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Evaluation of neurological deficiency in rats with cerebral ischaemia following the administration of omega polyunsaturated fatty acids

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ABSTRACT

Aim. The aim of the study was to assess the degree of neurological deficit in rats with experimental cerebral ischaemia following the administration of omega-3 polyunsaturated fatty acids.

Material and Methods. The experiments were conducted on 42 male outbred white rats weighing 260 ± 20 g. The modelling of cerebral ischaemia was performed with intravenous thiopental anaesthesia (40-50 mg / kg), and the research involved models of subtotal, partial and stepwise subtotal cerebral ischaemia. Subtotal cerebral ischaemia (SCI) was modelled by simultaneous ligation of both common carotid arteries (CCA). Partial cerebral ischaemia (PCI) was modelled by ligating one CCA on the right. Stepwise subtotal CI (SSCI) was performed by sequential ligation of both CCA within the intervals of 1 day (subgroup 1), 3 days (subgroup 2), or 7 days (subgroup 3). In order to investigate the effects of omega-3 polyunsaturated fatty acids (ω-3 PUFA), animals with CI were injected intragastrically with the drug "Omegamed" (SCI+ω-3 PUFA) at a dose of 5 g / kg of body weight for the duration of one week. The control group consisted of sham-operated rats of the same sex and weight. Neurological deficits were assessed with regard to the "muscle strength", "swimming test" and "open field" tests after 5-6 hours of the ischaemic period.

Results. Following a stepwise bilateral ligation of both common carotid arteries with a 1 day interval, neurological disorders were most prominent, which indicates an aggravation of neurological deficit with a reduction in the time between CCA dressings. In rats with SCI, the changes were more visible than with PCI, although they were less observable than in the group with SCI. The least noticeable changes were noted in the 3rd subgroup (with a 7 day interval between CCA dressings). Research has demonstrated a dependence of the severity of brain damage in SSCI on the interval between the blood supply cessation in both CCA. In the course of a 7 day interval between CCA dressings, the compensatory mechanisms were activated, which prevented the development of morphological changes and neurological deficits. When CCA was ligated within 1 day interval, the degree of neurological deficit was maximal, indicating an insufficient implementation of compensatory mechanisms. In comparison with the control group, the rats of the "SCI+ω3-PUFA" group retained neurological deficit, the muscle strength indicator was decreased by 86% ($p<0.05$), the swim-

ming duration by 63% (p<0.05), the number of crossed squares by 55% (p<0.05), the number of washes by 62% (p<0.05), the number of racks by 62.5% (p<0.05) and the number of bowel movements by 60% (p<0.05). However, the neurological deficit was less prominent as compared with the SCI group. In fact, an increase in muscle strength by 67% (p<0.05) was observed, in swimming duration by 37.5% (p<0.05) and in the number of squares crossed in the "open field" test by 31% (p<0.05), which indicates the presence of a corrective action in the ω3-PUFA preparation.

Conclusion. The administration of the preparation of ω-3 polyunsaturated fatty acids has a corrective effect in subtotal cerebral ischaemia, contributing to a reduced severity of the neurological deficit symptoms (an increase in muscle strength, duration of swimming and the number of squares crossed in the "open field" test).

Introduction

Acute disorders of cerebral circulation are one of the most pressing problems in modern medicine. The incidence of strokes varies in different regions of the world from 1 to 4 cases per 1000 individuals per year, increasing significantly with age. Cerebrovascular diseases of ischaemic aetiology tend to increase, rejuvenate, are associated with a severe clinical course, high rates of disability and mortality [1–4]. The primary mechanism by which stroke causes injury is the focal deprivation of blood supply to the cerebral parenchyma. Although a variety of phenomena can result in such ischaemia, large-artery atherosclerosis is most prevalent. In atherosclerosis, accumulations of fatty deposits in the arterial subintima aggregate platelet clumps. These, in turn, attract thrombin, fibrin, and erythrocyte debris which ultimately coagulate to a size which poses a stenotic risk to the cerebral vasculature. Local blood supply stagnation due to a low wall shear stress is thought to predispose certain areas of the vasculature, such as the carotid bulb, to atherosclerotic plaque development. Subsequently, the resulting thrombus deprives cells of the cerebral parenchyma of the required oxygen, resulting in the pathology. Nevertheless, the development of plaque and the consequent stenosis are not necessarily *in situ*. In fact, plaques can also travel to the cerebral circulation from another location, and then they are referred to as emboli. As a result of atrial fibrillation, the heart constitutes their most common source, although they can originate also in other diseased parts of the arterial system.

There are many other pathogenic routes to cerebral ischaemia. In addition to the previously discussed large-vessel infarcts, involving the carotid, vertebral, and basilar arteries, as well as major branches of the circle of Willis, small-vessel (or lacunar) infarcts are also a major aetiology. Most frequently as a result of lipohyalinosis or micro-atheroma, but also occasionally due to the same mechanism by which larger arteries are blocked, the blockage of these small, penetrating arteries running at right angles to the major branches produces the focal deficits characteristic of stroke. Some less frequently observed causes include acute arterial dissection secondary to fibromuscular dysplasia, hematologic disorders such as sickle cell anaemia, and recreational use of cocaine or amphetamines [5,6].

In order to study the degree of neurological and behavioural disorders in adult animals with cerebral ischaemia, a number of methods can be used: Bederson's test, the test for assessing the modified depth indicators of neurological deficit, the Garcia test, as well as the angular test, the leg extension test, and the "open field" test. They allow for the monitoring of the impaired motor function to register such elements as discoordination, trembling, paresis, paralysis. In terms of the Bederson's test, it is performed in the following manner: the rat is held by the tail at a distance of 1 meter above the floor and the mobility of the forelimbs is monitored. Normally, rats pull their extremities towards the floor. The test involves placing the rats on a slippery smooth surface and pressing gently from the side behind the shoulder until the forelimbs begin to slide, and the animals must equally resist sliding in both directions. In order to asses indicators of the depth of neurological deficit, a scale has been introduced which includes tests for detecting motor activity when hanging an animal by the tail, features of walking

on a horizontal plane, coordination of movements when walking on a beam, the severity of reflexes. On the other hand, the Garcia test includes an assessment of spontaneous activity in the cage for 5 min, the symmetry of the stretching of the forelimbs when the animals are suspended by the tail, the ability to climb the wall of the ethmoid cage, as well as the response to touching each side of the rat's body, and the response to touching vibrissae.

Angle test evaluates space perception disorders and gaze paresis. The rat is placed between two vertical planes. Intact rats easily turn both to the right and to the left, whereas in a number of pathologies, including cerebral ischaemia, neglect is observed, i.e. a state when the animal is not able to perceive a certain part of the space. Furthermore, the "paw extension" test allows to identify and evaluate disorders of the forelimb motor activity. The rat's extremities during the test should hang without support, then it is raised to the edge of the platform so that its vibrissae touch the surface of the plane. The animal is held by hands and pulled to the side on a smooth surface. The number of movements of the forelimbs performed on the side from which the rat is pushed is recorded. Simultaneously, intact rats perform many movements using their front paws.

The "open field" test comprises the assessment of the number of crossed squares, activity in the horizontal and vertical planes, grooming (washing), the number of bowel movements, and the search for depressions and holes for animals [7–13].

One of the most promising concepts of modern science is the search for new methods of stroke prevention and treatment. Omega-3 polyunsaturated fatty acids (ω-3 PUFA), such as eicosapentaenoic acid and docosahexaenoic acid, are widely regarded as vasoprotective. Several large-scale, randomized clinical trials have demonstrated that a dietary intake of omega-3 PUFAs improves the prognosis of patients with symptomatic heart failure, or after a recent myocardial infarction. Omega-3 PUFAs can be incorporated into the phospholipid bilayer of cell membranes and can affect membrane fluidity, lipid microdomain formation, and signalling across membranes. Furthermore, omega-3 PUFAs also modulate the function of membrane ion channels, such as Na and L-type Ca channels, to prevent lethal arrhythmias. Moreover, omega-3 PUFAs also inhibit arachidonic acid conversion into pro-inflammatory eicosanoids by serving as an alternative substrate for cyclooxygenase or lipoxygenase, which results in the production of less potent products. In addition, a number of enzymatically oxygenated metabolites derived from omega-3 PUFAs were recently identified as anti-inflammatory mediators. These omega-3 metabolites may contribute to the beneficial effects against vascular that are attributed to omega-3 PUFAs [14,16, 17,18,19].

Aim

The aim of this paper was to assess whether PUFA administration affects the degree of neurological deficit in rats with experimental cerebral ischaemia.

Methods

The experiments were performed on 42 male 3-month-old outbred white rats weighing 260 ± 20 g in compliance with the Directive of the European Parliament and of the Council No. 2010/63 / EU of 22.09.2010 on the protection of animals used for scientific purposes. Modelling of cerebral ischaemia (CI) was conducted with intravenous thiopental anaesthesia (40–50 mg / kg). The research involved models of subtotal (SCI), partial (PCI) and stepwise subtotal (SSCI) cerebral ischaemia. **Table 1** shows the experimental groups and the number of animals included.

Subtotal cerebral ischaemia (SCI) was modelled by simultaneous ligation of both common carotid arteries (CCA). Partial cerebral ischaemia (PCI) was modelled by ligating one CCA on the right. Stepwise subtotal CI (SSCI) was performed

Table 1.Experimental groups

Experimental groups		Number of animals
SCI		6
PCI		6
SSCI	subgroup 1 (1 day)	6
	subgroup 2 (3 days)	6
	subgroup 3 (7 days)	6
SCI + Omega-3 PUFAs		6
Control		6

by sequential ligation of both CCA with an interval of 1 day (subgroup 1), 3 days (subgroup 2), or 7 days (subgroup 3). In order to investigate the effects of ω-3 PUFA, animals with CI were injected intragastrically with the drug "Omegamed" (SCI + ω-3 PUFA) at a dose of 5 g / kg body weight for the duration of one week. The control group consisted of sham-operated rats of the same sex and weight. Neurological deficits were assessed in terms of the "muscle strength", "swimming test" and "open field" tests after 5–6 hours of the ischaemic period. The abovementioned methods were chosen as fairly simple to perform and providing a complete picture of the impairments to the animals' motor activity. "Muscle strength" and "swimming test" components were employed to study physical activity. The "muscle strength" is assessed by placing the rat on a horizontal 60 cm long metal mesh with a centimetre division scale which determines the retention time when the mesh is turned to a vertical position.

To conduct a "swimming test", the animal is placed in a glass reservoir with water (21° C) and the retention time the animal stays on the water surface is determined. The "open field" test is performed for 5 minutes on a flat surface, lined with 36 squares, enclosed around the perimeter.

On the basis of the "open field" test, disorders of motor activity can be observed, registering discoordination, the disappearance of voluntary movements or their limitation. The motor activity of animals in the horizontal plane includes movement in different direction, as well as walking in a circle. In this case, the participation in the movement of all extremities of the rat is evaluated. One crossed square is taken as a unit of movement for the visual registration of activity. Motor activity of rats in the vertical plane is represented by two types of racks: slimbing (climbing) – the hind extremities remain on the surface, and the front extremities rest against the wall of the "open field", and rearing ("rear" – "stand on their hind legs") – the front extremities remain on weight. Grooming can be short – in the form of quick circular movements of the front extremities around the nose and vibrissae, and long – washing the eyes, the area behind the ears, the entire head, paws, sides, back, anogenital region, and tail. Exploring holes in the floor is demonstrated by sniffing the edges or putting a muzzle into the holes.

The study was performed 6 hours after the simulation of the CI. Quantitative continuous data were obtained, which were processed using the licensed computer program Statistica 10.0 for Windows (StatSoft, Inc., USA). Since the experiment used small samples which presented an abnormal distribution, the analysis was conducted on the basis of nonparametric statistics. Data are presented as Me (LQ; UQ), where Me is the median, LQ is the lower quartile value; UQ is the upper quartile value. Differences between groups were considered significant at $p < 0.05$ (Kruskal-Wallis test with Bonferroni's correction).

Results

When assessing the neurological deficit in animals with SCI, a decrease in "muscle strength" by 95% ($p < 0.05$) was observed, and the retention time on the water surface in the "swimming test" decreased by 76% ($p < 0.05$). These data are presented in **Table 2**.

Furthermore, motor activity assessment in the "open field" test also confirmed the development of neurological deficits. When conducting this study, the number of crossed squares decreased by 64% ($p < 0.05$) in comparison with the indicators in animals of the control group, and the number of short washes was reduced by 67% ($p < 0.05$), the number of racks by 62.5% ($p < 0.05$), the number of defecations by 60% ($p < 0.05$). Compared to the control group rats, the animals with PCI demonstrated a decrease in the "muscle strength" indicator and the duration of swimming by 75% ($p < 0.05$) and 41% ($p < 0.05$), respectively. In terms of the "open field" test, the number of crossed squares decreased by 26% ($p < 0.05$), the number of short washes by 33% ($p < 0.05$), the number of "climbing" racks by 25% ($p < 0.05$), the number of defecations by 40% ($p < 0.05$). Prolonged washing and rearing was observed only in intact animals ($p > 0.05$).

The results of behavioural tests indicate the development of a minor neurological deficit in rats with PCI. In comparison with the "control" group, in the 3rd subgroup of SSCI (interval of 7 days), the "muscle strength" indicator decreased by 81% ($p < 0.05$), the duration of swimming by 45% ($p < 0.05$), the number of crossed squares in the "open field" test by 40% ($p < 0.05$), the number

Table 2. Indicators of changes in motor function in rats with cerebral ischaemia, Me (LQ; UQ)

Experimental groups		Muscle strength, min	Swimming test, min
Control		21(20; 23)	21,5(18;25)
SCI		1(1;1) *	5(4;5) *
SSCI	1 sg	1 (1;1) *	5 (4;5) *
	2 sg	3 (3;3) *+	8 (7;9) *+
	3 sg	4 (4;5) *+	12 (12;14) *+
PCI		5(4;5) *+	13(12;15) *+
SCI+ ω-3PUFA		3(2;3) *+	8(7;8) *+

Test "open field"

Experimental groups		Number of squares crossed	Number of short washes	Climbing	Number of defecations
Control		72(64;75)	6,5(5;8)	6,5(5;8)	5(4;6)
SCI		23 (21;23) *	2(1;2) *	3(3;3) *	2(2;2) *
SSCI	1 sg	23 (21;24) *	2 (1;2) *	3 (3;3) *	2 (1;2) *
	2 sg	33 (29;33) *+	3 (2;3) *	4 (3;4) *	3 (2;3) *
	3 sg	43 (41;45) *+	3 (3;4) *+	4 (4;4) *+	3 (2;3) *
PCI		53(52;55) *+	4(3;4) *+	6(5;6) *+	3(3;3) *
SCI+ ω-3PUFA		33 (30;33) *+	3 (2;3) *	4(3;4) *	2(1;3) *

* – p <0.05 compared with the control group; + – p <0.05 compared with SIGM; SCI – subtotal cerebral ischaemia; SSCI – subtotal stepwise cerebral ischaemia; PCI – partial cerebral ischaemia; ω-3PUFA – ω-3 polyunsaturated fatty acids; sg – subgroup

of washes by 54% (p < 0.05), the number of climbing racks by 50% (p < 0.05), the number defecations by 40% (p < 0.05). In the 2nd and 1st subgroups, the changes were more evident. So, the indicator of "muscle strength" decreased by 86 (p < 0.05) and 95% (p < 0.05), swimming duration by 63% (p < 0.05) and 77% (p < 0, 05), the number of squares crossed by 55% (p < 0.05) and 68% (p < 0.05), the number of washes by 54% (p < 0.05) and 69% (p < 0.05), the number of climbing racks by 57% (p < 0.05) and by 62.5% (p < 0.05), the number defecation by 50% (p < 0.05) and by 60% (p < 0.05), respectively. Compared with the 3rd subgroup of SSCI, in the 2nd subgroup the indicator of "muscle strength" decreased by 24% (p < 0.05), the duration of swimming by 33% (p < 0.05), the number of squares crossed in the test "open field" by 24% (p < 0.05), and in the 1st subgroup these indicators were reduced to the greatest extent by 75% (p < 0.05), by 58% (p < 0.05), by 47% (p < 0.05), respectively. In addition, in the 1st subgroup, there was a decrease in the number of washes by 33% (p < 0.05), the number of climbing racks by 25% (p < 0.05) and the number defecations by 33% (p < 0.05), and compared with the 2nd subgroup, there was a reduction in muscle strength by 67% (p < 0.05), swimming duration by 37.5% (p < 0.05) and the number of crossed squares in the "open field" test by 29% (p < 0.05). In the 3rd subgroup of SCI, the indicator of muscle strength and duration of swimming did not change (p > 0.05) in comparison with the "PCI" group, although during the "open field" test, a decrease in the number of crossed squares by 19% (p < 0.05) was observed and the number of racks by 33% (p < 0.05). Compared with SCI, in the 3rd subgroup of SCI with an interval between dressings of both CCA for 7 days, the muscle strength indicator was 75% higher (p < 0.05), the duration of swimming by 58% (p < 0.05), the number of crossed squares by 48% (p < 0.05), the number of washes and climbing racks by 33% (p < 0.05). In the 1st and 2nd subgroups of SCI, the manifestations of neurological deficit were more observable than in rats with PCI: muscle strength index by 40% (p < 0.05) and 80% (p < 0.05), swimming duration by 39% (p < 0.05) and 62% (p < 0.05), the number of squares crossed by 39% (p < 0.05) and 57% (p < 0.05), the number of racks climbing by 42% (p < 0.05) and 50% (p < 0.05), respectively. The number of washings and defecations in the 2nd subgroup did not differ from the values of indicators in the PCI group (p > 0.05), but in the 1st subgroup their number was 50% less (p < 0.05). In the 2nd subgroup of SSCI, in comparison with the group SCI, the indicator of muscle strength was 67% more (p < 0.05), the duration of swimming by 37.5% (p < 0.05), the number of squares crossed by 31% (p < 0.05) and washing by 33% (p < 0.05).

In comparison with the control group, the rats of the "SCI+ω3-PUFA" group retained neurological deficit, the muscle strength indicator was 86% less ($p < 0.05$), the swimming duration by 63% ($p < 0.05$), the number of crossed squares by 55% ($p < 0.05$), the number of washes by 62% ($p < 0.05$), the number of racks by 62.5% ($p < 0.05$) and the number of bowel movements by 60% ($p < 0.05$). However, in comparison with the SCI group, the neurological deficit was less evident. There was an increase in muscle strength by 67% ($p < 0.05$), swimming duration by 37.5% ($p < 0.05$) and the number of squares crossed in the "open field" test by 31% ($p < 0.05$), which indicates the presence of a corrective action in the ω3-PUFA preparation.

Discussion

In the study no differences in the degree of neurological deficit were observed between single-stage SCI and the 1st subgroup of SSCI with a 1 day interval between dressings ($p > 0.05$). With a stepwise bilateral ligation of both common carotid arteries with a 1 day interval, neurological disorders were most noticeable, which indicates an aggravation of neurological deficit with a reduction in the time between CCA dressings. In rats with SCI, the changes were more distinct than with PCI, but less than with SCI. The least prominent changes were noted in the 3rd subgroup (the interval between CCA dressings was 7 days). Studies demonstrated the dependence of the severity of brain damage in SSCI on the interval between the cessation of blood supply in both CCA. At a 7 day interval between CCA dressings, compensatory mechanisms were activated which prevented the development of morphological changes and neurological deficits. When CCA was ligated with an interval of 1 day, the degree of neurological deficit was maximal, which in turn indicates insufficient implementation of the compensatory mechanisms. Therefore, the rats with the experimental CI presented poorer muscle strength, less physical activity, although they showed behavioural disorders. The morphological basis of the revealed changes in CI is damage to the brain neurons as a result of the destabilization of nervous processes (the ratio of excitatory and inhibitory reactions), which affects the implementation of brain functions. In animals with SCI, as well as in the 1st "SSCI" subgroup, more evident disorders were observed in comparison with the 3rd "SSCI" subgroup and the "PCI" group. Therefore, it is possible to claim that with these methods of CI modelling, the adaptation processes occur which prevent the development of distinct morphological changes and allow neurons to adapt to conditions of moderate hypoxia. According to the literature, due to the development of compensatory mechanisms, 7 days after hypoxia resulting from CCA ligation, a tendency is observed to improve microcirculation: capillary patency is restored, their number and diameter increase, leading to an improvement in cerebral blood supply, which is one of the crucial compensation effects, and is based on an increase in the blood vessels density [3]. The corrective effect of polyunsaturated fatty acids on the state of neurons in subtotal cerebral ischaemia may stem from the improvement in the rheological properties of blood due to a decrease in the production of thromboxane A by platelets and to an increase in the tissue plasminogen activator, as well as to an improvement in the neuronal membrane fluidity, and a decrease in blood viscosity. Interestingly, not all Omega-3 PUFAs trials have shown reductions in vascular disorders. However, several adequately powered observation and intervention trials have strongly supported the efficacy of Omega-3 PUFAs for the prevention of vascular disorders. Furthermore, experimental studies have revealed multiple underlying molecular mechanisms, including membrane modification, attenuation of ion channels, regulation of pro-inflammatory gene expression, and the production of lipid mediators. It remains unclear which mechanism contributes most to the cardioprotective effects of Omega-3 PUFAs observed in vivo; nevertheless, the pleiotropic anti-inflammatory effects of Omega-3 PUFAs could be valuable, particularly in the setting of atherosclerosis and cardiac remodelling. Although further research is necessary to address the molecular relationship between Omega-3 PUFAs and vascular pathology, it might be useful to consider bioactive Omega-3 PUFA-derived metabolites, such as 18-hydroxyeicosapentaenoic acid, as endogenous anti-inflammatory molecules and potential new therapeutic targets for vascular disorders [17–19].

In addition, omega 3-PUFAs also show an anti-inflammatory effect due to their incorporation into the phospholipid layer in cell membranes of monocytes, leukocytes, endothelial cells, which is accompanied by a decrease in the production of inflammatory mediators and a decrease in the adhesion of leukocytes to the endothelial wall. Moreover, polyunsaturated fatty acids, affecting the synthesis of prostaglandins, regulate vascular tone and prevent catecholamine-induced vasoconstriction, which results in a moderate hypotensive effect [15,16].

Conclusion

Concluding, the severity of neurological deficit depends on the severity of the ischaemic injury. The most severe consequences occurred with subtotal one-stage ischaemia and stepwise ischaemia with a minimum 1 day interval between arterial ligation. In contrast, stepwise ischaemia with an interval between dressings of 7 days and partial ischaemia did not lead to such pronounced disorders of the neurological status. The introduction of the ω-3 polyunsaturated fatty acid preparation shows a corrective effect in subtotal cerebral ischaemia, contributing to a decreased severity of neurological deficit symptoms (an increase in muscle strength, duration of swimming and the number of squares crossed in the "open field" test).

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Conflict of interest statement
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