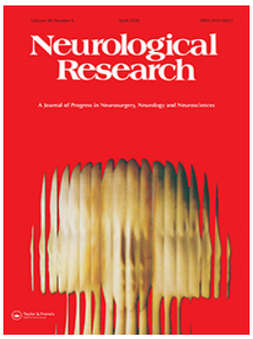

Taylor & Francis
Taylor & Francis Group

Neurological Research

A Journal of Progress in Neurosurgery, Neurology and Neurosciences

ISSN: 0161-6412 (Print) 1743-1328 (Online) Journal homepage: www.tandfonline.com/journals/yner20

Cortexin modulates OPG/RANK/RANKL and TRPC1 expression in cerebral ischemia-reperfusion injury

Cengiz Guven, Ahmet Türk, Seda Koçak, Busra Zencirci, Alper Yalcin, Hasan Aydın & Mevlut Doğukan

To cite this article: Cengiz Guven, Ahmet Türk, Seda Koçak, Busra Zencirci, Alper Yalcin, Hasan Aydın & Mevlut Doğukan (2026) Cortexin modulates OPG/RANK/RANKL and TRPC1 expression in cerebral ischemia-reperfusion injury, Neurological Research, 48:4, 510-521, DOI: 10.1080/01616412.2025.2536075

To link to this article: https://doi.org/10.1080/01616412.2025.2536075

NEUROLOGICAL RESEARCH
2026, VOL. 48, NO. 4, 510–521
https://doi.org/10.1080/01616412.2025.2536075

Taylor & Francis
Taylor & Francis Group

Cortexin modulates OPG/RANK/RANKL and TRPC1 expression in cerebral ischemia-reperfusion injury

Cengiz Guven[a], Ahmet Türk[b], Seda Koçak[c], Busra Zencirci[d], Alper Yalcin[e], Hasan Aydın[f] and Mevlut Doğukan[g]

[a]School of Medicine, Department of Cardiovascular Surgery, Adiyaman University, Adiyaman, Turkey; [b]School of Medicine, Department of Histology and Embryology, Adiyaman University, Adiyaman, Turkey; [c]Department of Physiology, School of Medicine, Kırşehir Ahi Evran University, Kırşehir, Turkey; [d]School of Medicine, Department of Anatomy, Adiyaman University, Adıyaman, Turkey; [e]School of Medicine, Department of Histology and Embryology, Kahramanmaraş Sutcu Imam University, Kahramanmaras, Turkey; [f]Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Adiyaman University, Adiyaman, Turkey; [g]School of Medicine, Department of Anesthesiology and Reanimation, Adiyaman University, Adiyaman, Turkey

ABSTRACT

Objective: Biomarkers such as Osteoprotegerin (OPG), Receptor Activator of Nuclear Factor κ-B (RANK), its ligand RANKL, and Transient Receptor Potential Canonical 1 (TRPC1) have been implicated in the neuroprotective response to neuronal injury, with their expression potentially influenced by antioxidant treatments. The objective of this study was to investigate the effects of Cortexin on the expression of these biomarkers in brain tissue following cerebral ischemia-reperfusion (I/R) injury.

Methods: Thirty-five male Wistar albino rats were divided into five groups: control, ischemia (45 minutes), I/R (7 days), I/R + 1 mg/kg Cortexin, and I/R + 2 mg/kg Cortexin. On day 8, rats were euthanized, and brain and serum samples were collected. Immunohistochemical staining was used to assess biomarker expression in brain tissue, while serum total oxidant status (TOS) and total antioxidant status (TAS) were measured using ELISA.

Results: Oxidative stress analysis showed significantly increased TOS levels ($p = 0.012$; $p = 0.005$) and decreased TAS levels ($p = 0.000$; $p = 0.000$) in the ischemia and I/R groups compared to control. Cortexin significantly reduced TOS ($p < 0.01$) and increased TAS, with 2 mg/kg Cortexin producing TAS levels higher than control ($p < 0.05$). Immunohistochemical analysis revealed significantly elevated OPG, RANK, RANKL, and TRPC1 expression in ischemia and I/R groups ($p < 0.001$). Cortexin treatment significantly decreased expression of all markers ($p < 0.01$). No significant difference was found between ischemia and I/R groups ($p > 0.05$), suggesting a sustained inflammatory response.

Conclusion: These findings suggest that Cortexin may exert neuroprotective effects by modulating oxidative stress and biomarker expression involved in inflammation and calcium signaling.

ARTICLE HISTORY
Received 18 April 2025
Accepted 14 July 2025

KEYWORDS
OPG; RANK; RANKL; TRPC1; cerebral ischemia

Introduction

Ischemic brain injury is emerging as a significant health issue worldwide. One of these health concerns, stroke, is projected to see a 10% increase in prevalence from 2021 to 2040 [1]. I/R injury happens when blood flow to a tissue or organ is blocked and then restored after a delay. I/R induces various pathophysiological events, including cell death, acidosis, inflammation, arachidonic acid formation, elevated intracellular Ca^{2+} concentrations, loss of cellular ion homeostasis, free radical-induced toxicity, energy failure, and leukocyte infiltration [2]. If not adequately treated, ischemic brain injury may result in permanent neurological deficits and a profound impairment of quality of life.

Cortexin is a polypeptide consisting of 82 amino acids derived from the cerebral cortex of cattle (*Bos taurus*) and pigs (*Sus scrofa*). It is known to support neuronal survival and repair, enhance memory formation, and regulate brain function in both acute and chronic cerebrovascular disorders [3]. Due to its small molecular weight (7 kDa), Cortexin can cross the blood–brain barrier. It has also been shown to

CONTACT Seda Koçak seda.kocak@ahievran.edu.tr Department of Physiology, School of Medicine, Kırşehir Ahi Evran University, Kırşehir, Turkey

exhibit antioxidant properties by reducing lipid peroxidation and possesses anti-apoptotic effects [4]. Cerebral ischemia/reperfusion (I/R) injury, associated with various pathological cellular processes including ROS generation, exacerbates neuronal damage [5]. Given its antioxidant and anti-apoptotic properties, Cortexin has the potential to mitigate some of the detrimental effects of oxidative stress in cerebral I/R injury.

Recent research has highlighted the involvement of the receptor activator of nuclear factor-κB (RANK) signaling axis, which includes RANK, receptor activator of nuclear factor kappa-B ligand (RANKL), and osteoprotegerin (OPG) in various physiological and pathological processes. Initially identified in bone and immune systems in the late 1990s, this signaling triad regulates cellular and metabolic processes that influence conditions such as osteoporosis, rheumatoid arthritis, certain cancers, and diabetes mellitus [6]. More recent studies have expanded our understanding of the role these proteins play in neurological conditions, particularly in neural tissue damage and the reparative processes following ischemic injury [7]. Studies have reported that biomarkers such as OPG, RANK, RANKL, and TRPC1 undergo changes due to the ROS that accumulate as a result of oxidative stress, and these biomarkers may serve as promising targets for therapeutic strategies. It has also been suggested that tissue damage could be mitigated by enhancing the levels of these biomarkers in tissues using antioxidant agents [8]. Given the involvement of oxidative stress in both I/R injury and the modulation of RANK, RANKL, and OPG, these biomarkers may serve as valuable targets for therapeutic strategies aimed at reducing damage and promoting recovery in cerebral ischemic conditions.

One of the plasma membrane cation channels belonging to the transient receptor potential (TRP) superfamily is TRP canonical (TRPC) channels. TRPC1 is a member of the TRPC sub-family and is the most widespread channel in the brain [9]. This channel is a non-selective, Ca^{2+} -permeable cation channel and plays a crucial role in regulating apoptotic processes within the brain [10]. Studies have demonstrated that TRPC1 contributes to the protection of neurons from injuries caused by cerebral I/R by suppressing reactive oxygen species (ROS) formation [11]. Pathway enrichment analysis of the selected targets (OPG, RANK, RANKL, and TRPC1) may offer deeper insight into the molecular mechanisms influenced by Cortexin. Furthermore, the potential for synergistic effects with other natural compounds warrants further investigation [12].

The aim of this study is to examine the effects of Cortexin, a compound known for its antioxidant and anti-apoptotic properties, on the expression levels of key biomarkers including OPG, RANK, RANKL, and TRPC1 in the brain tissues of rats subjected to experimental cerebral I/R. This investigation aims to elucidate the potential alterations in these biomarkers induced by oxidative stress and to assess the neuroprotective mechanisms that Cortexin may modulate in the context of cerebral I/R injury.

Materials and methods

Ethics committee approval

This study was approved by the Local Ethics Committee for the Use of Experimental Animals at Adıyaman University (Approval number: 2019–03–058). The experiments were carried out in compliance with the 'Guide for the Care and Use of Laboratory Animals'.

Cortexin application

Various doses of Cortexin have been used in previous studies, showing noticeable differences. These include 0.5 mg/kg [13], 1 mg/kg [14–17], 2 mg/kg [18], 3 mg/kg [16], and 4 mg/kg [19]. Notably, even a low dose of 0.5 mg/kg was found to reduce ischemic injury [13]. In our study, we selected 1 mg/kg and 2 mg/kg doses, which were administered intraperitoneally and acutely for a duration of 7 days.

Power analysis for sample size calculation

The required number of animals per group was determined using G*Power (version 3.1, Universität Düsseldorf, Germany), a statistical power analysis tool. Based on a one-way ANOVA with five groups, an effect size (f) of 0.60 (large), a significance level (α) of 0.05, and a statistical power (1-β) of 0.80, the minimum sample size required was calculated to be 6 animals per group. To account for potential losses or

unexpected variability, 7 animals were included in each group, totaling 35 rats. This calculation ensured sufficient statistical power to detect significant differences among experimental groups in the measured outcomes.

Experimental groups

In this study, 35 female Wistar Albino rats (Rattus norvegicus), aged 8–10 weeks and weighing between 200 ± 50 g, were divided into 5 groups, with 7 animals per group. During the 8-day experimental period, the rats were housed at a room temperature of 22 ± 2°C with a 12-hour light/dark cycle and were provided with food and water ad libitum. Prior to surgical procedures, all rats were anesthetized by intraperitoneal (i.p.) injection of 75 mg/kg Ketamine (Alfamine; Alfasan IBV, Woerden, The Netherlands), and 10 mg/kg Xylazine (Alfazine; Alfasan IBV, Woerden, The Netherlands).

Group I (Control group) ($n = 7$): On the first day of the experiment, rats in this group underwent only arterial clamping and intraperitoneal (i.p.) injections, without any additional surgical procedures. To replace the lost fluids, 5% of the rat's body weight in sterile saline (SS) was administered. On day 8, under anesthesia, blood samples were collected intracardiacally, followed by decapitation. Subsequently, brain tissues were extracted for further analysis.

Group II (Ischemia group) ($n = 7$): On the first day, rats in this group were fixed in a supine position on the operating table, and the midline of the neck was shaved. After disinfecting the surgical area, a midline incision was made. Following superficial microdissection, the right common carotid artery (CCA) was identified and further dissected. The trachea was exposed, and the paratracheal muscles were carefully dissected to access the internal carotid artery (ICA). A clamp was applied to the ICA, and it was kept clamped for 45 minutes to induce ischemia. After the ischemic period, blood samples were collected intracardiacally, and decapitation was performed to extract the brain tissues.

Group III (I/R group) ($n = 7$): On the first day, rats in this group were fixed in a supine position, the neck was shaved, and the surgical site was disinfected. After making a midline incision and performing superficial microdissection, the right common carotid artery (CCA) was identified and dissected to access the internal carotid artery (ICA). A clamp was placed on the ICA, and ischemia was induced for 45 minutes. Following the ischemic period, reperfusion was performed. The surgical incisions were sutured, and 5% of the rat's body weight in sterile saline was administered to compensate for fluid loss. No further interventions were performed until day 8, when the rats were anesthetized. Blood samples were collected intracardiacally, and decapitation was performed to extract brain tissues for analysis.

Group IV (I/R +1 mg/kg Cortexin group) ($n = 7$): On day 1, Cortexin (1 mg/kg/day) was administered intraperitoneally (i.p.). Following this, rats were subjected to the same surgical procedure as in Group III, with ischemia induced for 45 minutes by clamping the internal carotid artery (ICA). After the ischemic period, reperfusion was performed. The surgical incisions were sutured, and 5% of the rat's body weight in sterile saline (SS) was administered to replace the lost fluids. Cortexin was then administered daily at a dose of 1 mg/kg/day from day 1 until day 7. On day 8, under anesthesia, blood samples were taken intracardiacally, followed by decapitation, and brain tissues were extracted for further analysis.

Group V (I/R +2 mg/kg Cortexin group) ($n = 7$): On day 1, Cortexin (2 mg/kg/day) was administered intraperitoneally (i.p.). Rats were subjected to the same procedure as described for Group IV. Ischemia was induced for 45 minutes by clamping the internal carotid artery (ICA), followed by reperfusion. The surgical incisions were sutured, and 5% of the rat's body weight in sterile saline was administered to replace the lost fluids. Cortexin was administered daily at a dose of 2 mg/kg/day from day 1 until day 7. On day 8, under anesthesia, blood samples were taken intracardiacally, followed by decapitation, and brain tissues were extracted for further analysis.

After decapitation, brain tissues were immediately placed in 10% neutral formaldehyde for fixation. Following routine histological processing, the tissues were embedded in paraffin to form blocks. Sections of 3–5 µm thickness were obtained from these blocks and immunohistochemically stained to evaluate the immunoreactivity of OPG, RANK, RANKL, and TRPC1 in the brain cortex. Blood samples collected intracardiacally were centrifuged at 1107 G for 10 minutes (Hettich ROTİNA 380/380 R, Germany) to separate the serum. The antioxidant and oxidant capacity values of the serum were then measured using the ELISA method.

Biochemical analyses

Serum total antioxidant (TAS) and oxidant status (TOS) levels

The TOS and TAS levels in serum samples were measured by the ELISA method. TOS (Rat TOS Catalog No: YLA1392Ra, YL Biotechnology Co., Ltd., Shanghai, China) and TAS (Rat TAS Catalog No: YLA3389Ra, YL Biotechnology Co., Ltd., Shanghai, China) levels were determined following the procedures outlined in the kits. Measurements were carried out by Bio-Tek ELX800 (BioTek Instruments, U.S.A.) ELISA reader at a wavelength of 450 nm, as specified by the manufacturer.

Immunohistochemical analyses

Immunohistochemical staining was conducted with slight modifications using the avidin-biotin-peroxidase complex (ABC) method [20]. Paraffin blocks were sectioned into 4–6-µm-thick slices and deparaffinized. Primary antibodies (OPG, RANK, RANKL, and TRPC1) were diluted to 1/200 and applied using the Thermo Scientific ™ TP-015-HA commercial kit. The antibodies used included OPG (Osteoprotegerin Antibody, E-10: sc-390,518, Mouse monoclonal IgG, Santa Cruz Biotechnology, Inc., U.S.A.), RANK (Antibody B-8: sc-390,655, Mouse monoclonal IgG, Santa Cruz Biotechnology, Inc., U.S.A.), RANKL (Antibody G-1: sc-377,079, Mouse monoclonal IgG, Santa Cruz Biotechnology, Inc., U.S.A.), and TRPC1 (Polyclonal antibody, bs -10,404 R, Biossusa, U.S.A.). Positive and negative controls were carried out according to the directions of the manufacturer. After applying AEC chromogen, the slides were counterstained with Mayer's Hematoxylin and examined under a light microscope (Leica DM500 Nussloch, Germany). The slides were subsequently evaluated and photographed using a Leica DM500 microscope (Leica DFC295). Immunoreactivity was scored based on the intensity (0: none, +0.5: very weak, +1: weak, +2: moderate, +3: strong) and the extent (0.1: < 25%, 0.4: 26–50%, 0.6: 51–75%, 0.9: 76–100%) of staining. A histoscore was calculated by multiplying the intensity score by the extent score.

Statistical analyses

All statistical analyses were performed using GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, U.S.A.). Data were expressed as mean ± standard deviation (SD). The normality of the data distribution was assessed using the Shapiro–Wilk test. Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to identify pairwise differences. A p-value of less than 0.05 was considered statistically significant.

Results

Serum TAS and TOS levels assesments

The oxidative stress parameters measured in this study indicated a decrease in serum TAS levels in the ischemia and I/R groups compared to the control, I/R +1 mg/kg Cortexin, and I/R +2 mg/kg Cortexin groups, while TOS levels were found to be elevated. Serum TOS levels were comparable across the control, I/R +1 mg/kg Cortexin, and I/R +2 mg/kg Cortexin groups ($p = 0.850$; $p = 0.183$, respectively). Serum TOS levels were found to be higher in the ischemia and I/R groups than in the control group ($p = 0.012$; $p = 0.005$, respectively). In the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups, serum TOS levels were lower than in the ischemia group significantly ($p = 0.01$; $p = 0.004$, respectively). Furthermore, the I/R group exhibited higher serum TOS levels compared to both the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups ($p = 0.000$; $p = 0.000$, respectively) (Table I). However, no significant difference in serum TOS levels was observed between the ischemia and I/R groups ($p = 0.224$) (Table I). When compared to the control group, serum TAS levels were significantly lower in the ischemia and I/R groups ($p = 0.000$; $p = 0.000$, respectively). However, in the I/R +1 mg/kg Cortexin group, serum TAS levels were compatible with the control group; whereas they were higher in the I/R +2 mg/kg Cortexin group ($p = 0.710$; $p = 0.008$, respectively). Additionally, serum TAS levels were elevated in both the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups compared to the I/R group ($p = 0.008$; $p = 0.000$, respectively) (Table II). However, serum TAS levels were similar between the ischemia and I/R groups ($p = 0.201$) (Table I).

Table I. Effects of cortexin treatment on antioxidant and oxidant levels during the I/R injury.

	TOS		TAS	
Groups	Median (mean-max)	P value	Median (mean-max)	P value
Control	4,86 (3,04–6,30)[a]	< 0,05	15,27 [12, 55]- [16, 46][a]	< 0,05
Ischemia	12,25 [10, 01]- [18,21] [b]		6,25(4,23–7,36) [b]	
I/R	26,45 [8,22,23][bc]		1,36(1,10–2,36)[bc]	
I/R +1 mg/kg Cortexin group	3,65 (2,68–4,45) [ab]		10,01 [8,12,21] [a]	
I/R +2 mg/kg Cortexin group	2,75 (1,86–5,48)[cb]		13,04 [10, 87]- [8,14] [a]	

[a]compared to the control group, [b]compared to the ischemia group, [c]compared to the I/R ($p < 0,05$).

Table II. Comparison of the histoscore levels of TRPC1, OPG, RANK, and RANKL immunoreactivity across the groups.

	TRPC1		OPG		RANK		RANKL	
Groups	Median (mean-max)	P value	Median (mean-max)	P value	Median (mean-max)	P value	Median (mean-max)	P value
Control	0,1 (0,1–0,3)[a]	< 0,05	0,1(0,1–0,2)[a]	< 0,05	0,2(0,1–0,3)[a]	< 0, 05	0,2(0,1–0,4)[a]	< 0,05
Ischemia	0,9 (0,8–1,2) [b]		1,2(0,9–2,4) [b]		1,2(0,8–1,8) [b]		0,9(0,8–1,2) [b]	
I/R	1,2(0,2–2,4)[bc]		1,8(0,6–2,4)[bc]		1,8 [1,2,4][bc]		1,8 [1,2,7] [bc]	
I/R +1 mg/kg Cortexin group	0,2 (0,1–0,4) [a]		0,4(0,2–0,6) [a]		0,4(0,2–0,6) [a]		0,1(0,1–0,4) [a b]	
I/R +2 mg/kg Cortexin group	0,2 (0,1–0,3)[a]		0,4(0,2–0,6) [a]		0,1(0,1–0,2) [a]		0,3(0,1–0,4) [a b]	

[a]compared to the control group, [b]compared to the ischemia group, [c]compared to the I/R ($p < 0,05$).

Immunohistochemical analysis of OPG immunoreactivity in brain cortex

OPG immunoreactivity was comparable in the control group (Figure 1a), the I/R +1 mg/kg Cortexin group (Figure 1d), and the I/R +2 mg/kg Cortexin group (Figure 1e) ($p = 0.697$; $p = 0.112$, respectively). In contrast, OPG immunoreactivity was significantly higher in the ischemia (Figure 1b) and I/R groups (Figure 1c) compared to the control group ($p = 0.000$; $p = 0.000$, respectively). However, OPG immunoreactivity was significantly reduced in the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups compared to the ischemia group ($p = 0.022$; $p = 0.012$, respectively). Additionally, OPG immunoreactivity was higher in the I/R group than in both the I/R +1 mg/kg Cortexin and I/R

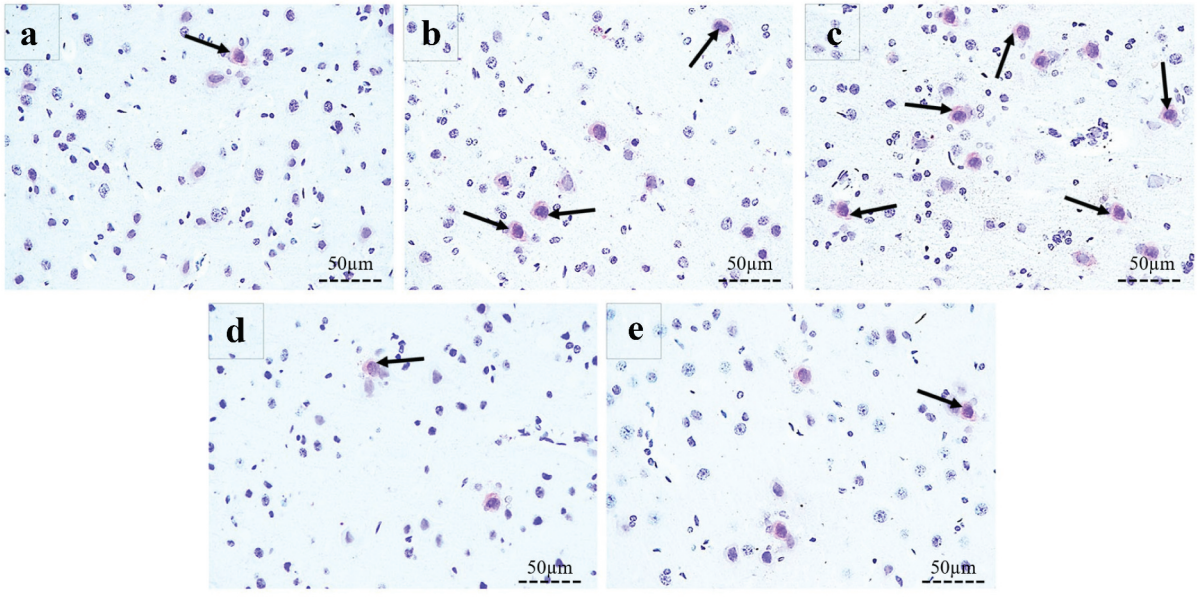

Figure 1. Immunoreactivity of OPG in brain tissue. (black arrow) (a): control group, (b): ischemia group,(c): IR group, (d): IR +1 mg/kg cortexin group, (e): IR +2 mg/kg cortexin group, immunohistochemical staining, Mayers Hematoxylen, AEC chromogen scala bar: 50 µm).

+2 mg/kg Cortexin groups ($p = 0.007$; $p = 0.003$). No significant difference in OPG immunoreactivity was observed between the ischemia and I/R groups ($p = 0.823$) (Figure 5).

Immunohistochemical analysis of RANK immunoreactivity in brain cortex

RANK immunoreactivity was similar in the control group (Figure 2a), the I/R +1 mg/kg Cortexin group (Figure 2d), and the I/R +2 mg/kg Cortexin group (Figure 2e) ($p = 0.145$; $p = 0.458$, respectively). Compared to the control group, RANK immunoreactivity was significantly higher in the ischemia (Figure 2b) and I/R groups (Figure 2c) ($p = 0.003$; $p = 0.000$). However, in the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups, RANK immunoreactivity was significantly reduced compared to the ischemia group ($p = 0.012$; $p = 0.000$, respectively). Furthermore, RANK immunoreactivity in the I/R group was higher than in both the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups ($p = 0.035$; $p = 0.000$). No statistically significant difference in RANK immunoreactivity was observed between the ischemia and I/R groups ($p = 0.557$) (Figure 5).

Immunohistochemical analysis of RANKL immunoreactivity in brain cortex

RANKL immunoreactivity was similar in the control group (Figure 3a), the I/R +1 mg/kg Cortexin group (Figure 3d), and the I/R +2 mg/kg Cortexin group (Figure 3e) ($p = 0.979$; $p = 0.256$, respectively). In contrast, RANKL immunoreactivity was significantly elevated in both the ischemia (Figure 3b) and I/R groups (Figure 3c) compared to the control group ($p = 0.006$; $p = 0.001$). However, RANKL immunoreactivity was significantly reduced in the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups when compared to the ischemia group ($p = 0.005$; $p = 0.004$). Furthermore, RANKL immunoreactivity was higher in the I/R group compared to both the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups ($p = 0.001$; $p = 0.028$). No significant difference in RANKL immunoreactivity was observed between the ischemia and I/R groups ($p = 0.568$) (Figure 5).

Immunohistochemical analysis of TRPC1 immunoreactivity in brain cortex

TRPC1 immunoreactivity was comparable in the control group (Figure 4a), the I/R +1 mg/kg Cortexin group (Figure 3d), and the I/R +2 mg/kg Cortexin group (Figure 3e) ($p = 0.264$; $p = 0.651$, respectively). In contrast, TRPC1 immunoreactivity was statistically elevated in both the ischemia (Figure 3b) and I/R groups (Figure 3c) compared to the control group ($p = 0.000$; $p = 0.000$). However, it was reduced expressively in the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups compared to the ischemia group ($p = 0.016$;

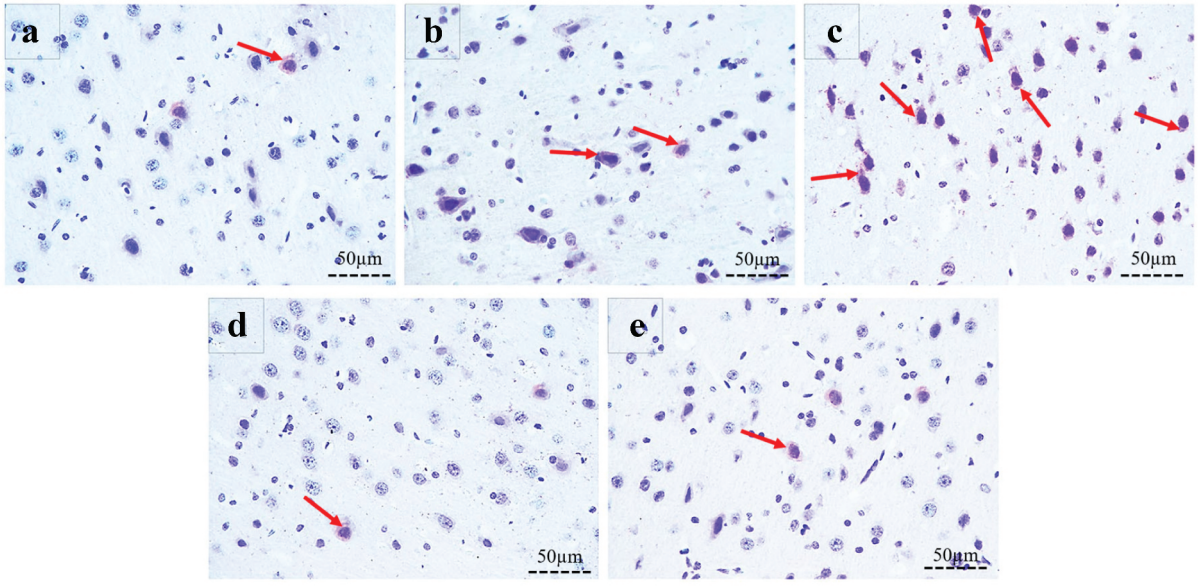

Figure 2. Immunoreactivity of RANK in brain tissue. (black arrow) (a): control group, (b): ischemia group,(c): IR group, (d): IR +1 mg/kg cortexin group, (e): IR +2 mg/kg cortexin group, immunohistochemical staining, Mayers Hematoxylen, AEC chromogen scala bar: 50 µm.

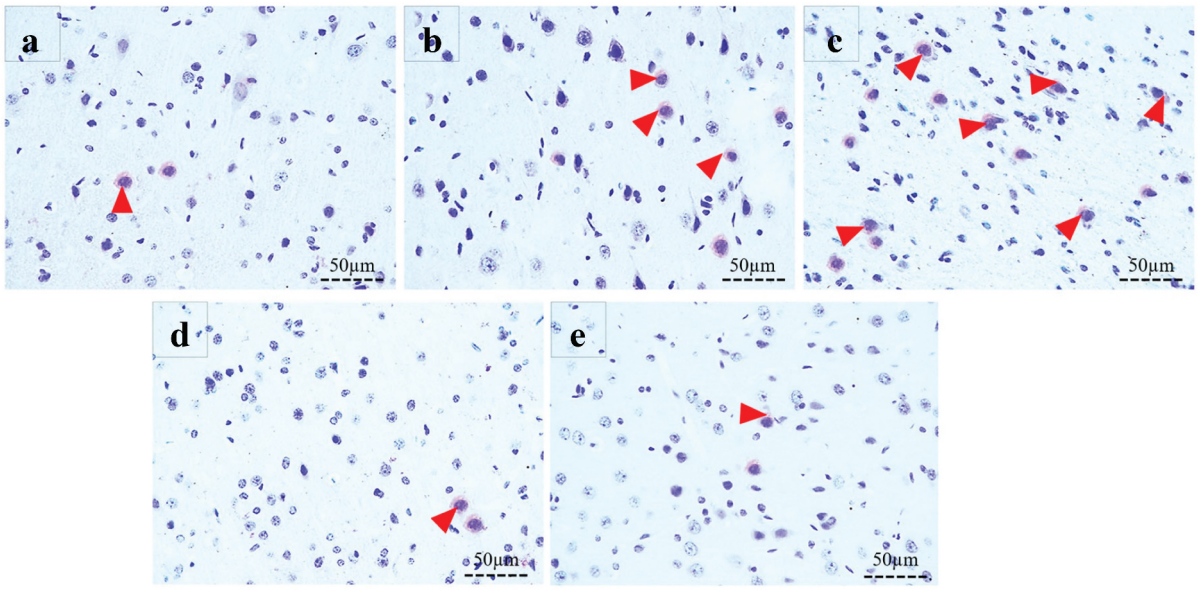

Figure 3. Immunoreactivity of TRPC1 in brain tissue.(black arrow) (a): control group, (b): ischemia group,(c): IR group, (d): IR +1 mg/kg cortexin group, (e): IR +2 mg/kg cortexin group, immunohistochemical staining, Mayers Hematoxylen, AEC chromogen scala bar: 50 µm).

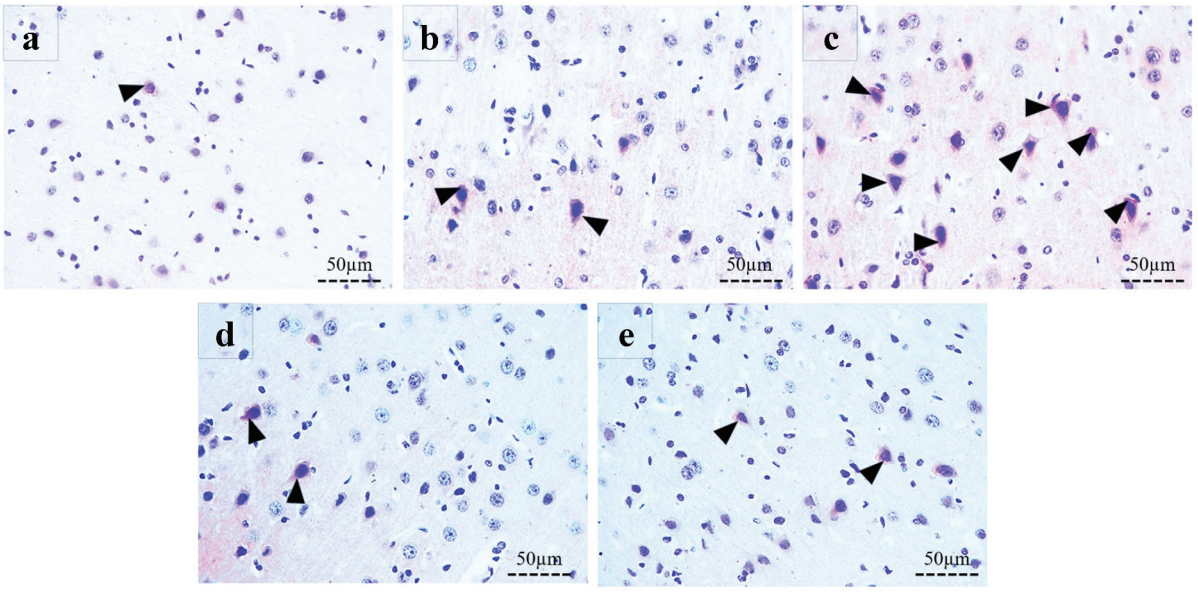

Figure 4. Immunoreactivity of RANKL in brain tissue. (black arrow) (a): control group, (b): ischemia group, (c): IR group, (d): IR +1 mg/kg cortexin group, (e): IR +2 mg/kg cortexin group, immunohistochemical staining, Mayers Hematoxylen, AEC chromogen scala bar: 50 µm.

$p = 0.002$). Moreover, TRPC1 immunoreactivity was elevated in the I/R group than in both the I/R +1 mg/kg Cortexin and I/R +2 mg/kg Cortexin groups ($p = 0.013$; $p = 0.002$). No significant difference in TRPC1 immunoreactivity was observed between the ischemia and I/R groups ($p = 0.947$) (Figure 5).

Discussion

The results of this study indicate that ischemia and ischemia/reperfusion (I/R) increased oxidative stress, as shown by elevated serum TOS levels and decreased TAS levels. However, in the I/R +1 mg/kg and I/R +2

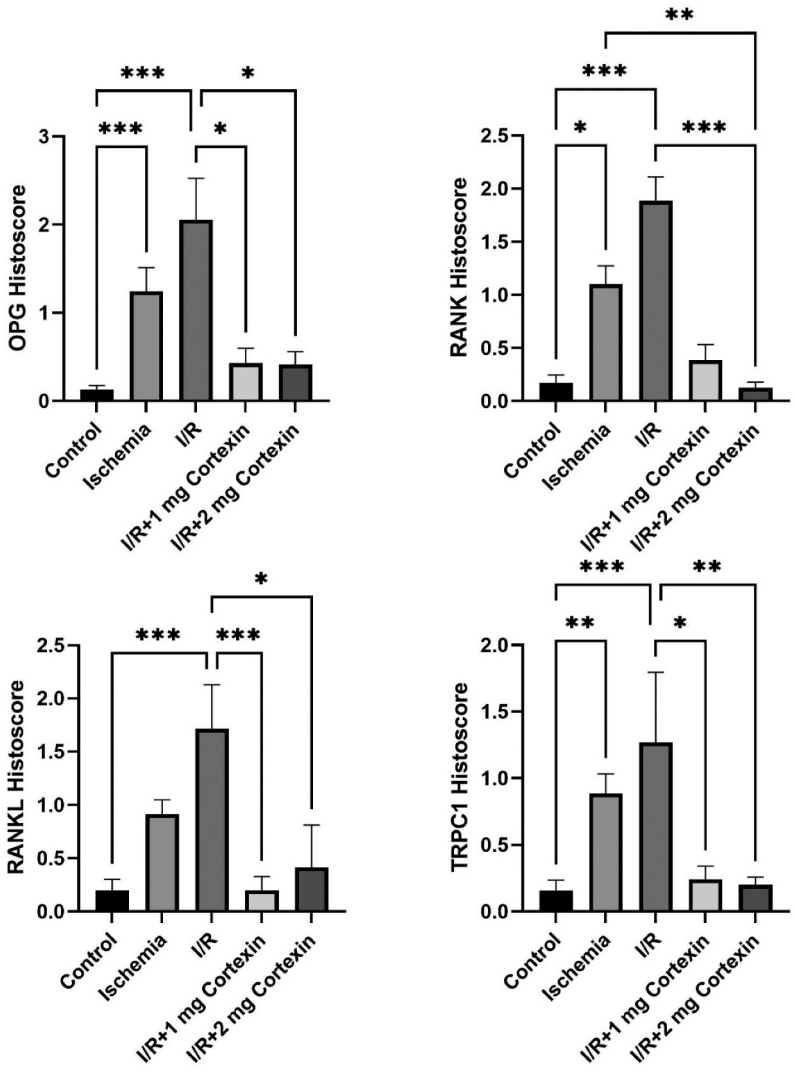

Figure 5. Histoscore analysis of OPC, RANK, RANKL, and TRPC1 expression in different experimental groups. Bar graphs represent the quantification of immunohistochemical staining for OPC (top left), RANK (top right), RANKL (bottom left), and TRPC1 (bottom right) across five groups: control, ischemia, I/R, I/R +1 mg Cortexin, and I/R +2 mg Cortexin. Data are shown as mean ± standard deviation. *< 0.05 , **< *0.01*, ***< *0.001*.

mg/kg Cortexin groups, serum TOS levels significantly decreased, and TAS levels increased, suggesting a protective effect of Cortexin in reducing oxidative stress. Additionally, similar improvements were observed in the expression of OPG, RANK, and RANKL, which are involved in inflammation and bone metabolism. Cortexin treatment appears to exert protective effects by reducing oxidative damage and inflammation. Moreover, it was observed that Cortexin reduced TRPC1 expression, potentially helping to regulate calcium homeostasis. These findings highlight the potential therapeutic benefits of Cortexin in alleviating oxidative stress and inflammation associated with ischemic injury.

After extended periods of ischemia or hypoxia, re-establishing blood flow to the tissue leads to over-production of reactive oxygen species (ROS), which is an important factor in cerebral I/R injury. This results in increased apoptosis, causing damage to brain tissue. Given the limited therapeutic approaches for ischemic stroke, research in this area continues to grow [21]. Study on ischemic stroke found that the patient group had lower circulating TAS levels and higher malondialdehyde (MDA) levels compared to the healthy control group [22]. In another study, it was explored whether serum TAS concentration could reflect the severity and outcome of ischemic stroke. The study reported that TAS concentration was lower in the ischemia group, suggesting that it could serve as a marker for the clinical outcome of ischemic stroke

[23]. A study investigating the potential of serum TAS concentrations in the first week of cerebral infarction as a predictor of mortality revealed a significant decrease in serum TAS levels [15]. Furthermore, another study demonstrated an increase in serum TOS levels during the acute phase in patients with hemorrhagic stroke [24]. The oxidative stress analysis revealed that serum total antioxidant status (TAS) levels were significantly reduced and total oxidant status (TOS) levels were elevated in both the ischemia and I/R groups compared to the control, indicating a pronounced oxidative stress response following cerebral injury. In contrast, treatment with Cortexin at both 1 mg/kg and 2 mg/kg effectively improved oxidative balance. Specifically, TOS levels in these treatment groups were significantly lower than in the ischemia and I/R groups and were statistically similar to the control group, suggesting that Cortexin mitigated the oxidative burden. TAS levels in the I/R +1 mg/kg Cortexin group were comparable to control values, while those in the 2 mg/kg Cortexin group were even higher than in the control, indicating a potential dose-dependent enhancement of antioxidant defense. The biochemical results of our study align with findings suggesting that serum TAS and TOS levels, resulting from excessive ROS production, contribute to oxidative stress. This emphasizes the importance of exploring potential therapeutic agents, such as Cortexin, which could mitigate oxidative damage and improve outcomes in ischemic stroke.

In ischemic stroke, oxidative stress can lead to the disruption of the blood–brain barrier (BBB) [8]. The BBB restricts the passage of pathogens and large or hydrophilic molecules. Moreover, the BBB structure limits the effectiveness of certain antioxidant agents for neuroprotection [25]. Cortexin, an antioxidant agent, has the ability to cross the blood–brain barrier [26]. In a study on spinal cord ischemia, cortexin was found to reduce spinal cord damage and exhibit protective effects [27]. Another study evaluating the efficacy of neurotrophic drugs in ischemic stroke models reported that cortexin demonstrated antioxidant activity and neuroprotective effects [28]. Prospective placebo-controlled studies on neonates with severe brain damage and encephalitis have confirmed the neuroprotective and stress-reducing effects of Cortexin. These studies demonstrated that the addition of Cortexin to early-stage treatment significantly enhanced recovery [22]. A study involving epileptic patients found that incorporating Cortexin into standard treatment protocols led to a substantial reduction in seizure frequency and an increased number of patients achieving remission. Furthermore, improvements were observed in symptoms such as headache, vertigo, fatigue, cognitive disorders, anxiety, depression, and arterial hypertension [29, 30]. A study examining the effects of cortexin on memory impairment found that 86.7% of children aged 7–12 years demonstrated improvement following Cortexin treatment. These findings further support the accumulating evidence that Cortexin may be a promising candidate for therapeutic strategies in ischemic stroke

Through controlling the neuroinflammatory response, the OPG/RANK/RANKL pathway contributes to neuroprotection in cases of cerebral ischemia and brain damage. Numerous investigations using animal and cell models have shown that after ischemia, there is an increase in the expression of RANK, RANKL, and OPG [31]. Although microglial cell activation of the RANK/RANKL system may be neuroprotective at first, over time, this system's dysregulation can worsen tissue damage. Notably, OPG expression that is either too high or too low may paradoxically have neurotoxic effects [32]. Given their established roles in neuroinflammation, ischemia-specific expression alterations, and the necessity to investigate their reactions in light of cortexin's possible neuroprotective effects, this research chose OPG, RANK, and RANKL for our investigation in order to learn more about their reactions with cortexin in ischemic brain injury. Shimamura et al. [33] determined that RANK mRNA levels increased in the infarcted area between 4 and 12 hours following brain ischemia. Additionally, RANKL mRNA expression was elevated at the 7th and 48th hours, while OPG mRNA expression showed an increase at the 12–24 hour and 72 hour time points. Another study suggested that elevated OPG levels may contribute to heightened post-ischemic inflammation through its modulation of the RANK/RANKL signaling pathway, thereby playing a critical role in the regulation of ischemic brain injury via the OPG/RANK/RANKL axis [34]. TRPC channels may exert neuroprotective or neurotoxic effects, varying according to the subtype involved and the nature of the pathological conditions. TRPC1 has been shown to exert a protective effect against cerebral ischemia/reperfusion-induced neuronal injury by suppressing ROS production, as evidenced in both MCAO and OGD models [5]. This effect is mediated not by Ca^{2+} influx itself, but through TRPC1's interaction with the NADPH oxidase component Nox4, promoting its degradation and preventing the assembly of the active oxidase complex [5, 35]. In an I/R mouse model, the absence of TRPC1 in the early stages was found to increase ROS production, thereby exacerbating damage caused by cerebral I/R [36]. Conversely, prolonged

overexpression of TRPC1 has been reported to exert neuroprotective effects against dopaminergic neurotoxins and brain hemorrhage [37]. Our research align with previous literature, demonstrating that in both ischemia and I/R groups, the expression of OPG/RANK/RANKL and TRPC1 biomolecules increased in the brain cortex region at 45 minutes of ischemia and after 7 days of reperfusion, compared to the control group. Cortexin treatment at both 1 mg/kg and 2 mg/kg doses effectively reduced the immunoreactivity of all four markers to levels that were comparable to or approaching those of the control group, with statistically significant differences observed when compared to the ischemia and I/R groups. Notably, no significant differences were observed between the ischemia and I/R groups for any of the markers, indicating that the inflammatory response persisted beyond the reperfusion phase without treatment As a final remark, while there were differences between the cortexin doses in terms of TAS and TOS, there were no differences in terms of immunoreactivities between different doses of cortexin.

Despite the promising results observed in this study, there are limitations. The study focused primarily on the biochemical markers of oxidative stress and inflammation, and did not explore other potential mechanisms by which cortexin might exert its neuroprotective effects. Future studies should investigate the molecular pathways involved in cortexin's neuroprotective properties in greater detail. Second, the study only investigated the effects of cortexin at specific doses and time points; additional research is needed to determine the optimal dosage and treatment duration for maximum efficacy. Third, one of the limitations of this study is the absence of behavioral assessments to evaluate functional neurological recovery. One important limitation of this study is the translational challenge associated with Cortexin. Since Cortexin is derived from animal brain tissue, concerns may arise regarding potential immunogenicity, batch-to-batch variability, and safety for human use. Lastly, the study did not include long-term follow-up to assess the sustainability of cortexin's effects on recovery and neuronal function, which warrants further investigation.

In conclusion, the results of this study suggest that cortexin exerts neuroprotective effects by reducing oxidative stress and inflammation in ischemic injury, as evidenced by improved serum TOS and TAS levels, and enhanced expression of biomarkers involved in inflammation and calcium homeostasis. These findings also suggest the potential therapeutic benefits of cortexin in mitigating the detrimental effects of ischemia and ischemia/reperfusion injury, offering a promising approach to stroke management. However, further research is necessary to elucidate the molecular mechanisms underlying cortexin's effects, establish optimal dosages, and assess the long-term sustainability of its neuroprotective properties.

Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

The author(s) reported that there is no funding associated with the work featured in this article.

ORCID

Seda Koçak http://orcid.org/0000-0003-1183-4847

Authors' contributions

C.G. and A.T. conceived and supervised the study, and contributed to manuscript revision.C.G. A.T. was responsible for the experimental design and methodology. C.G. A.T. B.Z. A.Y. M.D. S.K. conducted the experiments and collected the data B.Z. performed data analysis and interpretation. A.Y. contributed to statistical analysis and data visualization. S.K. drafted the initial manuscript. M.D. critically reviewed the manuscript for intellectual content. All authors read and approved the final version of the manuscript.

Availability of data and materials

Data will be made available on request.

Ethics approval and consent to participate

This study was approved by the Local Ethics Committee for the Use of Experimental Animals at Adıyaman University (Approval number: 2019–03–058). This study was designed and reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. [35]

References

[1] Li XY, Kong XM, Yang CH, et al. Global, regional, and national burden of ischemic stroke, 1990–2021: an analysis of data from the global burden of disease study 2021. eClinicalmedicine. 2024;75:102758. doi: 10.1016/j.eclinm.2024.102758
[2] Monteiro AM, Couteiro RP, Silva DFD, et al. Remote ischemic conditioning improves rat brain antioxidant defense in a time-dependent mechanism. Acta Cir Bras. 2021;36(7):e360707. doi: 10.1590/acb360707
[3] Fedin A, Belskaya G, Kurushina O, et al. Dose-dependent effects of cortexin in chronic cerebral ischemia (results of a multicenter randomized controlled study). Zh Nevrologii I Psikhiatrii Imeni SS Korsakova. 2018;118(9):35–42. doi: 10.17116/jnevro201811809135
[4] Zarubina I, Shabanov P. Cortexin and cortagen as correcting agents in functional and metabolic disorders in the brain in chronic ischemia. Eksperimental'naia i Klinicheskaia Farmakologiia. 2011;74(2):8–15.
[5] Xu N, Meng H, Liu T, et al. TRPC1 deficiency exacerbates cerebral ischemia/reperfusion-induced neurological injury by potentiating Nox4-derived reactive oxygen species generation. Cellular Physiol Biochem. 2018;51(4):1723–1738. doi: 10.1159/000495676
[6] Yasuda H, Shima N, Nakagawa N, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA. 1998;95(7):3597–3602. doi: 10.1073/pnas.95.7.3597
[7] Walsh MC, Choi Y. Biology of the RANKL–RANK–OPG system in immunity, bone, and beyond. Front Immunol. 2014;5:511. doi: 10.3389/fimmu.2014.00511
[8] Qin Y, Zhang Q, Liu Y. Analysis of knowledge bases and research focuses of cerebral ischemia-reperfusion from the perspective of mapping knowledge domain. Brain Res Bull. 2020;156:15–24. doi: 10.1016/j.brainresbull.2019.12.004
[9] Martinez-Galan JR, Verdejo A, Caminos E. TRPC1 channels are expressed in pyramidal neurons and in a subset of somatostatin interneurons in the rat neocortex. Front Neuroanat. 2018;12:15. doi: 10.3389/fnana.2018.00015
[10] Mizoguchi Y, Monji A. TRPC channels and brain inflammation. Adv Exp Med Biol. 2017;976:111–121.
[11] Xu N, Meng H, Liu T, et al. TRPC1 deficiency exacerbates cerebral ischemia/reperfusion-induced neurological injury by potentiating Nox4-derived reactive oxygen species generation. Cell Physiol Biochem. 2018;51(4):1723–1738. doi: 10.1159/000495676
[12] Ayaz H, Aşır F, Korak T. Skimmianine showed neuroprotection against cerebral ischemia/reperfusion injury. Curr Issues Mol Biol. 2024;46(7):7373–7385. doi: 10.3390/cimb46070437
[13] Demchenko A, Belenichev I. Efficiency of cortexin under the conditions of experimental chronic brain ischemia. Neurochem J. 2016;10(1):64–68. doi: 10.1134/S1819712416010050
[14] Stepanichev MY, Onufriev M, Peregud D, et al. The effects of cortexin on free-radical oxidation and inflammatory processes in rats with normal and accelerated aging. Neurochem J. 2018;12(2):184–194. doi: 10.1134/S1819712418020113
[15] Kuznetsov S, Kuznetsova N. Application of cortexin for correcting the consequences of hypoxic-ischemic damage in the brain of infant rats. J Evol Biochem Physiol. 2016;52(3):217–227. doi: 10.1134/S0022093016030042
[16] Kurkin DV, Bakulin DA, Morkovin EI, et al. Neuroprotective action of cortexin, cerebrolysin and actovegin in acute or chronic brain ischemia in rats. PLOS ONE. 2021;16(7):e0254493. doi: 10.1371/journal.pone.0254493
[17] Zarubina IV, Buznik GV, Shabanov PD. Neuroprotective effects of cortexin and ischemic preconditioning. Pediatrician (St Petersburg). 2015;6(3):56–61. doi: 10.17816/PED6356-61
[18] Eroğlu O, Karlıdağ T, Kuloğlu T, et al. The protective effect of cortexin on cisplatin-induced ototoxicity. J Int Adv Otol. 2017;14(1):27. doi: 10.5152/iao.2017.3825
[19] Sangadieva TI, Gulyaev SM, Lamazhapova GP. Nootropic effect of combined application of means from Baikal seal fat and cortexin at cerebral ischemia. Rev Clin Pharmacol Drug Ther. 2019;17(4):75–78. doi: 10.17816/RCF17475-78
[20] Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem. 1981;29(4):577–580. doi: 10.1177/29.4.6166661

[21] Xu L, Gao Y, Hu M, et al. Edaravone dexborneol protects cerebral ischemia reperfusion injury through activating Nrf2/HO-1 signaling pathway in mice. Fundamental & clinical pharmacology. Fundamental & clinical pharmacology. 2022;36:(5): 790–800. doi: 10.1111/fcp.12782
[22] Cojocaru IM, Cojocaru M, Sapira, V. Evaluation of oxidative stress in patients with acute ischemic stroke. Romanian journal of internal medicine = Revue roumaine de medecine interne. 2013; 51(2):97–106.
[23] Ghonimi Nam, Mahdy ME, Abdelsalam OA, et al. Total Antioxidant Capacity Predicts Outcome in Acute Ischemic Stroke Subtypes in Egyptian Patients. Journal of stroke and cerebrovascular diseases: the official journal of National Stroke Association. 2019;28(7):1911–7.
[24] Cevik MU, Acar A, Yucel Y, et al. Investigation of Total Oxidants/Antioxidants in Patients with İntracerebral Haemorrhage. Turk J Neurol. 2013;19(5):1–4. doi: 10.1111/fcp.12782
[25] Rashid K, Wachira FN, Nyabuga JN, et al. Kenyan purple tea anthocyanins ability to cross the blood brain barrier and reinforce brain antioxidant capacity in mice. Nutr Neurosci. 2014;17(4):178–185. doi: 10.1179/1476830513Y.0000000081
[26] Zarubina IV, Shabanov PD. Cortexin and cortagen as correcting agents in functional and metabolic disorders in the brain in chronic ischemia. Eksperimental'naia i Klinicheskaia Farmakologiia. 2011;74(2):8–15.
[27] Ponomarev GV, Shmonin AA, Shumeeva AG, et al. The administration of neurocytoprotectors in a rat model of experimental spinal cord ischemia. Zhurnal nevrologii i psikhiatrii imeni SS Korsakova. 2017;117(6):1342–6.
[28] Gromova OA, Torshin IY, Stakhovskaia LV, et al. Comparative studies of neurotrophic drugs based on brain hydrolysates. Zhurnal nevrologii i psikhiatrii imeni SS Korsakova. 2019;119(10):134–40.
[29] Belskaya G, Ponomareva I. New possibilities of pharmacological treatment of adult epilepsy. Zhurnal Nevrologii i Psikhiatrii Imeni SS Korsakova. 2014;114(10):59–62.
[30] Chutko L, Slu S, Iakovenko E, et al. Efficacy of cortexin in the treatment of memory disorders in children. Zhurnal Nevrologii i Psikhiatrii Imeni SS Korsakova. 2011;111(9 Pt 2):37–40.
[31] Freeman C, A S MD, A S MP. Unraveling the intricacies of OPG/RANKL/RANK biology and its implications in neurological disorders—a comprehensive literature review. Molecular Neurobiology. 2024;61(12):10656–10670.
[32] Shimamura M, Nakagami H, Shimizu H et al. Development of a novel RANKL-based peptide, microglial healing peptide1-AcN (MHP1-AcN), for treatment of ischemic stroke. Scientific reports. 2018;8(1):17770.
[33] Shimamura M, Nakagami H, Osako Mk, et al. OPG/RANKL/RANK axis is a critical inflammatory signaling system in ischemic brain in mice. Proceedings of the National Academy of Sciences. 2014;111(22):8191–6.
[34] Shamakov A, Kasymov V, and Kokhno V. Adjuvants to the treatment of acute cerebral insufficiency in newborns. Zhurnal Nevrologii i Psikhiatrii Imeni SS Korsakova. 2011;111(2):60–3.
[35] Jeon J, Bu F, Sun G, Tian J, Ting S, Li J, Aronowski J, Birnbaumer L, Freichel M, Zhu M X. (2020). Contribution of TRPC Channels in Neuronal Excitotoxicity Associated With Neurodegenerative Disease and Ischemic Stroke. Front Cell Dev Biol. 8:618663. doi: 10.3389/fcell.2020.618663
[36] Ashok A, Andrabi SS, Mansoor S, Kuang Y, Kwon BK, Labhasetwar V. Antioxidant therapy in oxidative stressinduced neurodegenerative diseases: Role of nanoparticle-based drug delivery systems in clinical translation. Antioxidants. 2022;11(2):408.
[37] Sukumaran P, Sun Y, Antonson N, Singh BB. Dopaminergic neurotoxins induce cell death by attenuating NF κB– mediated regulation of TRPC1 expression and autophagy. The FASEB Journal. 2017;32(3):1640.