

EXPERIMENTAL RESEARCHES

EFFECT OF MELATONIN ON NEUROLOGICAL STATUS AND OXIDATIVE MODIFICATION OF PROTEINS IN EXPERIMENTAL CEREBRAL ISCHEMIA

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ABSTRACT

Background. Ischemic stroke (IS) remains one of the leading causes of death and disability worldwide. Due to the limitations of oral and parenteral routes of neuroprotectant administration in post-stroke patients, the use of rectal suppositories with melatonin (MT) is a promising direction for neuroprotective therapy.

Aim. To evaluate the effect of rectal suppositories containing MT on the dynamics of neurological status and products of oxidative protein modification in experimental acute cerebral ischemia (EACI).

Materials and methods. Male Wistar rats ($n = 30$) were divided into 3 groups: I – sham-operated animals; II – animals with EACI according to the method of Chen S.T. et al.; III – animals with EACI that received rectal suppositories weighing 100 mg, containing 2.5 mg of MT. Neurological status was assessed on days 3 and 7 using the Garcia J.N. scale, the paw placement test, and the Bederson test. In the ischemic focus of the brain (IFB), the content of early and late products of protein oxidative modification (POM) was evaluated.

Results. In rats with EACI, neurological deficits developed on the corresponding day (decreased scores on the Garcia J.N. scale and the paw placement test, increased scores on the Bederson test) and an increase in the content of POM products in the IFB. Administration of MT suppositories contributed to the regression of these disorders: by day 7, there was an improvement in all neurological tests, and a decrease in POM products in the IFB.

Conclusions. The results obtained suggest that the neuroprotective effect of MT in the form of rectal suppositories in EACI is mediated through an antioxidant mechanism of action.

Key words: oxidative stress, melatonin, rectal suppositories, ischemic stroke

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ВЛИЯНИЕ МЕЛАТОНИНА НА НЕВРОЛОГИЧЕСКИЙ СТАТУС И ОКИСЛИТЕЛЬНУЮ МОДИФИКАЦИЮ БЕЛКОВ ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ЦЕРЕБРАЛЬНОЙ ИШЕМИИ

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РЕЗЮМЕ

Обоснование. Ишемический инсульт (ИИ) остается одной из ведущих причин смертности и инвалидизации населения в мире. В связи с ограничениями перорального и парентерального пути введения нейропротекторов у постинсультных пациентов, применение ректальных суппозиториев с мелатонином (МТ) представляет собой перспективное направление нейропротективной терапии.

Цель. Оценить воздействие ректальных суппозиториев, содержащих МТ, на динамику изменений неврологического статуса и продуктов окислительной модификации белков при экспериментальной острой церебральной ишемии (ЭОЦИ).

Материалы и методы. Крыс-самцов линии Wistar (30 особей) разделили на 3 группы: I – ложнооперированные животные; II – ЭОЦИ по методу Chen S.T. et al.; III – животные с ЭОЦИ, получавшие ректальные суппозитории массой 100 мг, содержащие 2,5 мг МТ. Неврологический статус изучали на 3-и и 7-е сутки по шкале Гарсиа Дж.Н., тесту с постановкой лапки, Bederson test; в очаге церебральной ишемии (ОЦИ) оценивали содержание продуктов окислительной модификации белков (ОМБ) (ранних и поздних).

Результаты. У крыс с ЭОЦИ на соответствующие сутки развивался неврологический дефицит (уменьшение баллов по Гарсиа Дж.Н. и тесту с постановкой лапки, рост — по Bederson test) и повышение содержания продуктов ОМБ в ОЦИ. Введение суппозиториев с МТ способствовало регрессу указанных нарушений: к 7 суткам фиксировалось улучшение показателей всех неврологических тестов и снижение продуктов ОМБ в ОЦИ.

Выявлена отрицательная корреляционная зависимость между показателями по шкале Гарсиа Дж.Н., тесту с постановкой лапки, Bederson test и продуктами ОМБ в ОЦИ.

Заключение. Полученные результаты позволяют предположить, что нейропротективное действие МТ в составе ректальных суппозиториев при ЭОИГМ реализуется через антиоксидантный механизм действия.

Ключевые слова: окислительный стресс, мелатонин, ректальные суппозитории, ишемический инсульт

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INTRODUCTION

Ischemic stroke (IS) is a significant medical and social challenge in healthcare, with 12 million cases registered globally each year [1]. In Russia, this number reaches 500,000 per year, placing a significant socioeconomic burden on the healthcare system due to high disability rates and prolonged rehabilitation [2]. The key pathogenetic mechanisms leading to neuronal death in ischemic brain lesions during IS include neuroinflammation, glutamatergic excitotoxicity, and oxidative stress. These mechanisms interact to determine the size of the ischemic lesion and the severity of neurological deficits [3]. Current therapeutic strategies for IS have limited efficacy and safety, owing to a narrow therapeutic window, high risk of hemorrhagic transformation, poor permeability of the blood-brain barrier (BBB) to neuroprotective agents, limited knowledge of pharmacokinetics, and frequent adverse effects. These limitations emphasize the need for the development of novel therapeutic approaches focused on neuroprotection [4, 5].

Recent studies have confirmed the significant neuroprotective potential of melatonin (MT) in IS, attributed to its pleiotropic effects. One such effect is its strong antioxidant activity, which is exerted both by directly scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS) and by activating endogenous antioxidant systems, such as superoxide dismutase, catalase, and glutathione peroxidase. In addition to its potent antioxidant activity, MT also has other neuroprotective mechanisms. These include anti-inflammatory effects, such as inhibition of the NF- κ B pathway and reduction in the synthesis of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6. It also has anti-apoptotic properties, such as caspase-3 suppression and stabilization of mitochondrial membranes. Furthermore, melatonin can reduce cerebral edema by regulating aquaporin-4 expression [6, 7, 8]. However, the clinical use of melatonin is limited by several factors. Oral formulations may not be suitable for patients with dysphagia, and parenteral administration has a short half-life (20–40 min), requiring frequent injections and potentially leading to local post-injection complications [9, 10].

In our previous study, we demonstrated the beneficial effects of melatonin incorporated into rectal suppositories on the neurological status, microcirculation, morphology, and morphometry of the ischemic brain lesion, as well as on lipid peroxidation parameters in ischemic brain tissue [11, 12]. This current study continues our research by comprehensively assessing the relationship between the neurological status and oxidative stress markers using melatonin-loaded rectal suppositories in experimental cerebral ischemia.

AIM

To assess the effect of melatonin-containing rectal suppositories on the severity of neurological deficits

and protein oxidative modification products in experimental acute cerebral ischemia.

MATERIALS AND METHODS

The study was conducted at the Department of Pathophysiology, Experimental Biological Clinic of South Ural State Medical University (SUSMU). A total of 32 adult Wistar male rats weighing 240 ± 20 g were used in the study. The animals were housed in standard conditions in individual polypropylene cages (Velaz, type 3, Czech Republic) with corn cob bedding and free access to food and water. They were fed a balanced complete feed (LBK) according to GOST R 50258–92. The facility maintained a day/night cycle, an air temperature of $20 \pm 2^\circ\text{C}$, and relative humidity of 60–80 %. The study was conducted in compliance with the guidelines of the European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and was approved by the Ethics Committee of SUSMU (Protocol No. 5, dated June 10, 2024).

The animals were randomly assigned to three groups: Group I ($n = 10$) was a sham-operated group; Group II ($n = 12$) consisted of animals with experimental acute cerebral ischemia (EACI); and Group III ($n = 12$) was treated with an original rectal suppositories (100 mg) containing 2.5 mg of melatonin administered every 24 hours for 7 days [13]. The observation period lasted 7 days. Two animals (one in Group II and one in Group III) died within the first 6 hours after surgery and were excluded from the analysis. Consequently, the final sample sizes were: Group I ($n = 10$), Group II ($n = 11$), and Group III ($n = 11$).

EACI was induced using a modified method developed by Chen S.T. et al., under combined Zoletil-xylazine anesthesia, in accordance with current guidelines for modeling cerebral ischemic stroke [14]. For Groups II and III, a 2-cm skin incision was made between the left ear and left eye. Soft tissues were dissected to the skull bones, and a 5-mm burr hole was drilled under continuous irrigation and cooling using a high-speed drill. Under a $\times 10$ operating microscope, selective diathermocoagulation of cortical pial vessels was performed in the middle cerebral artery representation zone. Group I animals underwent the same surgical procedures, except for the selective diathermocoagulation of pial vessels.

On days 3 and 7 of the experiment, the neurological status of all animal groups was assessed using the Garcia J.H. scale, the Placing test, and the Bederson test. The results were expressed as scores [15, 16].

For the preparation of a 10 % cerebral ischemic lesion homogenate, animals were euthanized by decapitation, the brain was extracted, and the ischemic zones were identified based through visual inspection and anatomic landmarks [17, 18]. Collected ischemic brain tissue was then placed in ice-cold 0.1 M phosphate buffer (pH 7.4). Subsequently, approximately 100 mg of this tissue was then homogenized at a ratio of 1:10 (w/v) for 3 minutes at 4°C , yielding 1 mL of the homogenate.

Protein oxidative modification (POM) products in the cerebral ischemic lesion homogenate were determined by measuring the reactivity of protein carbonyl derivatives with 2,4-dinitrophenylhydrazine, both spontaneously and under metal-induced conditions. Subsequently, aldehyde dinitrophenylhydrazones (ADNPH) and ketone dinitrophenylhydrazones (KDNPH) were recorded using a spectrophotometer in the ultraviolet (UV) and visible spectral regions. Results were reported as optical density units per mg of protein (arbitrary units/mg), and the reserve-adaptive potential (RAP, %) was calculated as the ratio of spontaneously formed oxidation products to those formed under induced conditions [19, 20].

Statistical analysis was conducted using IBM SPSS Statistics 19 (SPSS: An IBM Company, USA). The normality of distribution was assessed using the Shapiro – Wilk test. As most parameters did not follow a normal distribution, the data are presented as median (Me) and interquartile range [Q1; Q3]. Non-parametric methods were used to analyze the differences between groups, including Kruskal – Wallis, Mann – Whitney, and Wald – Wolfowitz tests. Correlations between parameters were assessed using Spearman's rank correlation coefficient (R). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Modeling of experimental acute cerebral ischemia (EACI) resulted in the development of right-sided hemiparesis and impaired statolocomotor function in rats. On days 3 and 7, animals in Group II showed a significant decrease in scores on the Garcia J.H. scale and the Placing test, as well as an increase in scores on the Bederson test, compared to animals in Group I. On day 7, when compared to day 3, there was a decrease in scores on the Garcia J.H. scale ($p = 0.042$) and the Placing test ($p = 0.041$) and an increase on the Bederson test ($p = 0.039$) (Table 1).

In animals of Group II, compared to Group I, there were increased levels of total protein oxidation modification

(POM) products in the homogenates from cerebral ischemic lesions on days 3 and 7 of the study under both spontaneous and induced conditions ($p < 0.001$ for all comparisons at the respective time points). When analyzing the dynamics of spontaneous parameters in animals with EACI, a significant decrease in total POM products was observed by day 7 compared to day 3 ($p = 0.042$). Similarly, the early ($p = 0.041$) and late ($p = 0.045$) POM fractions also showed a significant decrease. Under induced conditions, on days 3 and 7, Groups II also demonstrated higher levels of total POM products, as well as the early and late fractions, when compared to Group I ($p < 0.001$). Furthermore, the dynamics from days 3 to 7 in EACI animals showed a statistically significant decrease in total POM ($p = 0.038$), as well as in the early ($p = 0.035$) and late ($p = 0.043$) fractions.

The overall reserve-adaptive potential (RAP) of animals in Group II was significantly lower compared to that of Group I, both on day 3 ($p = 0.003$) and day 7 ($p = 0.002$). Within Group II, there was no significant difference in the overall RAP between days 3 and 7 ($p = 0.287$) (Table 2).

The use of rectal suppositories containing MT contributed to a decrease in the severity of neurological deficits, as assessed on days 3 and 7. Compared to Group II, animals of Group III showed a significant increase in scores on the Garcia J.H. scale (day 3: $p = 0.021$; day 7: $p = 0.008$) and the Placing test (day 3: $p = 0.015$; day 7: $p = 0.009$), as well as a significant decrease on the Bederson test (day 3: $p = 0.018$; day 7: $p = 0.006$).

In the homogenates of cerebral ischemic lesions, animals in Group III compared to Group II showed a statistically significant decrease in the levels of total POM products on days 3 and 7, both under spontaneous and induced conditions. This reduction was observed in both the early and late fractions. On day 3, the differences were: for total POM products $p = 0.012$, for early fractions $p = 0.015$, for late fractions $p = 0.018$; on day 7: $p = 0.006$, $p = 0.008$, and $p = 0.009$, respectively. Analysis of the dynamics within Group III from days 3 to 7 revealed a significant decrease

TABLE 1
ASSESSMENT OF THE IMPACT OF MT THERAPY ON THE SEVERITY OF NEUROLOGICAL DEFICIT IN EACI (ME [Q1; Q3])

Parameter	Group I		Group II		Group III	
	Day 3 (n=5)	Day 7 (n=5)	Day 3 (n=6)	Day 7 (n=5)	Day 3 (n=6)	Day 7 (n=5)
Garcia J.H. scale, scores	18.00 [18.00; 18.00]	18.00 [18.00; 18.00]	7.50 [7.00; 8.00]*	4.50 [3.00; 6.00]*&	11.50 [10.00; 13.00]**	15.00 [14.00; 16.00]**&
Bederson test, scores	0.00 [0.00; 0.00]	0.00 [0.00; 0.00]	2.00 [2.00; 3.00]*	4.00 [4.00; 4.00]*&	1.00 [1.00; 2.00]**	0.00 [0.00; 1.00]**&
Placing test, scores	9.50 [9.00; 10.00]	9.50 [9.00; 10.00]	4.00 [3.00; 4.00]*	2.00 [1.00; 2.00]*&	6.00 [6.00; 7.00]**	8.50 [8.00; 9.00]**&

Note. Significant differences according to the Mann – Whitney U-test are indicated as follows: * – compared with Group I; # – compared with Group II; & – significant differences between day 3 and day 7 within the corresponding group ($p < 0.05$).

in the concentrations of total POM products ($p = 0.024$), total early ($p = 0.021$) and total late ($p = 0.027$) POM products. The overall RAP in rats from Group III was higher than in Group II on both day 3 ($p = 0.031$) and day 7 ($p = 0.017$) (Table 2).

A comparative study of the neurological status of animals in Group III compared to Group I showed a decrease in scores on the Garcia J.H. scale (day 3: $p = 0.034$; day 7: $p = 0.042$) and the Placing test (day 3: $p = 0.027$; day 7: $p = 0.038$), as well as an increase on the Bederson test (day 3: $p = 0.029$; day 7: $p = 0.031$) (Table 1).

When assessing POM products in the homogenates of cerebral ischemic lesions in animals of Group II compared with Group I on days 3 and 7, both under spontaneous and induced conditions, there was a statistically significant increase in total POM products as well

as early and late POM fractions ($p < 0.001$ for all parameters at the respective time points). Overall RAP in animals from Group III compared to Group I was significantly reduced on days 3 and 7 ($p = 0.034$ on day 3; $p = 0.022$ on day 7) (Table 2).

Correlation analysis conducted on days 3 and 7 revealed a negative correlation between scores on the Garcia J.H. scale and the Placing test and lipid peroxidation (LPO) product levels in the ischemic lesion homogenate. Additionally, a correlation was observed between scores on the Garcia J.H. scale and the Placing test, and POM product levels in the ischemic lesion homogenate. The reduction in neurological deficit among animals with EACI treated with MT-containing rectal suppositories was associated with a decrease in both LPO and POM product levels in the ischemic lesion (Table 3).

TABLE 2
THE EFFECT OF MT ON THE CONTENT OF POM IN THE SITE OF CEREBRAL ISCHEMIA IN EACI (ME [Q1; Q3])

Parameter	Group I		Group II		Group III	
	Day 3 (n=5)	Day 7 (n=5)	Day 3 (n=6)	Day 7 (n=5)	Day 3 (n=5)	Day 7 (n=5)
Total POM products (spontaneous), arb. units/mg	65.0 [62.0; 68.0]	65.0 [62.0; 68.0]	220.0 [210; 230]*	200.0 [190; 210]*&	170.0 [160; 180]**	150.0 [140; 160]**&
ADNPH (spontaneous), arb. units/mg	55.0 [52.0; 58.0]	55.0 [52.0; 58.0]	190.0 [180; 200]*	175.0 [165; 185]*&	150.0 [140; 160]*	130.0 [120; 140]**&
KDNPH (spontaneous), arb. units/mg	11.0 [10.0; 12.0]	11.0 [10.0; 12.0]	25.0 [23.0; 27.0]*	22.0 [20.0; 24.0]*&	20.0 [18.0; 22.0]**	18.0 [16.0; 20.0]**&
UV fraction (spontaneous), arb. units/mg	0.018 [0.016; 0.020]	0.018 [0.016; 0.020]	42.0 [40.0; 44.0]*	38.0 [36.00; 40.00]*&	30.0 [28.0; 32.0]**	25.0 [23.0; 27.0]**&
Visible fraction (spontaneous), arb. units/mg	0.030 [0.028; 0.032]	0.030 [0.028; 0.032]	3.2 [3.0; 3.4]*	2.8 [2.6; 3.0]*&	2.2 [2.0; 2.4]**	1.8 [1.6; 2.0]**&
Total POM products (induced), arb. units/mg	70.0 [67.0; 73.0]	70.0 [67.0; 73.0]	420.0 [400.0; 440.0]*	380.0 [360.0; 400.0]*&	300.0 [280.0; 320.0]**	240.0 [220.0; 260.0]**&
ADNPH (induced), arb. units/mg	20.0 [18.0; 22.0]	20.0 [18.0; 22.0]	250.0 [240.0; 260.0]*	220.0 [210.0; 230.0]*&	180.0 [170.0; 190.0]**	150.0 [140.0; 160.0]**&
KDNPH (induced), arb. units/mg	15.0 [14.0; 16.0]	15.0 [14.0; 16.0]	120.0 [115.0; 125.0]*	100.0 [95.0; 105.0]*&	80.0 [75.0; 84.0]**	60.0 [55.0; 60.0]**&
UV fraction (induced), arb. units/mg	10.0 [9.0; 11.0]	10.0 [9.0; 11.0]	30.0 [28.0; 31.0]*	25.0 [23.0; 27.0]*&	20.0 [18.0; 22.0]**	15.0 [14.0; 16.0]**&
Visible fraction (induced), arb. units/mg	25.0 [23.0; 27.0]	25.0 [23.0; 27.0]	20.0 [19.0; 21.50]*	35.0 [33.0; 37.0]*&	20.0 [18.0; 22.0]**	15.0 [14.0; 16.0]**&
RAP, %	92.9 [89.7; 96.0]	92.9 [89.7; 96.0]	52.4 [50.0; 54.8]*	52.6 [50.0; 55.3]*	56.7 [53.3; 60.0]**	62.5 [58.3; 66.7]**&

TABLE 3

CORRELATIONS BETWEEN INDICATORS OF FREE RADICAL OXIDATION IN BRAIN TISSUE AND NEUROLOGICAL STATUS DURING MT THERAPY (RECTAL SUPPOSITORIES) IN EXPERIMENTAL CEREBRAL ISCHEMIA

Parameter	Total POM products (spontaneous), arb. units/mg	Total POM products (induced), arb. units/mg
Garcia J.H. scale, scores	R= <u>-0.76</u> -0.71	R= <u>-0.81</u> -0.79
Placing test, scores	R= <u>-0.66</u> -0.62	R= <u>-0.78</u> -0.74

Note. R – Spearman's rank correlation coefficient; numerator – day 3, denominator – day 7.

DISCUSSION

We hypothesize that simultaneous diathermocoagulation, damaging pial vessels, induced cerebral hypoperfusion. This reduction in cerebral blood flow, in turn, provoked mitochondrial respiratory chain dysfunction, accompanied by electron leakage and formation of reactive oxygen species (ROS), such as hydroxyl radical and peroxynitrite. These ROS exerted damaging effects on neuronal cell proteins and lipids [21]. Proteins in neuronal membranes and cytoskeletons are primary targets for ROS due to their presence of highly reactive amino acids, such as lysine, arginine, proline, cysteine, and methionine, leading to formation of carbonyl derivatives (ADNPH/KDNPH) [22]. These carbonyl derivatives, interacting with neuronal cytoskeletal proteins, such as β -actin and tubulin, disrupt axonal transport, cause loss of neuritic processes, and ultimately lead to cytoskeletal disintegration. Oxidation of cysteine residues in the active sites of ion-transporting proteins disrupts ion homeostasis, leading to membrane depolarization and increased intracellular Ca^{2+} concentration in neurons. This can activate excitotoxicity and lead to neuronal death [23]. Several previous studies have demonstrated that the stability of carbonyl derivatives, as opposed to rapidly metabolized LPO products, makes POM a reliable marker of oxidative damage in nervous tissue [24, 25].

The use of melatonin-containing rectal suppositories in animals with EACI has resulted in a partial reduction in neurological deficits, as measured by increased scores on the Garcia J.H. scale and the Placing test and decreased scores on the Bederson test. This may be due to the pleiotropic effects of melatonin, which is incorporated into rectal suppositories and has lipophilic properties that ensure its unhindered penetration through the intestinal mucosa and into the bloodstream [26]. The antioxidant effect of melatonin is achieved through its direct neutralization of ROS, forming non-toxic metabolites. By activating nuclear transcription factors that encode heme oxygenase-1 and thioredoxin-1, melatonin enhances the synthesis of antioxidant enzymes, such as heme oxygenase-1, quinone oxidoreductase-1, glutamate-cysteine ligase, superoxide dismutase,

and glutathione peroxidase. Furthermore, the primary melatonin metabolite, cyclic 3-hydroxymelatonin, exhibits a pronounced antioxidant activity that is superior to that of ascorbate and α -tocopherol. The final metabolite, N1-acetyl-5-methoxykynuramine, is also a potent ROS scavenger and a cyclooxygenase inhibitor, further enhancing the antioxidant effects of melatonin [27]. Melatonin prevents programmed cell death in the penumbra via mitochondria-mediated and receptor-dependent mechanisms. It induces tyrosine kinase-dependent phosphorylation of STAT3, which dimerizes and translocates to the nucleus, enhancing transcription of the Bcl-2 family of genes (Bcl-2 and Bcl-xL), thereby inhibiting mitochondrial permeability, release of cytochrome c, and activation of caspase-9 and caspase-3, suppressing the intrinsic apoptotic pathway. Melatonin suppresses the expression of death receptors (Fas/CD95) on cell surfaces and reduces the sensitivity of neurons to the Fas ligand, thereby inhibiting the apoptotic pathway dependent on these receptors [28].

CONCLUSION

The results of the study demonstrated the development of a marked neurological deficit in rats with EACI on days 3 and 7. This was objectively recorded using the Garcia J.H. scale, the Placing test, and the Bederson test. At the same time, a significant increase in the levels of all studied fractions of POM products (early and late) was observed in the cerebral ischemic lesion homogenate under both spontaneous and induced conditions in both groups of animals.

The use of melatonin-containing rectal suppositories contributed to a significant improvement in neurological function, as demonstrated by improved scores on all neurological tests. At the same time, a statistically significant reduction in the concentration of POM products was observed in the area of cerebral ischemia, although levels still did not reach those seen in the sham-operated group. Correlation analysis confirmed a strong relationship between the reduction in neurological deficit and the decrease in POM product levels in the cerebral ischemic lesion.

Conflicts of interest

The authors declare no conflicts of interest.

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