig cells varied in size and staining intensity. More often than in Int, testicular macrophages and mesenchymal cells were encountered (Fig. 1H).

Thus, the conducted observations revealed that chronic severe cerebral hypoperfusion in the rats results in atrophic changes in the endocrine apparatus of the testes. At the same time, the number of functionally active Leydig cells progressively decreases. Logically, under such conditions, testosterone production will decrease, as demonstrated by several studies [3, 4, 10], which is caused by depression of the hypothalamic–pituitary–testicular axis [3]. Testosterone, however, exerts a neuroprotective effect by regulating neuronal plasticity, promoting the formation of neurotrophic factors, reducing cell death, apoptosis, inflammation, excitotoxicity, oxidative and nitrosative stress, and brain edema in ischemic stroke [4].

Instead, in brain lesions a pathological circle is formed, in which impairment of brain function is accompanied by depression of the hypothalamic–pituitary–testicular axis and a decrease in testosterone production. The low level of the latter reduces the effectiveness of compensatory and restorative reactions in the brain. Based on the above, we attempted to stimulate the hypothalamic–pituitary–testicular axis directly or indirectly through neuroprotection in rats with severe vascular brain damage, to compensate for the atrophic changes in the testicular endocrine apparatus.

Testosterone replacement therapy is a traditional method of treating hypoandrogenism, but it has potential side effects, primarily associated with suppression of the hypothalamic–pituitary–testicular axis. Clomiphene citrate, a drug originally developed for the treatment of female infertility, has recently attracted attention as a means of increasing testosterone production in men. By blocking the negative feedback of estrogen on the hypothalamus and pituitary, clomiphene stimulates the secretion of gonadotropins, which leads to increased production of endogenous testosterone [11].

Clomiphene competitively binds to estrogen receptors (ERα and ERβ) [12]. The ER receptor, when bound to clomiphene, predominantly recruits corepressors of nuclear receptors (NCoR, SMRT) in the hypothalamus, leading to decreased transcription of estrogen-sensitive genes that normally mediate the negative feedback on the secretion of gonadotropin-releasing hormone. This leads to increased expression of the gene of the latter and enhances its production. Gonadotropin-releasing hormone stimulates the transcription of the subunits of pituitary gonadotropins (FSHβ and LHβ), thereby increasing the level of gonadotropins in the blood [13]. Thus, by altering ER-mediated transcription, clomiphene indirectly regulates genes involved in steroidogenesis (CYP19A1/aromatase, StAR) and gonadotropin subunits, enhancing its role in stimulating testosterone production [14].

Thus, the increase in the size of the testicular interstitial islets in rats, primarily due to the

restoration of the morphofunctional state of Leydig cells under conditions of severe chronic cerebral hypoperfusion in rats treated with clomiphene, can be attributed to the restoration of the hypothalamic–pituitary–testicular axis. Moreover, a pronounced response of the testicular interstitium to clomiphene was also observed in intact rats.

Metformin is a widely known first-line drug for the treatment of type 2 diabetes mellitus. However, its effects are not limited to lowering blood sugar levels, but are realized through various mechanisms in all organs and tissues. One of the properties of metformin is neuroprotective action [15, 16]. Its mechanisms of action include activation of AMPK signaling, reduction of oxidative stress, modulation of neuroinflammation, stimulation of autophagy/lipophagy, and regulation of mitochondrial function, prevention of apoptosis, and improvement of endothelial function [17, 18].

Metformin has also attracted attention for its influence on reproductive health. Available data indicate that metformin affects oxidative stress, insulin sensitivity, and the activity of the hypothalamic–pituitary–gonadal axis, thereby improving male fertility in diabetes mellitus [19, 20].

Although metformin demonstrates protective effects in many studies (especially among men with obesity and diabetes), there is also convincing evidence of adverse outcomes, including a decrease in testosterone levels [21, 22], dysfunction of Leydig cells, and impaired steroidogenesis [23].

Our studies showed that in conditionally intact animals, administration of metformin for 42 days revealed a tendency toward a decrease in the size of interstitial islets. And its combined use with clomiphene reduced the effectiveness of the latter.

Under conditions of hypoperfusion, the state of the interstitial islets in the rats treated with metformin after 28 and 42 days of the experiment did not differ significantly from that in the rats of the BCCAO group. But after 56 days, an increase in the volume of islets and signs of Leydig cell activity were observed. It can be assumed that gradual pathological changes led [3, 4] to metabolic disorders in the testes, which were corrected due to the effects of metformin [15].

The combined use of clomiphene and metformin resulted in a depression of the clomiphene effect up to the 42nd day of observation. Only by the 56th day did the size of interstitial islets and the condition of Leydig cells significantly approach the level of intact animals.

CONCLUSIONS

Cerebral hypoperfusion leads to atrophic changes in the endocrine apparatus of the testes. This, by causing a decrease in testosterone production, may reduce the effectiveness of compensatory–restorative processes. Clomiphene proved to be a promising drug for correcting atrophic changes in the endocrine apparatus of the testes under chronic cerebral hypoperfusion. In contrast, metformin showed lower or negative efficacy in this regard, and its combined use with clomiphene was less effective than clomiphene alone.

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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АТРОФІЯ ЕНДОКРИННОГО АПАРАТУ СІМ'ЯНИКІВ ПРИ ГІПОПЕРФУЗІЇ МОЗКУ У ЩУРІВ ТА ЇЇ КОРЕКЦІЯ

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Ураження мозку (інсульт, травма, гіпоперфузія) супроводжуються не тільки неврологічними розладами, а й

ендокринним дефіцитом. Він може виявлятися в різних формах – як ледь помітних, так і таких, котрі кардинально змінюють життя, впливають на регуляцію метаболізму, сексуальне здоров'я, психологічний добробут та реабілітаційний потенціал. Мета нашої роботи – оцінити атрофію ендокринного апарату яєчок при гіпоперфузії мозку у щурів та можливість її фармакологічної корекції. У щурів моделювали гіпоперфузію мозку перев'язуванням двох загальних сонних артерій. Вивчали атрофічні зміни в інтерстиції сім'яників та влив на їх перебіг кломіфену та метформіну. Кломіфен, який є активатором гіпоталамо-гіпофізарно-тестикулярної осі, виявляє ці властивості й при тяжкій хронічній гіпоперфузії мозку у щурів і попереджає/відновлює морфофункціональний стан ендокринного апарату сім'яників. Метформін у інтактних тварин виявляє тенденцію до його депресії. На пізніх етапах спостережень гіпоперфузії мозку у щурів він, можливо, покращує стан клітин Лейдіга через компенсацію метаболічних порушень, що прогресивно накопичувалися. Комбіноване застосування кломіфену та метформіну при гіпоперфузії мозку показало меншу ефективність у відновленні морфофункціонального стану ендокринного апарату яєчка, ніж кломіфен. Враховуючи останнє, слід зіставити результати змін стану сім'яників з редукцією неврологічної симптоматики, що розвивається при гіпоперфузії мозку, при дії кломіфену та метформіну для остаточного висновку щодо доцільності корекції змін, які розвиваються. Таким чином, гіпоперфузія головного мозку призводить до атрофічних змін ендокринного апарату сім'яників. Це, спричиняючи зниження продукції тестостерону, може зменшує ефективність компенсаторно-відновлювальних процесів. Кломіфен виявився перспективним препаратом для корекції атрофічних змін ендокринного апарату сім'яників при хронічній гіпоперфузії мозку, тоді як метформін показав меншу/негативну ефективність у цьому напрямку, а їх комбіноване застосування було менш дієвим, ніж кломіфену.

Ключові слова: гіпоперфузія мозку; двобічна оклюзія загальних сонних артерій; сім'яники; інтерстицій; клітини Лейдіга; кломіфен; метформін.

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