EFFECT OF PUMPKIN (CUCURBITA PEPO L.) AND MARIGOLD (TAGETES PATULA L.) EXTRACTS ON HIPPOCAMPAL MITOCHONDRIA FUNCTIONAL ACTIVITY WITHIN CONDITIONS OF EXPERIMENTAL ACUTE BRAIN HYPOMETABOLISM


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The aim of the study is to evaluate the effect of pumpkin (Cucurbita pepo L.) and marigold extracts (Tagetes patula L.) on the hippocampal mitochondria functional activity within the conditions of experimental acute brain hypometabolism.

Materials and methods. The work was performed on 50 male Wistar rats, which reproduced an acute brain hypometabolic state by administration of a 3M sodium azide solution in hippocampus (n = 40 and n = 10 – a group of sham-operated animals). The test extracts and the reference drug – EGb 761 – were prophylactically administered at the dose of 100 mg/kg per os for 10 days. 24 hours after the last administration, sodium azide was injected, the brain was taken, the hippocampus was isolated to obtain a supernatant and determine the parameters of mitochondrial respiration, the intensity of anaerobic processes, the concentration of the apoptosis-inducing factor, endonuclease G, and β-amyloid.

Results. The carried out study established that the prophylactic administration of pumpkin and marigold extracts contributed to the restoration of a mitochondrial function and a decrease in the intensity of anaerobic processes. In the group of the rats treated with pumpkin and marigold extracts, an increase of ATP concentration in the hippocampal supernatant by 65.7% (p<0.002) was observed; it was 66.2% (p<0.002) relative to the animals deprived of pharmacological support. When the rats were treated with pumpkin and marigold extracts, a decrease in the concentration of apoptosis-inducing factor (by 33% (p<0.002) and 38.3% (p<0.002), respectively) and endonuclease G (by 3.6 times (p<0.002) and 4.4 times (p<0.002), respectively) was also noted. The administration of pumpkin and marigold extracts reduced the amyloid β-peptide concentration in the rats’ hippocampus by 54.4% (p<0.0002) and 54.4% (p<0.0002), respectively. The test-extracts had an equivalent therapeutic efficacy with the reference drug.

Conclusion. On the basis of the obtained data, it is possible to suggest the prospect of a further study of pumpkin and marigold extracts as the drugs of a targeted correction of cerebral hypometabolism.

Keywords: plant extracts, hypometabolism, hippocampus, mitochondria


Alzheimer’s disease (AD) is a worsening neurodegenerative disease which accounts for 50–70% of dementia cases, comprising more than 12 million people [1]. Generally, clinical manifestations of AD are observed in the late stages of the disease and are associated with deposition of cytotoxic β-amyloid in the brain structures [2]. Amyloid β-peptide is formed as a result of proteolysis of transmembrane protein – amyloid precursor protein (APP) during catalysis of secretase enzymes. To date, it has been established that the accumulation of β-amyloid is one of the most reliable and early markers of irreversible neurodegeneration [3]. In the processes of neuronal degradation, various β-amyloid isoforms form two types of cytotoxic conglomerates: the aggregates not stabilized by metal ions (usually soluble in water and having little cytotoxic potential) and associates in which divalent metal ions are present (these conglomerates form covalently cross-linked oligomers, which are then deposited in the cytoplasm of neurons (mainly the hippocampus) in the form of amyloid plaques) [4]. The excess of amyloid β-peptide in brain cells leads to irreversible phosphorylation of tau protein, resulting in the increased production of 4-hydroxynonenal – membrane-toxic aldehyde, which, in its turn, initiates oxidative processes in neuronal membranes, accompanied by a deterioration of the ion transport ATPases, functioning as glucose and glutamate transporters [5]. As a result,
for the protection of vertebrate animals used for experimental and other scientific purposes [19] and taking into account the International recommendations of the European Convention for the protection of vertebrate animals used in experimental studies [97] [24, 25].

**Test objects. Experiment design**

In this work, a thick pumpkin extract (*Cucurbita pepo L.*) and a thick marigold extract (*Tagetes patula L.*) were used as test objects. Marigold inflorescences (*Tagetes patula L.*) of the «Carmen» variety were collected in the botanical garden of Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. The pumpkin fruits (*Cucurbita pepo L.*) of the «Atlant srednepozdnij» variety were collected in the «Aleksandrovskij» district, Stavropol region. The raw materials samples were harvested in the period from June to September 2016–2018. Fresh pumpkin fruits and marigold inflorescences, dried in shadow, were used as raw materials.

EGB 761 (a standardized *Ginkgo biloba* extract manufactured by Hunan Warrant Pharmaceuticals, China) was a reference drug. The reference drug was administered *per os* at the dose of 100 mg/kg [10] before a surgery operation for 10 days. The test objects were administered similarly to the reference drug (prophylactically, at the dose of 100 mg/kg for 10 days). During the experiment, the following experimental groups of animals were formed: sham-operated rats (SO, n = 10); a negative control group of rats, deprived of pharmacological support (NC, n = 10); groups of animals treated with the reference drug and test–objects (n = 10 in each experimental group). The study design is shown in Fig. 1.

**Experimental model of cerebral hypometabolism**

Cerebral hypometabolism was modeled by an intrahippocampal injection of a 3M sodium azide solution, for which the animals had been anesthetized (chloral hydrate 350 mg/kg, intraperitoneally), the rats’ heads were fixed, the pelage was removed and the skull was scalped. Then, in the left and right hemispheres, trepanation holes were drilled with a 1 mm diameter trepanation bur with the stereotactic coordinates P: −3.0; ML: ± 3.0, V: −3.0 (from Bregma), which corresponded to the dorsal part of the hippocampus [11]. Then, a 30G needle was slowly inserted into the trepanation hole to the depth of 1.5 mm; a 3 M sodium azide solution (pH = 7.4) was injected in 0.5 μl volume, alternating with the right and left hemispheres (totally, there were 4 injections made). The needle was removed after 30 seconds since the insertion. The wound was sutured, the seam treated with a 5% iodine solution. Biomaterial was taken 24 hours after the surgery [12].
Figure 1 – Experiment design

Note: SO – a sham-operated rats group; NC – a negative control animals group; ATP – adenosine triphosphate; AIF – apoptosis-inducing factor; ENDOG – endonuclease G.

Biomaterial sampling
In the work, the rat hippocampus was used as biomaterial. The hippocampus was removed according to the standard procedure. The isolated hippocampus was divided into 2 parts: the first was homogenized in the following medium: 1 mmol EDTA + 215 mmol mannitol + 75 mmol sucrose + 0.1% BSA solution + 20 mmol HEPES (pH 7.2), followed by a double centrifugation in modes of 1,400g → 3 min. at 4°C (supernatant was removed) and 13000g → 10 min. The resulting secondary supernatant was removed for a respirometric analysis. The second part of the hippocampus was homogenized in PBS with pH 7.4 in a ratio of 1:7, centrifuged in the mode of 10000g → 5 min, and then the resulting supernatant was taken for ELISA.

Respirometric analysis
In the work, the previously described approach to assessing mitochondria respirometric functions had been used. The study was performed according to the SEAHORSE protocol on a laboratory AKPM-01L respirometer (Alfa-Bassens, Russian Federation). During the analysis, the change in oxygen consumption in the medium containing native mitochondria, was assessed against the background of the injection of mitochondrial respiration disconnectors: oligomycin 1 μg/ml; 4-(trifluoromethoxy) phenyl) hydrazono) malononitrile (FCCP – 1 μM); rotenone – 1 μM; sodium azide – 20 mmol. Glucose (15 mmol) was used as an oxidation substrate in the process of the study of the intensity of anaerobic processes. At the same time, the following parameters characterizing the mitochondria respiratory function were calculated: ATP-generating capacity; maximum respiratory rate, respiratory capacity. The glycolysis intensity, glycolytic capacity, and glycolytic reserve were also determined [13].

Enzyme-linked immunosorbent assay immunoassay
The standard ELISA reagents kits manufactured by Cloud clone (USA) was used. During the study, the changes in the following parameters were evaluated: β-amyloid concentration, apoptosis-inducing factor (AIF), endonuclease G (ENDOG) and ATP concentration. The analysis was carried out in accordance with the manufacturer’s recommendations (a protocol analysis instruction was attached to each kit). The results measurement was carried out on a microplate reader Infinite F50 (Tecan, Austria).

Methods of statistical analysis
Statistical analysis of the obtained results was performed in the STATISTICA 6.0 software package (StatSoft, USA). The data were expressed as M ± SEM. The comparison of means was carried out by the one-way analysis of the variance method with the post-hoc Newman-Keuls test.
RESULTS

The influence of the test objects and the reference drug on the change of the β-amyloid concentration in the rats’ hippocampus within the conditions of experimental acute brain hypometabolism

During this part of the work it was found out that the β-amyloid concentration (Fig. 2) in the SO group was 9.46 ± 0.09 pg/ml. At the same time, in the NC group of the animals within the conditions of the experimental cerebral hypometabolism, an increase of the amyloid β-peptide concentration in comparison with the SO group of the animals was by 9 times (p<0.0001).

The use of Egb 761 reduced the content of cytotoxic β-amyloid in the rats’ hippocampus in relation to the NC group of the animals by 54.4% (p<0.0002). Against the background of the test pumpkin and marigold extracts administration, the β-amyloid concentration decreased relative to the NC group of the animals by 54.4% (p<0.0002) and by 49% (p<0.0002), respectively (Fig. 2).

The influence of the test objects and the reference drug on the change of the rats’ hippocampal mitochondria respirometric function within the conditions of the experimental acute brain hypometabolism

When assessing the effect of the test extracts and Egb 761 on the change of the hippocampal mitochondria respirometric function, it was found out that in the NC group within the conditions of the experimental brain hypometabolism compared with the SO group of rats, a decrease in the ATP-generating capacity was by 10.8 times (p<0.0001), the maximum respiratory rate was by 19.4 times (p<0.0001) and the respiratory capacity was by 9.9 times (p<0.0001) (Fig. 3).

In the animals deprived of pharmacological support, in relation to the SO group of rats, the glycolysis intensity increased by 8.5 times (p <0.0001), and the glycolytic capacity and glycolytic reserve decreased by 5.8 times (p<0.0001) and 6.1 times (p <0.0001), respectively (Fig. 4). As a result, the ATP concentration in the hippocampal supernatant of the NC group decreased by 2.2 times (p<0.0001) relative to the SO group (Fig. 5).
Figure 3 – Effect of the test objects and the reference drug on the change of the mitochondrial respiration in the rats’ hippocampus within the conditions of the experimental acute brain hypometabolism

Note. SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; Egb 761 – a group of rats treated with Egb 761; # – statistically significant relative to the SO group of animals (Newman-Keusle test, p<0.0001); statistically significant relative to the NC group of animals (Newman-Keusle test * – p<0.02; γ – p<0.001; α–p<0.004).

Figure 4 – The effect of the test objects and the reference drug on the change of the anaerobic processes activity in the rats’ hippocampus within the conditions of the experimental acute brain hypometabolism

Note. SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; Egb 761 – a group of rats treated with Egb 761; # – statistically significant relative to the SO animals’ group (Newman-Keusle test, p<0.0001); * – statistically significant relative to the NC group of animals (Newman-Keusle test, * – p<0.005; γ–p<0.002; α–p<0.007; Δ- p<0.03; μ–p <0.01; η–p <0.004).
Against the background of EGB 761 administration in rats, an increase (relative to the NC group of animals) of ATP-generating capacity (Fig. 3) by 6.7 times (p<0.002), the maximum respiratory rate by 9.7 times (p<0.001) and respiratory capacity – by 5.1 times (p<0.001) was noted. In the rats treated with EGB 761, there was also an increase in glycolytic capacity and glycolytic reserve (Fig. 4) in comparison with the same parameters of the NC animals' group by 2.6 times (p<0.007) and by 4.3 times (p<0.03), respectively, while the intensity of glycolysis decreased by 1.9 times (p<0.005). In addition, the concentration of ATP in the animals treated with EGB 761 was 65.7% (p<0.002) higher than that in the rats without any pharmacological correction.

In the animals treated with the pumpkin extract, relative to the NC rats' group, an increase in ATP-generating capacity was by 8.2 times (p<0.001); the maximum level of respiration was by 10.9 times (p<0.001) and respiratory capacity was by 5.9 times (p<0.004) (Fig. 3). At the same time, the intensity of glycolysis in the animals that were administrated pumpkin extract, was 4.7 times (p<0.002) lower than that in the NC group of rats, while the glycolytic capacity and glycolytic reserve (Fig. 4) in the animals treated with the test pumpkin extract, increased in comparison with the rats deprived of pharmacological support by 2.4 times (p<0.01) and twice (p<0.004), respectively. The administration of the pumpkin extract in the animals contributed to an increase of ATP content (Fig. 5) in the hippocampus by 66.2% (p<0.002) in relation to the NC group of rats.

When the marigold extract was administrated in the animals, an increase (relative to the NC rats’ group) of ATP-generating capacity, the maximum respiratory rate and respiratory capacity (Fig. 3) by 6.2 times (p<0.001); by 9.4 (p<0.001) and 4.7 times (p<0.004), respectively, were noted. In addition, the intensity of glycolysis in the animals treated with marigold extract was 3.3 times lower (p<0.002) than that in the NC group of rats, while the glycolytic capacity and glycolytic reserve (Fig. 4) increased in the animals treated with marigold extract compared with the rats deprived of pharmacological support by 3.3 (p<0.01) and 4.2 times (p<0.004), respectively. As a result, the ATP concentration in the hippocampus of the animals treated with the marigold extract was 60.7% (p<0.002) higher than that of the rats of the NC group (Fig. 5).

**Figure – 5. The effect of the test-objects and the reference drug on the change of the ATP concentration in the rats’ hippocampus within the conditions of the experimental acute brain hypometabolism**

Note. SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; EGB 761 – a group of rats treated with EGB 761; # – statistically significant relative to the SO group (Newman-Keusle test, p<0.0001); * – statistically significant relative to the NC group (Newman-Keusle test, p<0.002).

The influence of the test objects and the reference drug on the change of the internal apoptosis pathway activity within the conditions of experimental acute brain hypometabolism

In the course of this part of the study it was found out that the concentration of AIF and ENDOG in the animals of the NC group exceeded the similar values of the SO rats’ group by 3 (p<0.0001) and 21.7 times (p<0.002), respectively (Table 1). At the same time, the animals treated with EGB 761 showed a decrease in AIF concentration by 36.8% (p<0.002) and ENDOG by 2.7 times.
Against the background of using the test pumpkin extract in the animals, the contents of AIF and ENDOG in the hippocampus decreased (relative to the NC group of rats) by 33% (p<0.002) and 3.6 times (p<0.002), respectively. A decrease in the concentration of proapoptotic markers of the internal apoptosis pathway was also observed while using the marigold extract. So, in the animals that were administrated the test marigold extract, the concentration of AIF and ENDOG was 38.3% (p<0.002) and 4.4 times (p<0.002), respectively, lower than the similar parameters of the NC rats’ group (Table 1).

Table 1 – The influence of the test objects and the reference drug on the change of the internal apoptosis pathway activity within the conditions of experimental acute brain hypometabolism

<table>
<thead>
<tr>
<th>Group</th>
<th>SO</th>
<th>NC</th>
<th>EGB 761</th>
<th>ET</th>
<th>EB</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF, ng/ml</td>
<td>6.16±0.413</td>
<td>18.69±0.04#</td>
<td>11.81±0.043*</td>
<td>12.61±0.426*</td>
<td>11.54±0.446*</td>
</tr>
<tr>
<td>ENDOG, ng/ml</td>
<td>72.03±1.069</td>
<td>1564.42±63.59#</td>
<td>578.41±60.45*</td>
<td>433.09±42.41*</td>
<td>352.35±10.796*</td>
</tr>
</tbody>
</table>

Note. AIF – apoptosis-inducing factor; ENDOG – endonuclease G; SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; EGB 761 – a group of rats treated with EGB 761; \# – statistically significant relative to the SO animals’ group (Newman-Keuls test, p<0.0001); \* – statistically significant relative to the NC group of animals (Newman-Keuls test, p<0.0002).

In addition, people with cerebral hypometabolism are, as a rule, β-amyloid-positive patients whose prognosis is most unfavorable [21].

In this regard, it can be assumed that the correction of hippocampal hypometabolism can be a promising approach to preventive pharmacotherapy of AD, which was partially confirmed by Villain et al., 2008 [22]. However, in spite of the probable expediency of early correction of metabolic disorders, provided they are diagnosed in a timely manner, currently existing AD treatment methods are aimed at reducing the cytotoxicity of β-amyloid, and the spectrum of compounds which can restore the metabolic activity of cells, is quite limited [23]. In addition, the developed drugs of targeted AD correction often have undesirable toxicological parameters. An example of this agent is semagacestat, a γ-secretase inhibitor, a potentially effective drug for the treatment of AD, which reduces the toxicity of β-amyloid, but has a high oncogenic potential, and therefore its further study has been stopped [24].

In this regard, a study to evaluate the effect of pumpkin (Cucurbita pepo L.) and marigold extracts (Tagetes patula L.) on the functional activity of hippocampal mitochondria within the conditions of the experimental acute brain hypometabolism was conducted. During the study, it was found out that the prophylactic administration of the tests pumpkin and marigold extracts contributed to the restoration of the mitochondrial function and a decrease of the anaerobic processes intensity equally with the reference drug – a standardized Ginkgo biloba extract (EGB 761).

Moreover, in the groups of the rats treated with marigold, pumpkin extracts and EGB 761, there was an increase of ATP concentration in the hippocampal supernatant compared to the same indicator in the group of the rats deprived of pharmacological support by 65.7% (p<0.002); 66.2% (p<0.002); and 60.7% (p<0.002), respectively. It may indicate normalization of bioenergetic...
processes in the hippocampus within the conditions of acute cerebral hypometabolism when using the test extracts and the reference drug [25].

In addition, the restoration of the optimal ATP synthesis can prevent the destabilization of mitochondrial membranes, which prevents their decomposition and release of AIF, thereby inhibiting internal apoptotic cascade reactions [26]. That was also established during the study. So, when the animals were treated with pumpkin and marigold extracts, a decrease of the AIF concentration (by 33% (p<0.002) and 38.3% (p<0.002), respectively), and ENDOG (by 3.6 times (p <0.002) and by 4.4 times (p<0.002), respectively), was noted.

Against the background of the test extracts and Egb 761, a decrease in the concentration of amyloid β-peptide in the rats' hippocampus by 54.4% (p<0.0002); by 54.4% (p<0.0002) and by 49% (p<0.0002), respectively, was noted. As a whole with a decrease of apoptosis intensity, it can prevent neuronal destruction [27].

**CONCLUSION**

On the basis of the obtained data, it can be assumed that the prophylactic administration of pumpkin extract and marigold extract helps to normalize bioenergetic processes, decrease the concentration of proapoptotic markers and β-amyloid in the rats' hippocampus under acute hypometabolism caused by intrahippocampal administration of a 3M sodium azide solution.

At the same time, the test extracts showed an equivalent therapeutic efficacy with the reference drug – a standardized extract of *Ginkgo biloba* (Egb 761), which makes these extracts promising objects for a further study in order to create drugs for targeted correction of AD in the early hypometabolic stage of the disease.

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**AUTHOR CONTRIBUTIONS**

All authors had equally contributed to the research work.

**CONFLICTS OF INTEREST**

The authors and peer reviewers of this paper report no conflicts of interest.

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