Research Article

Prophylactic Zinc and Therapeutic Selenium Administration Increases the Antioxidant Enzyme Activity in the Rat Temporoparietal Cortex and Improves Memory after a Transient Hypoxia-Ischemia

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In the cerebral hypoxia-ischemia rat model, the prophylactic administration of zinc can cause either cytotoxicity or preconditioning effect, whereas the therapeutic administration of selenium decreases the ischemic damage. Herein, we aimed to explore whether supplementation of low doses of prophylactic zinc and therapeutic selenium could protect from a transient hypoxic-ischemic event. We administrated zinc (0.2 mg/kg of body weight; ip) daily for 14 days before a 10 min common carotid artery occlusion (CCAO). After CCAO, we administrated sodium selenite (6 μg/kg of body weight; ip) daily for 7 days. In the temporoparietal cerebral cortex, we determined nitrites by the Griess method and lipid peroxidation by the Gerard-Monnier assay. qPCR was used to measure mRNA of nitric oxide synthases, antioxidant enzymes, chemokines, and their receptors. We measured the enzymatic activity of SOD and GPx and protein levels of chemokines and their receptors by ELISA. We evaluated long-term memory using the Morris-Water maze test. Our results showed that prophylactic administration of zinc caused a preconditioning effect, decreasing nitrosative/oxidative stress and increasing GPx and SOD expression and activity, as well as eNOS expression. The therapeutic administration of selenium maintained this preconditioning effect up to the late phase of hypoxia-ischemia. Ccl2, Ccr2, Cxcl12, and Cxcr4 were upregulated, and long-term memory was improved. Pyknotic cells were decreased suggesting prevention of neuronal cell death. Our results show that the prophylactic zinc and therapeutic selenium administration induces effective neuroprotection in the early and late phases after CCAO.

1. Introduction

Zinc plays a dual role in the cerebral hypoxia-ischemia depending on its concentration in the cerebral stroke area; this concentration is known to be determined by zinc serum levels [1]. Accordingly, low serum levels of zinc have long been considered as a risk factor for stroke [2]. In contrast, the input of low concentration of zinc chloride ($ZnCl_2$) or zinc protoporphyrin (ZnPP) reduces the size of postischemic brain damage [3]. Several mechanisms can be accounted for the latter effect. For instance, the decrease in interleukin-1 (IL-1) and IL-23 expression [4], increase in chemokine and growth factor levels [5], and decrease in oxidative stress are because of the antioxidant activity of Cu and Zn superoxide dismutase (SOD1 and 3) [6]. However, excessive accumulation of zinc can also cause neuronal degeneration in the hippocampus and cerebral cortex [7]. Therefore, hypothermia by preventing the accumulation of zinc decreases the cell death [8–10], the degeneration of hippocampal neurons, and the loss of memory after hypoxia-ischemia [11]. The mechanism underpinning the hypothermia effect is the reduction of zinc transport from the presynaptic neurons into the postsynaptic neurons during experimental global ischemia.

Selenium has been shown to preserve mitochondrial function, stimulate mitochondrial biogenesis, and reduce infarct volume after focal cerebral ischemia [12]. Selenium treatment in a rat ischemia model decreases oxidative stress [13]. Furthermore, the oral administration of selenium improves learning and memory in an Alzheimer's disease rat model [14]. Administration of sodium selenite together with melatonin 30 min before medial cerebral artery occlusion (MCAO) and for 3 days postreperfusion decreases oxidative stress [15]. A mechanism for the antioxidant effect of selenium is the inhibition of inducible nitric oxide synthases (iNOS) and COX-2 expression through the inactivation of p38 MAPK and NF-κB [16]. Another mechanism is the incorporation of selenium into selenoproteins such as glutathione peroxidase and thioredoxin reductase, thus making the removal of peroxides more efficient [17].

Our group has reported opposite effects of zinc administration in the 10 min common carotid artery occlusion (CCAO) rat model, where CCAO causes cell death by apoptosis and necrosis without producing an ischemic core [11, 18]. The subacute administration of zinc (2.5 mg/kg) before CCAO exerts a neuroprotective effect by increasing the expression of CCL2/CCR2, FGF2, and IGF-1 in the temporoparietal rat cortex [5]. In contrast, chronic zinc administration at a low dose (0.5 mg/kg body weight) before CCAO decreases CCL2/CCR2, CCL3/CCR1, CCL4/CCR8, and CXCL13/CXCR5 and increases CXCL12/CXCR4, but does not prevent cell death in the late phase [19]. The antioxidant effect of selenium has been used to exert neuroprotection alone [12] or in combination with other antioxidants such as melatonin [15], *Ginkgo biloba* [13], and alpha-tocopherol [20]. Therefore, we propose that the combination of the prophylactic effect of zinc with the therapeutic effect of selenium can maintain the neuroprotection on neuroinflammation and neurodegeneration induced by transient CCAO.

To test that hypothesis, we administered zinc (0.2 mg/kg body weight; ip) for 14 days before CCAO, followed by sodium selenite (6 μg/kg body weight; ip) administration for 7 days after CCAO. We determined nitrosative-oxidative stress (nitrites, lipid peroxidation, NOSs, and antioxidant enzymes), markers of neuroinflammation (chemokines and their receptors) and cell death, over time after CCAO. We also measured neuronal plasticity using the Morris-Water maze test. Because the temporoparietal cortex resulted to be more affected than the hippocampus, we only reported the findings in the temporoparietal cortex. Our results demonstrate that the combined treatment of zinc with selenium extends the effective neuroprotection against CCAO-induced hypoxia-ischemia.

2. Materials and Methods

2.1. Experimental Animals. Male Wistar rats (body weight 190 to 240 g) were obtained from the vivarium of CINVESTAV and maintained in suitable rooms with controlled conditions of temperature ($22 \pm 3°C$) and light-dark cycles (12 h–12 h; light onset at 07:00). Five rats per cage (acrylic; 34 cm × 44 cm × 20 cm) were housed. Food (Laboratory Autoclavable Rodent Diet 5010, 130 ppm of zinc, and 0.47 ppm of selenium) and drinking water were provided ad libitum. All procedures were by the current Mexican legislation, NOM-062-ZOO-1999 (SAGARPA), which in turn is based on the Guide for the Care and Use of Laboratory Animals, NRC. The Institutional Animal Care and Use Committee approved the experimental procedures with the protocol number 09-102. All efforts were made to minimize animal suffering.

2.2. Zinc and Selenium Administration. Zinc was administered as $ZnCl_2$ (0.2 mg/kg of body weight in water for injection, ip; Sigma-Aldrich; Saint Louis, MO, USA) every day for 14 days (chronic zinc administration). Sodium selenite (6 μg/kg of body weight in the water for injection, ip Sigma-Aldrich; Saint Louis, MO, USA) administration started 24 h after the last dose of Zn and continued every day for 7 days. Rats were grouped as follows: (1) control, healthy rats without treatment and surgery, (2) zinc, chronic zinc administration, (3) Zn + Se, chronic zinc administration followed by a single selenium administration on day 15, (4) CCAO, common carotid artery occlusion for 10 min, (5) Zn + CCAO, chronic zinc administration before CCAO, and (6) Zn + CCAO + Se, chronic zinc administration before CCAO followed by selenium administration for 7 days. The rats of groups (1), (2), and (3) were euthanized on day 15 to dissect out their brains. The brains of groups (4), (5), and (6) were obtained at different times (3, 6, 24, and 168 h) after reperfusion. All the variables studied were measured in the temporoparietal cortex, and all rat groups were age-matched.

2.3. Common Carotid Artery Occlusion (CCAO). The asepsis procedures were performed in the surgical instruments and surgical area. The animals were anesthetized with a mixture of ketamine (70 mg/kg) and xylazine (6 mg/kg) at a dose of 200 μL/100 g of body weight, ip. A 0.5 cm-long midline

skin incision was made in the neck area, and the left common carotid artery was carefully dissected. Then, the artery was occluded for 10 min with a clamp (Bulldog Clamps, INS6000119; Kent Scientific Corporation; Torrington, CT, USA). Upon completion of the occlusion, the reperfusion of the artery was visually verified, and the incision was sutured with a 3-0 silk thread (Atramat; Ciudad de Mexico, Mexico). The animals were kept in an individual cage under a 100-Watt, yellow light source until their complete recovery. The animals were euthanized and beheaded in the corresponding time postreperfusion using ketamine (70 mg/kg) and xylazine (6 mg/kg) at a dose of $200 \mu L/100 g$ of body weight. The ipsilateral temporoparietal cortex from the different groups was obtained for the biochemical, cellular, and molecular assays.

2.4. Nitrites.
The temporoparietal cortex ($n = 5$ rats in each group) was mechanically homogenized in phosphate-buffered saline solution (PBS), pH 7.4, and centrifuged at 12,500 rpm for 30 min at 4°C by using a Z 216 MK microcentrifuge (HERMLE Labortechnik; Wehingen, Germany). The production of NO was assessed through the accumulation of nitrites (NO_2^-) in the supernatants as described elsewhere [21]. Briefly, the nitrite concentration in $100 \mu L$ of the supernatant was measured by using a colorimetric reaction generated by the addition of $100 \mu L$ of Griess reagent, composed of equal volumes of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1.32% sulfanilamide in 60% acetic acid. The absorbance of the samples was determined at 540 nm with a SmartSpec 3000 spectrophotometer (Bio-Rad; Hercules, CA, USA) and interpolated by using a standard curve of $NaNO_2$ (1 to $10 \mu M$) to calculate the nitrite concentration.

2.5. Lipid Peroxidation.
Malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAD) were measured in the same supernatant where the nitrites were measured ($n = 5$ rats in each group), following the procedure described elsewhere [22]. The colorimetric reaction was made using $200 \mu L$ of the supernatant after the subsequent addition of $650 \mu L$ of 10.3 mM N-methyl-2phenyl-indole (Sigma-Aldrich; Saint Louis, MO, USA) diluted in a mixture of acetonitrile : methanol (3 : 1) and $150 \mu L$ of methanesulfonic acid (Sigma-Aldrich; Saint Louis, MO, USA). The reaction mixture was vortexed and incubated at 45°C for 1 h and afterward centrifuged at 3000 rpm for 10 min. The absorbance in the supernatant was read at 586 nm with a SmartSpec 3000 spectrophotometer (Bio-Rad; Hercules, CA, USA). The absorbance values were compared to a standard curve in the concentration range of 0.5 to $5 \mu M$ of 1,1,3,3-tetramethoxypropane (10 mM stock) to calculate the content of malondialdehyde + 4-hydroxyalkenal (MDA + 4-HAD) in the samples.

2.6. Glutathione Peroxidase Activity.
GPx activity was measured using the Glutathione Peroxidase Assay Kit (ab102530; Abcam; Cambridge, UK) following the manufacturer's instructions. A sample of 50 mg of the temporoparietal cortex was washed with cold PBS and homogenized in $200 \mu L$ cold assay buffer with a mechanic homogenizer on ice for 15 passes and centrifuged at $10000 g$ for 15 min at 4°C. The supernatants were collected into a fresh corresponding tube and samples of $50 \mu L$ were distributed in the respective wells of ELISA microplates (Costar, Corning Incorporated; NY, USA). Then, $40 \mu L$ of the fresh reaction mix was added to the well, mixed, and incubated at room temperature for 15 min. Then, $10 \mu L$ of cumene hydroperoxide solution was added to each well, mixed, and read at 340 nm on a plate reader (Bio-Rad; Hercules, California, USA). Then, the plates were incubated in the dark at 25°C for 5 min and read again at 340 nm. The activity of GSH-Px in nmol/mL was calculated according to the manufacturer's instructions.

2.7. Superoxide Dismutase Activity.
SOD activity was measured using the Superoxide Dismutase Activity Colorimetric Assay kit (ab65354, Abcam; Cambridge, UK) following the manufacturer's instructions. A temporoparietal cortex sample of 50 mg from each rat group was washed in cold PBS and homogenized with a mechanic homogenizer on ice for 15 passes in cold 0.1 M Tris/HCl, pH 7.4 (containing 0.5% Triton X-100, 5 mM β-ME, and 0.1 mg/mL PMSF). Then, the homogenates were centrifuged for 5 min at 4°C at $14000 g$. The supernatants were collected into a fresh corresponding tube, and samples of $20 \mu L$ were distributed in the respective wells of ELISA microplates (Costar, Corning Incorporated; NY, USA). Then, $200 \mu L$ of WST working solution was added into each well followed by the addition of $20 \mu L$ of Enzyme Working Solution. The plates were mixed and incubated for 20 min at 37°C before reading using a microplate reader (Bio-Rad; Hercules, California, USA) at 450 nm. The OD of each sample was normalized with untreated control (group 1).

2.8. Enzyme-Linked Immunosorbent Assay (ELISA).
CCL2/CCR2, CXCL12/CXCR4, and CXCL13/CXCR5 levels were measured by ELISA in homogenates of the temporoparietal cortex ($n = 5$ for each group). Protein content was determined using the Sedmak and Grossberg method [23]. Aliquots containing $5 \mu g$ of total protein were placed into wells of ELISA plates. Subsequently, $100 \mu L$ of 0.1 M carbonate buffer was added into each well and the plates were incubated at 4°C for 18 h. To block nonspecific binding sites, $200 \mu L$ of 0.5% bovine serum albumin (IgG free) was added into each well at room temperature. After 30 min incubation, the wells were washed thrice with PBS-Tween 20 (0.1%) solution. The primary antibodies were rabbit monoclonal antibodies to CCL2 (Bio-Rad/AbD Serotec Cat. number AAR31, RRID:AB_2071792, 1 : 500 dilution), and the following antibodies were obtained from Abcam (Abcam; Cambridge, UK), CCR2 (Abcam Cat. number ab21667, RRID:AB_446468, 1 : 500 dilution), CXCL12 (Abcam Cat. number ab25118, RRID:AB_448630, 1 : 500 dilution), CXCL13 (Abcam Cat. number ab112521, RRID:AB_10863283, 1 : 500 dilution), CXCR4 (Abcam Cat. number ab2074, RRID:AB_302814, 1 : 500 dilution), and CXCR5 (Abcam Cat. number ab10405, RRID:AB_2089665, 1 : 500 dilution). The primary antibodies were added to each well and incubated for 2 h at room temperature. After three washes with PBS-Tween 20

TABLE 1: List of chemokines and receptors assessed in qPCR: TaqMan probe used was obtained from Thermo Fisher Scientific Inc.

Gene	Gene name	Assay
Ccl2	Chemokine (C-C motif) ligand 2	Rn00580555_m1
Ccr2	Chemokine (C-C motif) receptor 2	Rn01637698_s1
Cxcl12	Chemokine (C-X-C motif) ligand 12	Rn00573260_m1
Cxcr4	Chemokine (C-C motif) receptor 4	Rn00573522_s1
Cxcl13	Chemokine (C-X-C motif) ligand 13	Rn01450028_m1
Cxcr5	Chemokine (C-C motif) receptor 5	Rn02132880_s1
Nos1	Nitric oxide synthase 1, neuronal	Rn00583793_m1
Nos2	Nitric oxide synthase 2, inducible	Rn00561646_m1
Nos3	Nitric oxide synthase 3, endothelial	Rn02132634_s1
Sod1	Superoxide dismutase 1, cytosolic	Rn006566938_m1
Sod2	Superoxide dismutase 2, mitochondrial	Rn00690588_g1
Sod3	Superoxide dismutase 3, extracellular	Rn00563570_m1
Gpx4	Glutathione peroxidase 4	Rn00820188_g1

(0.1%), a horseradish-peroxidase-conjugated goat anti-rabbit or mouse IgG (1 : 1000 dilution; Dako North America Inc; Carpinteria, CA, USA) was added into the wells and incubated for 2 h at room temperature. The antibody-antigen complex was revealed by addition of $100\,\mu$L of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) containing 0.3% H_2O_2 into each well. After 15 min, optical density (OD) was determined using a Benchmark multiple reader at 415 nm (Bio-Rad; Hercules, CA, USA). All samples were processed under the same experimental conditions and time.

2.9. Retrotranscription. Total RNA was isolated from 100 mg of temporoparietal cortex using 1 mL of TRIzol (Invitrogen Corporation; Carlsbad, CA, USA) and then RNA-treated with RNase-free DNase I and quantified using a NanoDrop Spectrophotometer (Thermo Scientific NanoDrop Technologies; Wilmington, DE, USA). cDNA was obtained from 5 μg of total RNA using 1 μL of SuperScript III reverse transcriptase kit (Catalog 18080093, Invitrogen; Carlsbad, CA, USA),1 μL of Oligo dT 50 μM, 1 μL of dNTP mix 10 mM, and water grade molecular biology to 13 μL. Retrotranscription conditions were denaturation at 70°C for 10 min, hybridization at 42°C for 5 min, synthesis of cDNA at 55°C for 50 min and then 70°C for 15 min, and removal of RNA at 37°C for 20 min. Finally, 1 μL of RNase H (Invitrogen; Carlsbad, CA, USA) was added and samples were incubated at 37°C for 20 min.

2.10. qPCR. Fresh cDNA was used to amplify each gene using TaqMan probes (Table 1) obtained from Thermo Fisher (Thermo Fisher Scientific; Waltham, MA, USA). The amplification reactions contained 0.25 μL of the respective TaqMan probe, 2.5 μL of Master Mix (TaqMan Universal Master Mix; Life Technologies; Carlsbad, CA, USA), and 2.25 μL of cDNA in a final volume of 5 μL. The conditions for qPCR were 10 min for denaturation at 95°C, followed by 45 cycles of amplification of 15 s at 95°C and 1 min at 60°C.

Rat β-actin was used as internal control and for normalization. The amplification assays were made using a 7900HT Fast Real-Time PCR System (Applied Biosystems; Foster City, CA, USA). The $2^{-\Delta\Delta Ct}$ analyses were used to calculate the relative transcript levels expressed as fold change for gene expression.

2.11. Spatial Reference Learning and Memory. The Morris water maze was used to measure the spatial reference memory. The measurements were conducted in a round tank, 150 cm in diameter and 80 cm deep, filled with water, and divided into four imaginary quadrants. Water was maintained at a temperature of 23 ± 2°C. Several distal visual cues were placed on both walls of the Morris water maze and the room in which it had been installed. This evaluation consisted of five test days of four consecutive trials per day. During the trial, each animal was placed in the tank facing the wall and allowed to swim freely to an escape platform (40 cm in height and 15 cm in diameter), which was submerged by 2 cm under the water surface and conserved to the center of the southeast (SE) quadrant of the tank. If the animals did not find the platform during a period of 60 s in the first trial of each test day, they were gently guided to it, allowed to remain on the platform for 30 s, and then removed from the tank. This procedure was used to ensure that the animals retained the visual-spatial information of the maze online during the execution of the swimming task [24]. Long-term memory was evaluated in the absence of the platform on day 7 after learning. The latency to reach the platform and the number of times that rats pass by the platform location were measured.

2.12. Histopathological Study. The animals were anesthetized with ketamine (70 mg/kg) and xylazine (6 mg/kg) at a dose of 200 μL/100 g of body weight, ip, and intracardially perfused with 200 mL of PBS followed by 100 mL of 4% paraformaldehyde. The brains were obtained and kept in 4% paraformaldehyde at 4°C for 24 h. The brains were embedded in paraffin using a Histokinette (Leica Microsystems; Wetzlar, Germany). The steps of the tissue processing were consecutive dehydration in different ethanol concentrations (80% for 1 h, 96% for 3 h, and 100% for 3), clearance in pure xylol for 2 h, and inclusion in paraffin at 56°C for 2 h. The tissues were placed in blocks using metallic cassettes. The histological sections of 3 μm were made in a rotary microtome-type Minot (Leica RM2135; Wetzlar, Germany) and placed on slides recovered with poly-L-lysine and finally fixed with heat at 60°C for 30 min. The slices were deparaffinized in an oven at 60°C and placed in xylene 2 times for 15 min. The hydration of slices was made by two consecutive passages in decreasing concentration of ethanol (100%, 96%, and 80%) and finally in tap water for 5 min. Hematoxylin staining was performed for 5 min or less until sections look blue. After washing with tap water, the differentiation was carried out with 1% acid alcohol (1% HCl in 70% alcohol) for 1 dip. The slices were washed in running tap water and dipped again in an alkaline solution (i.e., a saturated solution of lithium carbonate) followed by a tap water washing. The staining in 1% eosin Y was done for 10 minutes. The slices were
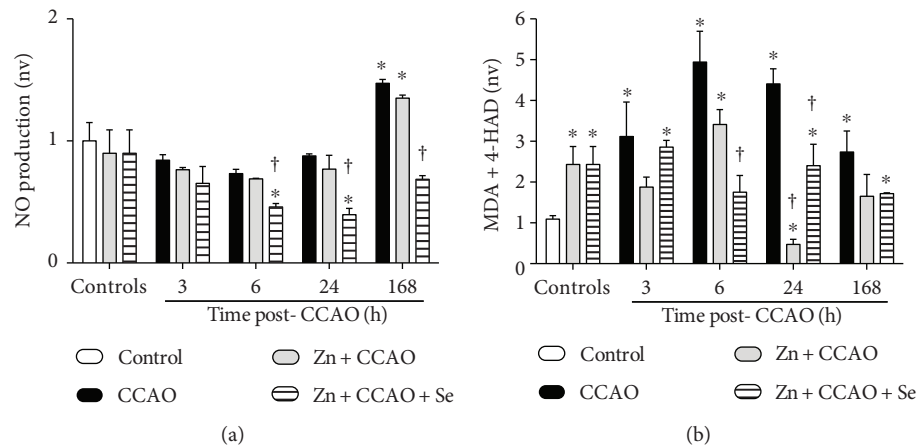
(a)

(b)

FIGURE 1: The combined treatment with zinc and selenium decreases the CCAO-induced nitrosative-oxidative stress in the temporoparietal cortex. (a) Nitrosative stress and (b) lipid peroxidation. CCAO: common carotid artery occlusion for 10 min; Zn + CCAO: chronic zinc administration before CCAO; Zn + CCAO + Se: chronic zinc administration before CCAO followed by selenium administration. The values were normalized against the control untreated group; nv: normalized values. Each value represents mean ± SEM of 5 independent experiments made in triplicate. $^*P < 0.05$, one-way ANOVA with post hoc Dunnett's test when compared to the control untreated group. $^†P < 0.05$, Student's t-test when compared with the respective CCAO group.

washed again with tap water for 1 min. The dehydration of slices was made by ten consecutive dippings in increasing concentration of alcohols (80%, 96%, and 100%) and clearing with xylene. Finally, the slices were mounted on glass slides using Entellan (Merck KGaA; Darmstadt, Germany) and protected with coverslips. The slides were then examined with a light microscope equipped with 10x objective (Leica Microsystems; Wetzlar, Germany). The count of pyknotic cells in 20 fields of 4 brains in layer 5 of the temporoparietal cortex was made using ImageJ software (RRID:SCR_003070, National Institutes of Health).

2.13. Experimental Design and Statistical Analysis. All values were expressed as mean ± SEM from 5 independent experiments including the controls. The values of each variable studied were normalized concerning group 1 (untreated control), except the qPCR values that were expressed as a fold change ($2^{-\Delta\Delta Ct}$). One-way ANOVA and Dunnett's post hoc test were used to compare all groups with the untreated control group. Student's t-test was used to compare the treated groups concerning the CCAO group. All statistical analyses were performed using the GraphPad Prism 6 software. The results of learning memory were analyzed with Kruskal-Wallis one-way analysis of variance for comparison of multiple groups, and the Mann-Whitney U test was used for the statistical analysis of two groups. P values <0.05 were considered statistically significant. All statistical analyses were performed using data analysis software (GraphPad Prism, RRID:SCR_0158070).

3. Results

CCAO increased NO production at 168 h (43 ± 2%, $P =$ 0.0001) postreperfusion in the temporoparietal cortex when compared with the untreated control (Figure 1(a)). The Zn + CCAO group did not prevent the CCAO-induced increase at 168 h in nitrite levels (35 ± 2%, $P = 0.0001$) when

compared with the untreated control (Figure 1(a)). In contrast, the combined treatment with zinc and selenium (Zn + CCAO + Se) completely normalized the nitrite levels when compared with the CCAO group (Figure 1(a)), suggesting a reduction of nitrosative stress. In addition, this combined treatment reduced nitrite levels at 6 h (27 ± 4%, $P = 0.0036$), 24 h (54 ± 6%, $P = 0.0009$), and 168 h (53 ± 2% $P = 0.0004$) after CCAO, when compared to the respective CCAO group (Figure 1(a)).

CCAO also increased lipid peroxidation in the temporoparietal cortex from 3 h (211 ± 84%, $P = 0.0405$), with a maximum peak at 6 h (340 ± 37%, $P = 0.0010$) postreperfusion (Figure 1(b)). The zinc group caused a preconditioning effect, generating an increase in MDA + 4-HAD levels (123 ± 30%, $P = 0.0130$) when compared with the untreated control (Figure 1(b)). The Zn + CCAO group decreased the CCAO-induced increase in lipid peroxidation (89 ± 2%, $P = 0.0492$) only at 24 h, whereas the combined treatment Zn + CCAO + Se decreased lipid peroxidation levels as early as 6 h after CCAO until the end of the study as compared with the respective CCAO group (Figure 1(b)). The reduction in MDA + 4-HAD levels in the Zn + CCAO + Se group at 6 h was 64 ± 8% ($P = 0.0260$), at 24 h was 44 ± 12% ($P = 0.0362$), and at 168 h was 37 ± 1% ($P = 0.0285$).

We evaluated the transcription and expression of NOSs to determine their participation in the NO production in the temporoparietal cortex (Figure 2). CCAO increased mRNA levels for *Nos1* (Figure 2(a)) and *Nos3* (Figure 2(e)), but not for *Nos2* (Figure 2(c)). The increase was statistically significant at 3 h (3.89 ± 0.72, $P = 0.0157$) for *Nos1* and 6.43 ± 3.05, $P = 0.0358$, for *Nos3* at 168 h (3.3 ± 0.4, $P = 0.0499$ for *Nos1* and 7.7 ± 1.1 for *Nos3*, $P = 0.0147$) after CCAO (Figures 2(a) and 2(e), resp.). An upregulation of *Nos1* (3.4 ± 0.69 fold change, $P = 0.0497$) and *Nos3* (12.1 ± 1.25 fold change, $P = 0.0289$) mRNAs was induced by the Zn + Se group; the latter administration also upregulated Nos2 mRNA (6.82 ± 0.1 fold change, $P = 0.0001$) (Figure 2(c)).
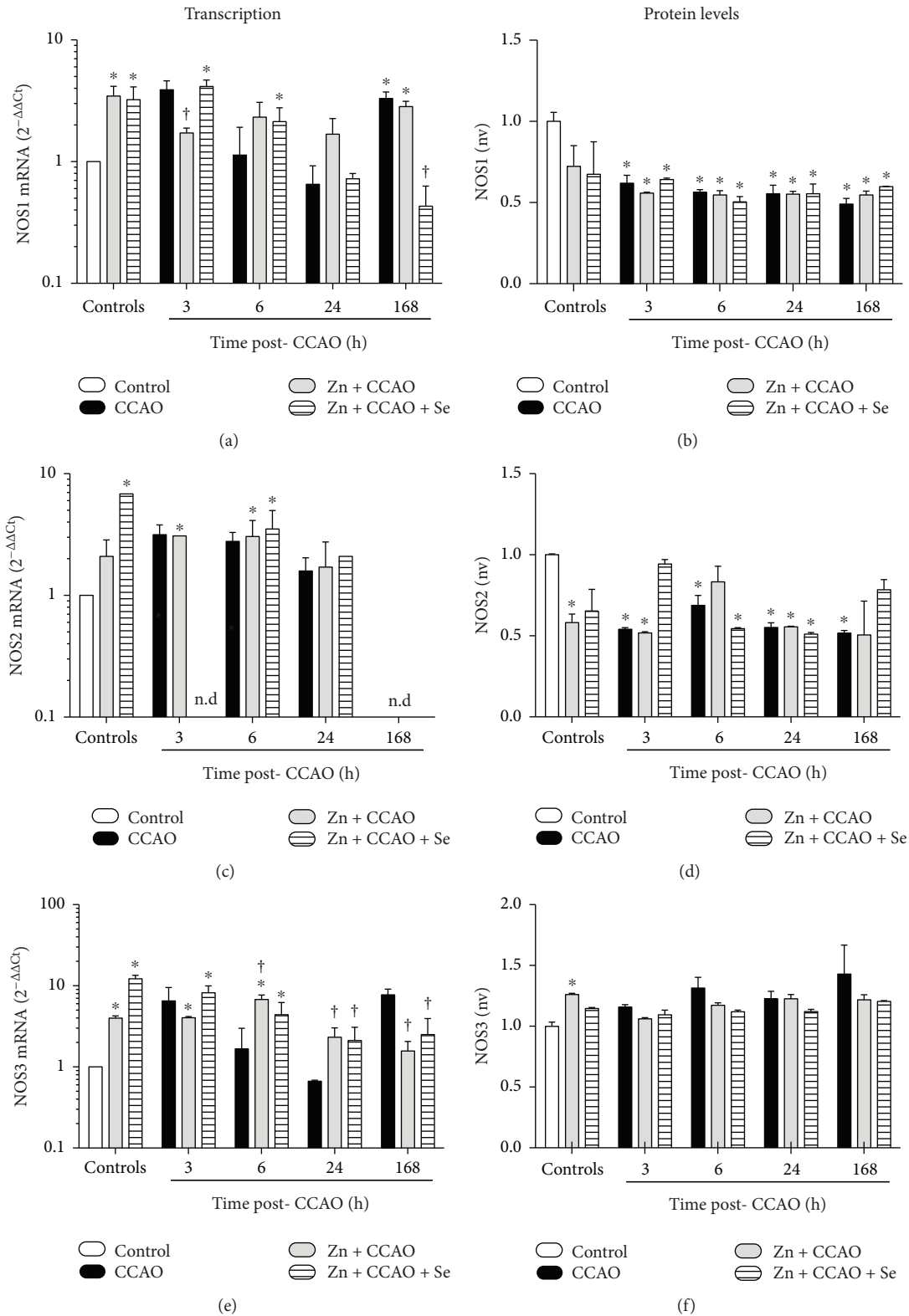
FIGURE 2: Effect of the combined treatment with zinc and selenium on nitric oxide synthase transcription and translation in the temporoparietal cortex of ischemic rats. (a), (c), and (e) mRNA levels; (b), (d), and (f) protein levels. CCAO: common carotid artery occlusion for 10 min; Zn + CCAO: chronic zinc administration before CCAO: Zn + CCAO + Se: chronic zinc administration before CCAO followed by selenium administration. The values were normalized against the control group. nv: normalized values. Each value represents mean ± SEM of 5 independent experiments made in triplicate. $^*P < 0.05$, one-way ANOVA with post hoc Dunnett's test when compared to the control untreated group; $^\dagger P < 0.05$, Student's t-test when compared with the respective CCAO group.
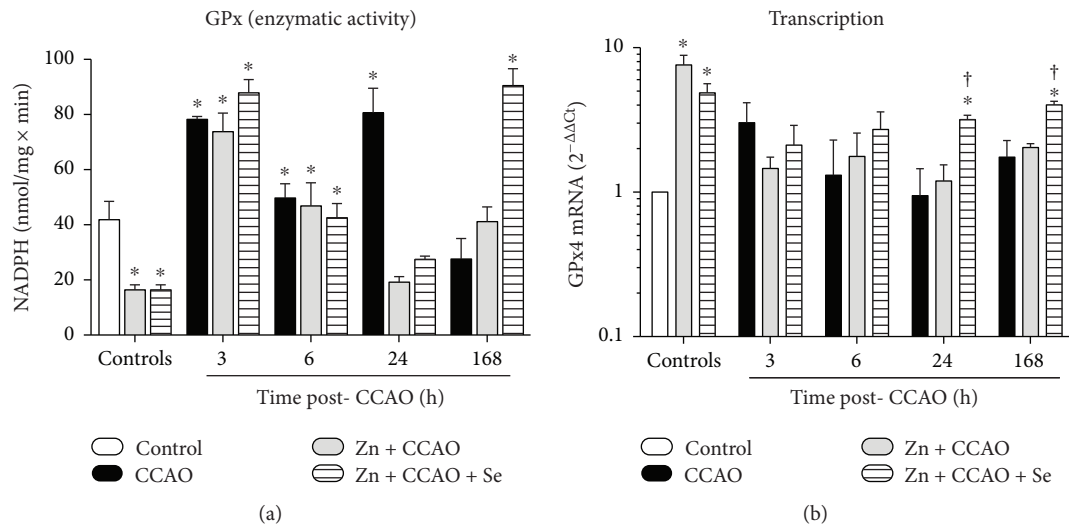
(a)

(b)

FIGURE 3: The combined treatment with zinc and selenium increased the enzymatic activity and transcription of glutathione peroxidase in the late phase of hypoxia-ischemia in the rat. (a) Enzymatic activity of GPx and (b) mRNA levels of GPx4. CCAO: common carotid artery occlusion for 10 min; Zn + CCAO: chronic zinc administration before CCAO; Zn + CCAO + Se: chronic zinc administration before CCAO followed by selenium administration. The values were normalized against the control group. Each value represents mean ± SEM of 5 independent experiments made in triplicate. $^*P < 0.05$, one-way ANOVA with post hoc Dunnett's test when compared to the control untreated group; $^\dagger P < 0.05$, Student's t-test when compared with the respective CCAO group.

Zn + CCAO only prevented the CCAO-induced increase in *Nos1* mRNA at 3 h after CCAO and in *Nos3* mRNA at 168 after CCAO (Figure 2(a) and Figure 2(e)). The combined treatment Zn + CCAO + Se prevented the CCAO-induced increase in *Nos1* and Nos3 mRNAs only at 168 h after CCAO (Figures 2(a) and 2(e)). It is interesting to note that *Nos2* mRNA was not detected in the Zn + CCAO + Se group at 3 h and 168 h after CCAO (Figure 2(c)). A similar effect occurred in the CCAO group and Zn + CCAO group at 168 h after CCAO (Figure 2(c)).

Concerning NOSs protein expression, the CCAO do not modify NOS3 (Figure 2(f)). NOS1 (Figure 2(b)) and NOS2 (Figure 2(d)) in the temporoparietal cortex decreased their expression over time in all groups when compared with the untreated controls, and there was no statistical difference among the groups. The average of decrease was 43% ($P < 0.004$) for NOS1 and 44% ($P < 0.005$) for NOS2.

CCAO increases GPx activity in the temporoparietal cortex at 3 h ($86 \pm 2\%$, $P = 0.0132$) and at 24 h postreperfusion ($92 \pm 11\%$, $P = 0.0100$) when compared with the untreated controls (Figure 3(a)). The Zn + Se group significantly decreased at the same levels ($60 \pm 4\%$, $P = 0.0311$) for GPx activity (Figure 3(a)). A significant decrease in GPx activity was observed at 24 h postreperfusion (Figure 3(a)) in the Zn + CCAO ($76 \pm 3\%$, $P = 0.0027$) and Zn + CCAO + Se groups ($65 \pm 2\%$, $P = 0.0043$). At 168 h postreperfusion, only the Zn + CCAO + Se group increased by $227 \pm 22\%$ for GPx ($P = 0.0028$) activity when compared with the respective CCAO group (Figure 3(a)), showing an antioxidant effect in the late phase.

In the absence of CCAO, *Gpx4* mRNA was upregulated with zinc administration (7.6 ± 1.25, $P = 0.0008$) and Zn + Se administration (4.8 ± 0.7, $P = 0.0190$) when compared with the untreated controls (Figure 3(b)). CCAO did not affect

the basal levels of *Gpx4* mRNA (Figure 3(b)). The combined treatment Zn + CCAO + Se increased *Gpx4* mRNA at 24 h (3.35 ± 0.25, $P = 0.0459$) and at 168 h (2.3 ± 0.13, $P = 0.0485$) postreperfusion (Figure 3(c)) as compared with the CCAO group (Figure 3(b)).

SOD activity in the temporoparietal cortex was increased at $258 \pm 12\%$ ($P = 0.0021$) by the zinc or Zn + Se group when compared with untreated controls (Figure 4(a)). CCAO decreases SOD activity ($65 \pm 11\%$, $P = 0.0486$) at 168 h when compared with the untreated controls (Figure 4(a)). At this time, only Zn + CCAO increased SOD activity ($78 \pm 20\%$, $P = 0.0457$) when compared with the respective CCAO group (Figure 4(a)).

The zinc or Zn + Se group is differentially regulated in the *Sod* isoforms. The zinc group did not affect *Sod1* transcript levels (Figure 4(b)), whereas it upregulated *Sod2* (3.0 ± 0.69, $P = 0.0453$) and Sod3 (2.9 ± 0.76, $P = 0.0280$) transcripts when compared with the untreated controls (Figures 4(c) and 4(d)). The increases in mRNA levels were 2.78 ± 0.23-fold ($P = 0.0030$) for *Sod1*, 2.8 ± 0.2-fold ($P = 0.0395$) for *Sod2*, and 2.4 ± 0.3-fold ($P = 0.0383$) for *Sod3*. CCAO did not affect *Sod1* transcripts. Whereas CCAO upregulated *Sod2* (2.9 ± 0.46, $P = 0.0422$) at 3 h (Figure 4(c)) and *Sod3* at 3 h (2.8 ± 0.28, $P = 0.0038$) and 168 h (2.5 ± 0.39, $P = 0.0065$), CCAO did not modify when compared with the untreated controls (Figure 4(d)). None of the treatments modified *Sod1* transcripts in the times studied when compared with the respective CCAO group (Figure 4(b)). Zn + CCAO + Se only upregulated *Sod2* (5.3 ± 1.3, $P = 0.0051$) and *Sod3* (6.13 ± 0.9, $P = 0.0290$) at 24 h postreperfusion when compared to the respective CCAO group (Figures 4(c) and 4(d)).

Chemokine transcription levels in the temporoparietal cortex are shown in Figure 5. CCAO did not affect transcription of *Ccl2* and its receptor *Ccr2* in the time studied
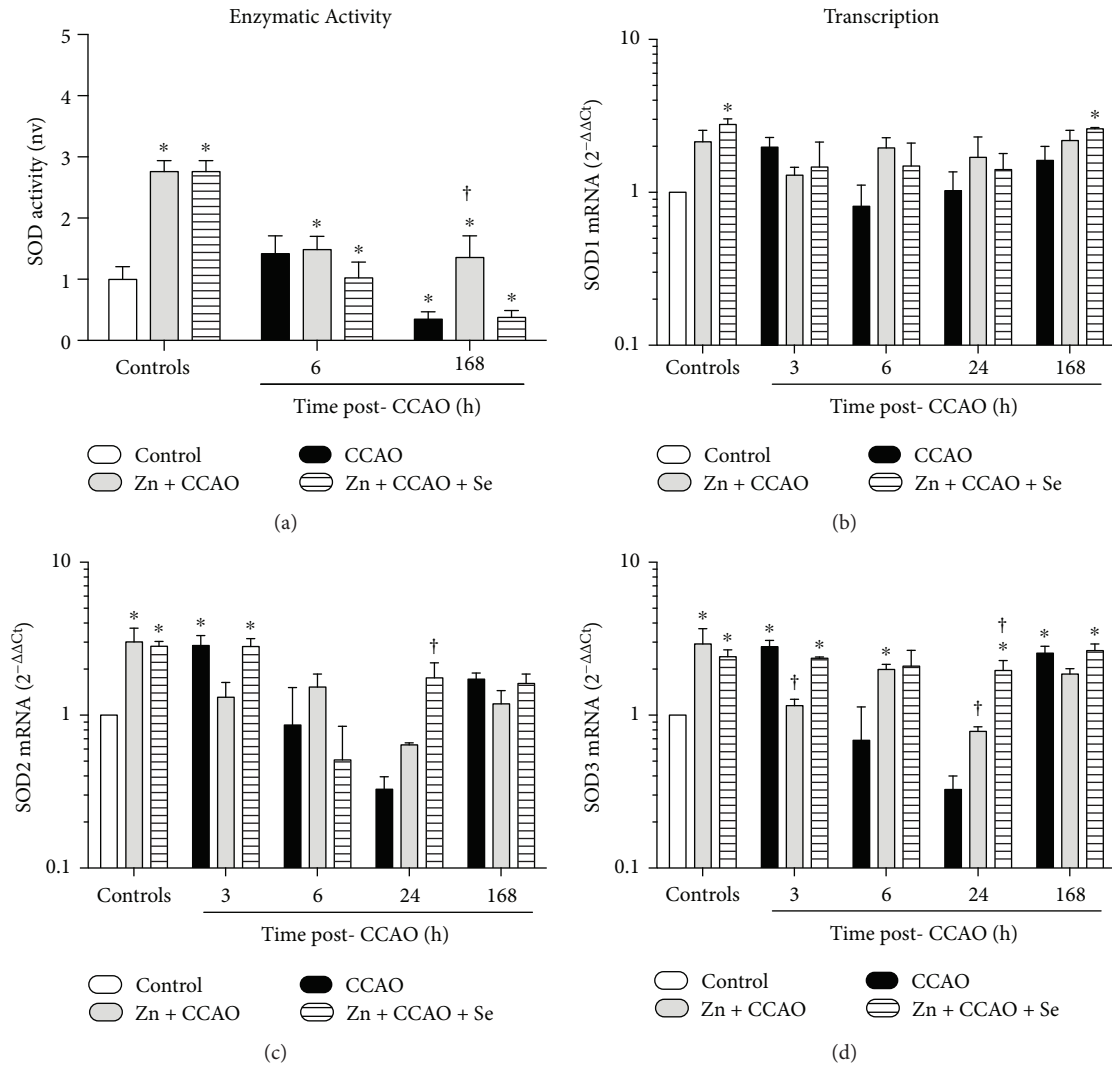
(a)

(b)

(c)

(d)

FIGURE 4: Differential effect of the combined treatment with zinc and selenium on the enzymatic activity and transcription of superoxide dismutase in the rat hypoxia-ischemia model. (a) Enzymatic activity of SOD, (b) mRNA levels of Sod1, (c) mRNA levels of Sod2, and (d) mRNA levels of Sod3. CCAO: common carotid artery occlusion for 10 min; Zn + CCAO: chronic zinc administration before CCAO; Zn + CCAO + Se: chronic zinc administration before CCAO followed by selenium administration. The values were normalized against the control group. Each value represents mean ± SEM of 5 independent experiments made in triplicate. $^{*}P < 0.05$, one-way ANOVA with post hoc Dunnett's test when compared to the control untreated group; $^{†}P < 0.05$, Student's t-test when compared with the CCAO group.

(Figures 5(a) and 5(b)). As compared with the control group, CCAO upregulated the following chemokines and their receptors: Cxcl12 at 3 h (3 ± 0.7, $P = 0.0426$; Figure 5(c)), its receptor Cxcr4 at 3 h (2.7 ± 0.61, $P = 0.0475$; Figure 5(d)) and 6 h post-CCAO (3.9 ± 0.9, $P = 0.0354$; Figure 5(d)), and Cxcl13 at 3 h (3.84 ± 0.0204, $P = 0.01$; Figure 5(e)) and its receptor Cxcr5 at 3 h (16.6 ± 2.2, $P = 0.0005$; Figure 5(f)) and at 6 h (8.0 ± 2.3, $P = 0.0316$; Figure 5(f)).

Zinc caused an upregulation only of Cxcr4 (5.5 ± 0.9, $P = 0.0354$; Figure 5(d)), Cxcl13 (3.5 ± 0.8, $P = 0.0218$; Figure 5(e)), and Cxcr5 (8.3 ± 1.4, $P = 0.0261$; Figure 5(f)), whereas Zn + Se upregulated Ccr2 (3.19 ± 0.77, $P = 0.0164$, Figure 5(b)), Cxcl12 (11.2 ± 1.56, $P = 0.0001$; Figure 5(c)), Cxcr4 (8.8 ± 1.8, $P = 0.0006$, Figure 5(d)), Cxcl13 (3.6 ± 0.13, $P = 0.0268$; Figure 5(e)), and Cxcr5 (9.1 ± 1.6, $P = 0.0085$; Figure 5(f)).

As compared with CCAO effect, Zn + CCAO upregulated the following chemokines and their receptors: Ccl2 at 168 h (2.4 ± 0.2, $P = 0.0307$; Figure 5(a)), Cxcl12 at 6 h (3.8 ± 0.69, $P = 0.0376$; Figure 5(c)), and Cxcr4 at 24 h (4.4 ± 0.2, $P = 0.0001$; Figure 5(d)). Upregulation and downregulation were observed in Cxcl13 at 3 h (0.38 ± 0.12, $P = 0.0321$) and at 6 h (3.49 ± 0.44, $P = 0.0428$; Figure 5(e)) and in Cxcr5 at 3 h (0.23 ± 0.07, $P = 0.0075$) and at 168 h (2.3 ± 0.55, $P = 0.0473$; Figure 5(f)).

As compared with the CCAO group, Zn + CCAO + Se caused an upregulation of Ccl2 at 168 h (3.7 ± 0.69, $P = 0.0226$; Figure 5(a)) and Cxcl12 at 3 h (2.5 ± 0.43, $P = 0.0367$) and 6 h (4 ± 1, $P = 0.0314$, Figure 5(c)). Cxcr4 was upregulated since 24 h (5.5 ± 1.1, $P = 0.0162$) to 168 h (2.65 ± 0.49, $P = 0.0413$; Figure 5(d)) post-CCAO. Cxcl13 was upregulated at 24 h (5.9 ± 1.6, $P = 0.0357$; Figure 5(e)), and
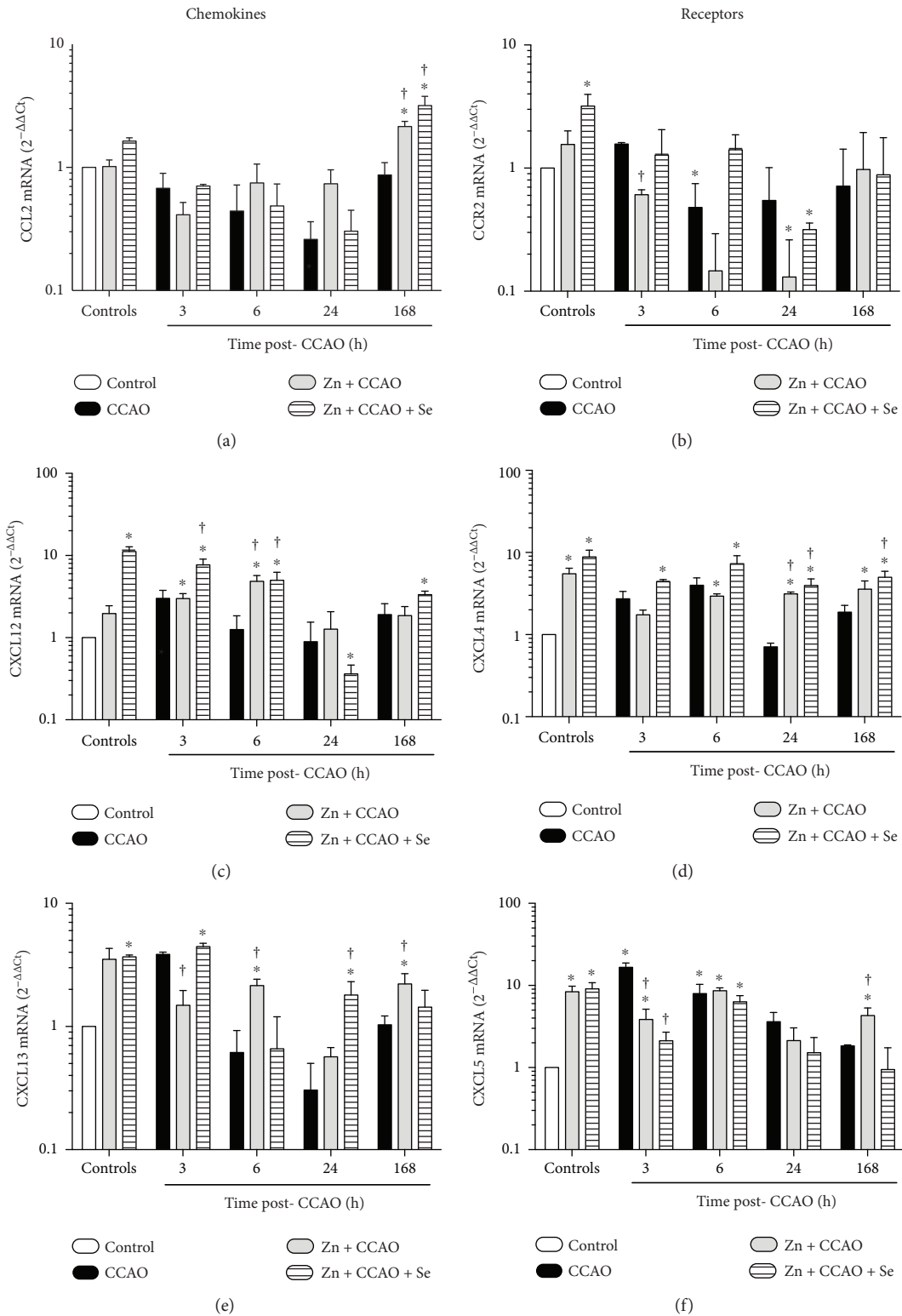
Figure 5: Differential effect of the combined treatment with zinc and selenium on chemokine and receptor transcription in the rat hypoxia-ischemia model. (a), (c), and (e) Chemokines levels; (b), (d), (f) receptors of the respective chemokines. CCAO: common carotid artery occlusion for 10 min; Zn + CCAO: chronic zinc administration before CCAO; Zn + CCAO + Se: chronic zinc administration before CCAO followed by selenium administration. The values were normalized against the control group. Each value represents mean ± SEM of 5 independent experiments made in triplicate. $*P < 0.05$, one-way ANOVA with post hoc Dunnett's test when compared to the control untreated group; $^{\dagger}P < 0.05$, Student's t-test when compared with the respective CCAO group.

Cxcr5 was downregulated at 3 h (0.12 ± 0.03, $P = 0.0031$; Figure 5(f)).

Protein levels of CCR2 were increased by CCAO at 24 h ($17 \pm 6\%$, $P = 0.0457$; Figure 6(b)) in the temporoparietal cortex. CXCL12 levels were increased by Zn + CCAO at 24 h ($24 \pm 4\%$, $P = 0.0114$; Figure 6(c)) or Zn + CCAO + Se at 24 h ($32 \pm 6\%$, $P = 0.0026$; Figure 6(c)), whereas CXCL13 levels were increased by Zn + CCAO + Se ($16 \pm 1\%$, $P = 0.0425$; Figure 6(e)) at 168 h post-CCAO.

The histopathology studies showed the presence of pyknotic cells at 168 h after CCAO (Figure 7(b)) as compared with the control group (Figure 7(a)). Zinc (Figure 7(c)) or Zn + Se (Figure 7(e)) did not modify the histological morphology as compared with the untreated control. On the contrary, CCAO caused a significant increase in the number of pyknotic cells (indicative of apoptosis) by $1460 \pm 188\%$ ($P = 0.0001$) at 168 h postreperfusion as compared with the untreated control (Figures 7(b) and 7(h)). Zn + CCAO reduced the number of pyknotic cells by $80\% \pm 3\%$ ($P = 0.001$) at 168 h, as compared with the CCAO group (Figures 7(d) and 7(h)), but there was a statistical difference as compared with the untreated control group (Figure 7(g)). Zn + CCAO + Se significantly decreased the number of pyknotic cells by $90 \pm 2\%$ ($P = 0.0001$) as compared with CCAO (Figure 7(f) and 7(h)) and reached the basal values of the untreated control (Figure 7(g)).

The functional recovery from CCAO was assessed through the learning and memory test using the Morris water maze. There was not any statistical difference in information acquisition among the groups (Figure 8(a)). In memory evaluation, 7 days after the training, CCAO increased the escape latency by $53.9 \pm 17\%$ ($P = 0.0238$; Figure 8(b)) and decreased the crossing by the platform location by $37 \pm 10\%$ ($P = 0.0272$; Figure 8(c)) as compared with the controls. On the contrary, Zn + CCAO or Zn + CCAO + Se significantly decreased the latency time to remember the localization of the escape platform by $44 \pm 11\%$ ($P = 0.0197$) and $56 \pm 7\%$ ($P = 0.0046$), respectively, as compared with CCAO, suggesting improvement of consolidation of information. Only the Zn + CCAO + Se group showed a crossing by the platform location ($65 \pm 14\%$ $P = 0.0348$) higher than CCAO, thus confirming that this treatment favors the neuronal functionality and exerts effective neuroprotection against hypoxia-ischemia.

4. Discussion

Our results show that the combined prophylactic of zinc and therapeutic of selenium administration had better effective protection against a transient hypoxic-ischemic event in the temporoparietal cortex, unlike other strategies we have tested such as the prophylactic administration of Se alone or combined with Zn (data not shown). This neuroprotection can be mainly explained by the increase in transcription and enzymatic activity of GPx and SOD, which prevented lipid peroxidation, and the significant decrease in neuronal cell death that is shown by the improvement of long-term memory.

Several studies have shown that chronic prophylactic administration of zinc shows a preconditioning effect [25–27]. This preconditioning effect can be explained by the induction of antioxidant enzymes, chemokines, and DNA methylases through zinc finger proteins [28–30]. Selenium has also been involved in the epigenetic regulation at least of antioxidant enzymes and DNA methylases [31]. Accordingly, our results show that the administration of those elements caused an upregulation of N*os3*, *Gpx4*, and *Sod* and the chemokines *Ccl2*, *Cxcl12/Cxcr4*, and *Cxcl13/Cxcr5*; the translation of NOS3; and the increase in the enzymatic activity of GPx and SOD. However, the protein levels of these chemokines and their receptors were not modified by zinc or selenium administration in the period studied. Therefore, the major contributor to the preconditioning effect in the transient hypoxia-ischemia model was the antioxidant effect and the preservation of NO bioavailability through NOS3 expression. As reported in a similar hypoxia-ischemia model, NOS3 is essential in the preservation and maintenance of microcirculation, inhibiting platelet aggregation, leukocyte adhesion, and migration and decreasing the inflammatory response [32]. The increased expression of NOS3 induced by zinc might be associated with the zinc finger protein ZFP580 [33]. Also, zinc stabilizes the dimerization of NOS3, which can prevent the production of superoxide anion and promote the increase in nitric oxide (NO) in the early phase of the hypoxia-ischemia process [34, 35]. Interestingly, selenium enhanced NOS3 expression similarly to only zinc administration at the early phase of CCAO, thus recovering the endothelial function as shown in an endothelial dysfunction model [36].

Our results showed that the prophylactic chronic zinc administration in the transient hypoxia-ischemia model increased the enzymatic activity of SOD and the levels of the transcripts of *sod1*, *sod2*, and *sod3*. These enzymes are known to play a major role in protecting from intracellular, extracellular, and mitochondrial oxidative stress, as reported in the cerebral cortex and hippocampus [37–39]. A mechanism that accounts for the increase in SOD1 and SOD3 activity is their stabilization by zinc [40]. Also, the antioxidant effect of zinc might be due to the induction of metallothioneins, which are involved in the homeostasis of zinc and ROS [41]. Our results also show that the therapeutic administration of selenium maintains the level of enzymatic activity and transcription of SOD2 in the early phase, SOD1 in the late phase, and SOD3 in the complete period of the study post-CCAO. These three enzymes could have provided an effect of resistance/tolerance to ischemia in the early and late phases of cerebral hypoxia-ischemia, as reported in transgenic mice [42–45]. Furthermore, the effects of SOD on preventing the disruption of the blood-brain barrier [46], decreasing karyorrhexis, and attenuating the activation of NF-κB [47] might also explain the neuroprotection induced by the combined treatment with zinc and selenium. Results in *sod1* [48], *sod2*, and *sod3* knockout models [37, 49, 50] also confirmed the neuroprotective effect of SOD. Accordingly, the deficiency of SOD enzymatic activity in the late phase of ischemia has been associated with increases in the size of the infarction, the release
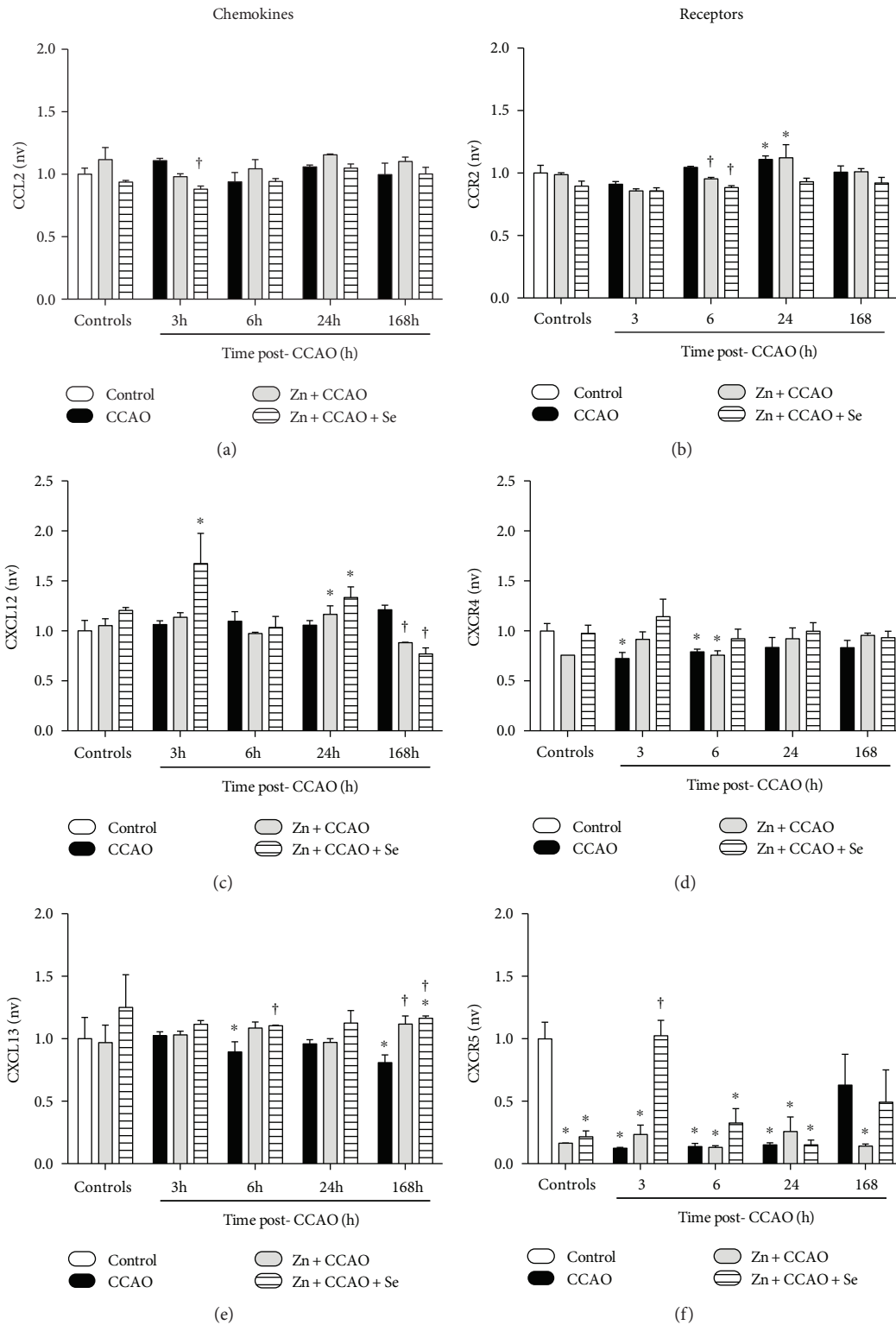
FIGURE 6: The administration of the combined treatment with zinc and selenium modifies the protein levels of chemokines and receptors in the rat hypoxia-ischemia model. (a), (c), and (e) Chemokine levels, and (b), (d), (f) receptors of the respective chemokines. CCAO: common carotid artery occlusion for 10 min; Zn + CCAO: chronic zinc administration before CCAO. Zn + CCAO + Se: chronic zinc administration before CCAO followed by selenium administration. The values were normalized against the control group. nv: normalized values. Each value represents mean ± SEM of 5 independent experiments made in triplicate. $^{*}P < 0.05$, one-way ANOVA with post hoc Dunnett's test when compared with the untreated control group; $^{†}P < 0.05$, Student's t-test when compared with the respective CCAO group.
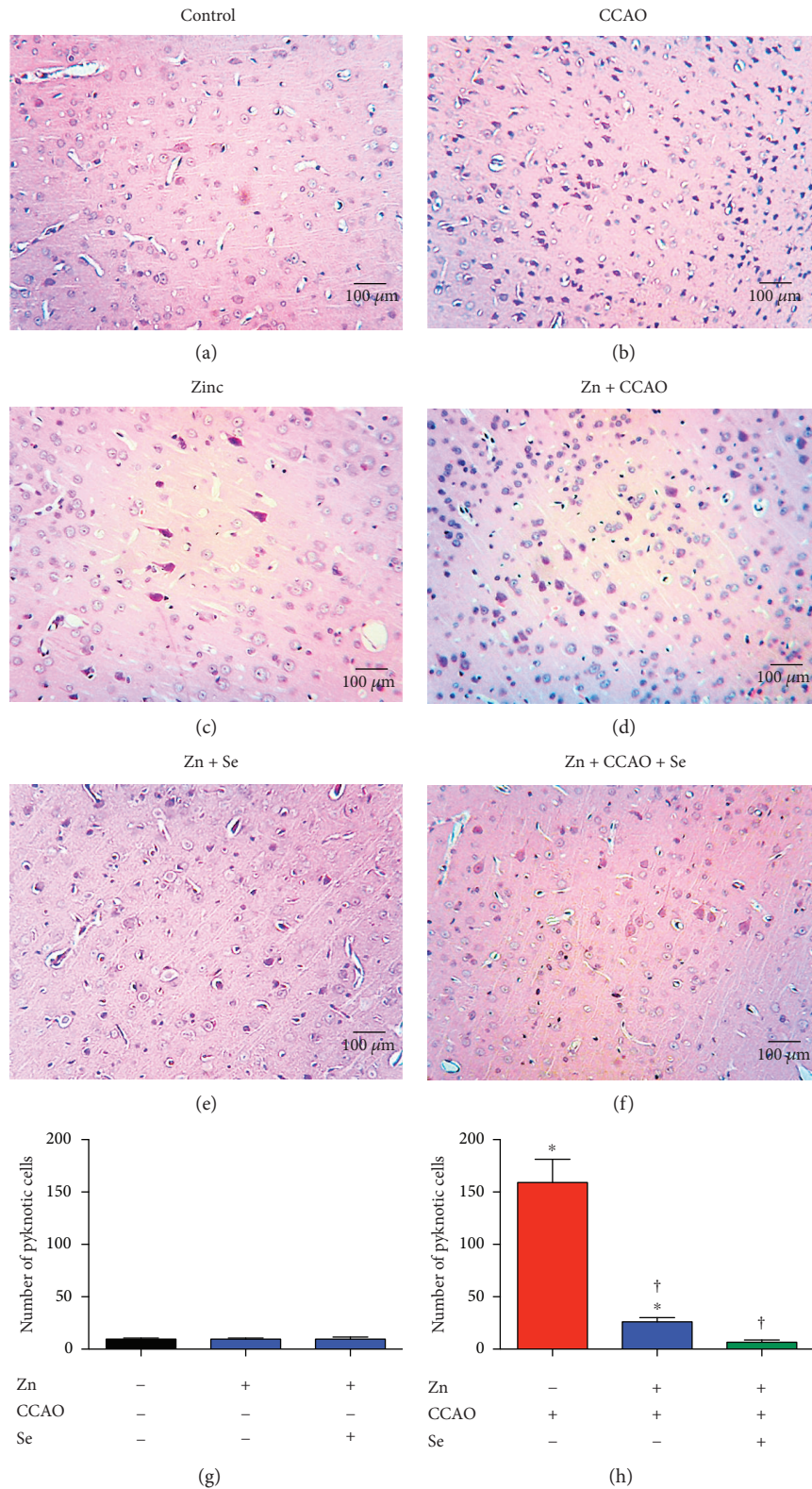
FIGURE 7: The administration of the combined treatment with zinc and selenium decreases the number of pyknotic cells in the temporoparietal cortex of hypoxia-ischemic rats. Representative micrographs with hematoxylin-eosin staining. The headings indicate the different experimental conditions. (g) Counting of pyknotic cells in the control groups treated with zinc or zinc + Se. (h) Counting of pyknotic cells in the groups with CCAO and treated with zinc (zinc) or the combined treatment with zinc (zinc + CCAO) and selenium (zinc + CCAO + Se). Each value represents mean ± SEM of 5 independent experiments made in triplicate. $^{*}P < 0.05$, one-way ANOVA with post hoc Dunnett's test when compared with the untreated control group; $^{†}P < 0.05$, Student's t-test when compared with the CCAO group.
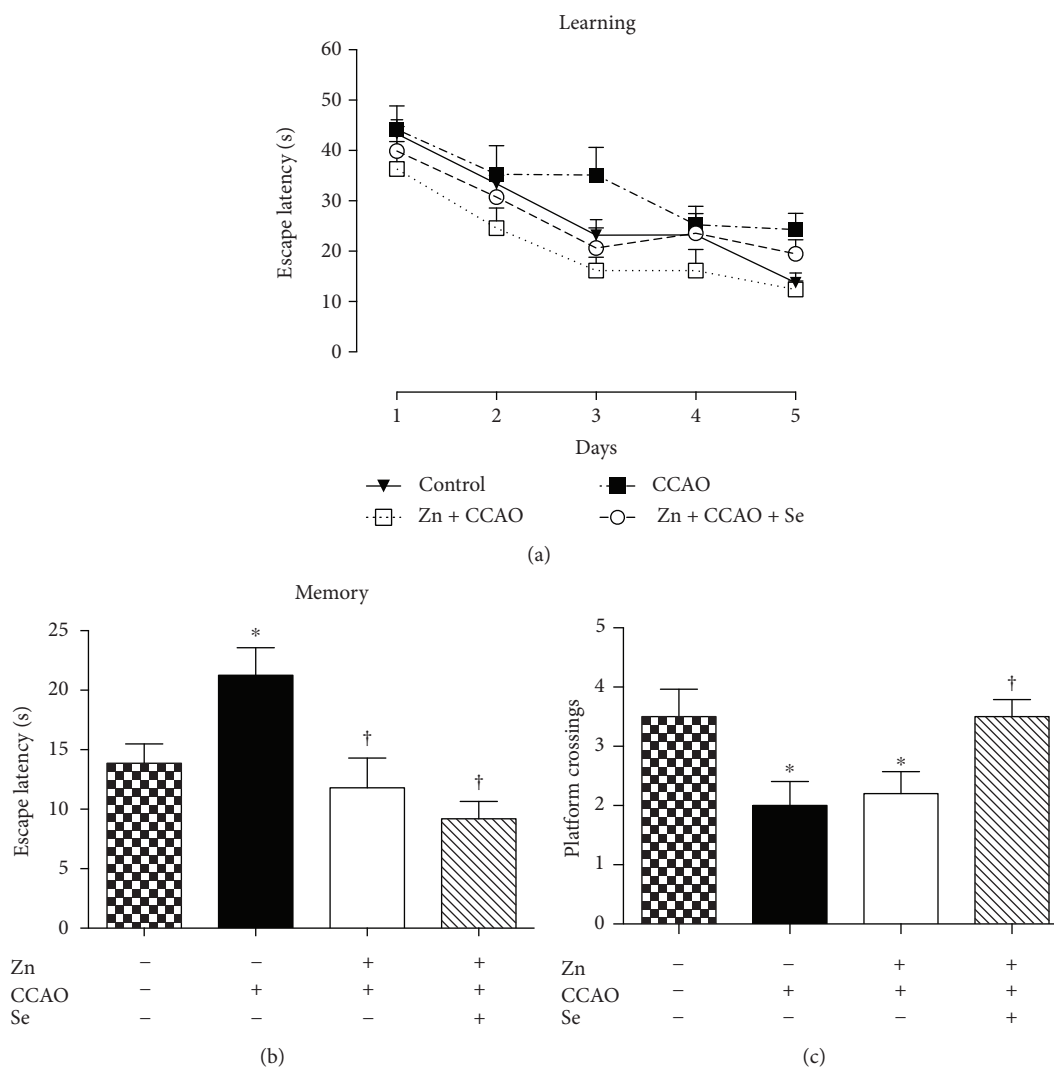
(a)

(b)

(c)

FIGURE 8: The administration of the combined treatment with zinc and selenium improves information consolidation in the hypoxia-ischemia model in rats. Control = untreated rats; Zn = zinc; CCAO = common carotid artery occlusion; Zn + CCAO = chronic zinc administration before CCAO; Zn + CCAO + Se = chronic zinc administration before CCAO followed by selenium (Se) administration. Each value represents mean ± SEM of 5 independent experiments made in triplicate. $^*P < 0.05$, one-way Kruskal-Wallis analysis of variance with post hoc Dunnett's test when compared with the untreated control group; $^{\dagger}P < 0.05$, Mann-Whitney U test when compared with the CCAO group.

of cytochrome c, and the production of mitochondrial superoxide radicals [51]. Of the three SOD isoforms, SOD2 is thought to be the primary contributor to the protective effect in both transient and permanent occlusion [52, 53]. Our results support this proposal.

We found that the combined treatment with zinc and selenium also causes upregulation of *Gpx4* in the early and late phases of CCAO. This result suggests that GPx4 also contributes to the neuroprotective effect of zinc and selenium, removing peroxides from cell membranes and macromolecules such as lipids, proteins, and DNA [54, 55]. Another mechanism of neuroprotection by GPX4 is to prevent apoptosis, counteracting the activity of lipoxygenase (LOX) [56] and promoting survival and proliferation [57]. In agreement with the antiapoptotic effect, *Gpx* knockout mice develop an increased volume of myocardial infarction in a hypoxia-ischemia event [58]. Furthermore, selenium

can inhibit TRPM2 and TRPV1 receptors (activated by increasing H_2O_2), thus preventing the entry of calcium into the cell that is known to detonate oxidative stress and inflammation [59].

Our results show that CCAO upregulated the mRNA for *Cxcl12/Cxcr4* and *Cxcl13/Cxcr5* without modifying their protein levels, although it decreased CXCL13 protein levels. In contrast with our transient CCAO model for 10 min, the permanent occlusion of the middle cerebral artery (MCAO) increases CCL2, CXCL2, and CXCL13 levels after 2 days, gradually decreasing after 7 days of MCAO [60, 61]. Therefore, the transient effect of hypoxia-ischemia might be insufficient to alter the protein levels of chemokines and receptors in the time points we have studied. Moreover, the lack of the effect in protein levels can be explained by posttranscriptional regulation of miRNAs [62, 63] or a posttranslational regulation at the level of

degradation after receptor-ligand desensitization [64]. In this latter case, those chemokines could have exerted their function before their degradation. Then, the upregulated *Ccl2/Ccr2* by the combined zinc and selenium administration in the late phase of hypoxia-ischemia might be neuroprotective because they are known to decrease cell death and improve memory [65]. Furthermore, CCL2 also stimulates the migration of neuronal precursor cells to the damaged area [66]. We have previously reported that high levels of CCL2 by a subacute prophylactic administration of zinc are associated with a preconditioning process [5]. However, the combined treatment with zinc and selenium did not maintain the preconditioning effect of zinc but exerted the therapeutic effect of selenium in the late phase. This effect is reflected by a decrease in cell death and recovery of long-term memory.

CXCL12 and CXCL13 have been associated with a deleterious role during cerebral ischemia [67] attracting lymphocytes [68]. Nevertheless, CXCL12 and CXL13 can also attract neuronal precursor cells mainly through interaction with CXCR4 or CXCR5, respectively [69–72]. CXCL12 and CXCL13 promote the migration of neuroblasts from the subventricular zone in neonatal mice [73], although the main promoter of neuroblast migration is CXCL12 [74]. In this study, we found that the combined treatment with zinc and selenium upregulated *Cxcl12* and *Cxcl13* and increased CXCL13 protein levels. Therefore, these chemokines can be associated with the neuroprotective effect of the combined treatment with zinc and selenium.

The effect of selenium on memory consolidation has been shown in other models different from hypoxia-ischemia like Alzheimer's disease [75]. The facilitation of learning and improvement of cognitive development have been associated to the neuroprotective effect of zinc, which decreases free radicals produced by cerebral ischemia [76]. Our group showed similar results in the preconditioning effect of subacute zinc administration in the CCAO rat model [5]. However, the prophylactic chronic zinc administration (0.2 mg/kg of body weight/days) exerted a partial effect because there was no memory consolidation as reported previously with a higher dose of zinc [19]. In contrast, the therapeutic administration of selenium improved the long-term memory consolidation, which is consistent with the significant decrease in neuronal cell death induced by cerebral ischemia in the temporoparietal cortex.

In summary, the combination of the chronic prophylactic zinc administration with the therapeutic selenium administration exerts effective neuroprotection against transient hypoxia-ischemia. In this effect, GPx and SOD seem to be the key players in reducing oxidative stress and cell death, suggesting possible participation in neuroregeneration. The perspective of this work consists of challenging the present therapeutic strategy with a longer time of common carotid artery occlusion as it happens in humans.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no competing financial interest, personal or other relationships with other people or organizations within five years of beginning the submitted work.

Acknowledgments

References

[1] N. Tonder, F. F. Johansen, C. J. Frederickson, J. Zimmer, and N. H. Diemer, "Possible role of zinc in the selective degeneration of dentate hilar neurons after cerebral ischemia in the adult rat," *Neuroscience Letters*, vol. 109, no. 3, pp. 247–252, 1990.

[2] A. Bhatt, M. U. Farooq, S. Enduri et al., "Clinical significance of serum zinc levels in cerebral ischemia," *Stroke Research and Treatment*, vol. 2010, Article ID 245715, 4 pages, 2010.

[3] Y. Yamasaki, T. Suzuki, H. Yamaya, N. Matsuura, H. Onodera, and K. Kogure, "Possible involvement of interleukin-1 in ischemic brain edema formation," *Neuroscience Letters*, vol. 142, no. 1, pp. 45–47, 1992.

[4] T. Doi, H. Hara, M. Kajita, T. Kamiya, and T. Adachi, "Zinc regulates expression of IL-23 p19 mRNA via activation of eIF2α/ATF4 axis in HAPI cells," *Biometals*, vol. 28, no. 5, pp. 891–902, 2015.

[5] V. M. Blanco-Alvarez, G. Soto-Rodriguez, J. A. Gonzalez-Barrios et al., "Prophylactic subacute administration of zinc increases CCL2, CCR2, FGF2, and IGF-1 expression and prevents the long-term memory loss in a rat model of cerebral hypoxia-ischemia," *Neural Plasticity*, vol. 2015, Article ID 375391, 15 pages, 2015.

[6] F. Lopez, J. Hernandez-Palazon, R. Lopez, E. Alarcon, and J. F. Martinez-Lage, "Activity of copper-zinc superoxide dismutase in a global ischemic brain lesion model without arterial hypotension," *Neurocirugía*, vol. 15, no. 2, pp. 151–158, 2004.

[7] D. R. Morris and C. W. Levenson, "Zinc in traumatic brain injury: from neuroprotection to neurotoxicity," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 16, no. 6, pp. 708–711, 2013.

[8] F. F. Johansen, N. Tonder, M. Berg, J. Zimmer, and N. H. Diemer, "Hypothermia protects somatostatinergic neurons in rat dentate hilus from zinc accumulation and cell death after cerebral ischemia," *Molecular and Chemical Neuropathology*, vol. 18, no. 1-2, pp. 161–172, 1993.

[9] D. Tsuchiya, S. Hong, S. W. Suh, T. Kayama, S. S. Panter, and P. R. Weinstein, "Mild hypothermia reduces zinc translocation, neuronal cell death, and mortality after transient global ischemia in mice," *Journal of Cerebral Blood Flow & Metabolism*, vol. 22, no. 10, pp. 1231–1238, 2002.

[10] K. Matsushita, K. Kitagawa, T. Matsuyama et al., "Effect of systemic zinc administration on delayed neuronal death in the gerbil hippocampus," *Brain Research*, vol. 743, no. 1-2, pp. 362–365, 1996.

[11] V. M. Blanco-Alvarez, P. Lopez-Moreno, G. Soto-Rodriguez et al., "Subacute zinc administration and L-NAME caused an increase of NO, zinc, lipoperoxidation, and caspase-3 during

a cerebral hypoxia-ischemia process in the rat," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 240560, 10 pages, 2013.

[12] S. L. Mehta, S. Kumari, N. Mendelev, and P. A. Li, "Selenium preserves mitochondrial function, stimulates mitochondrial biogenesis, and reduces infarct volume after focal cerebral ischemia," *BMC Neuroscience*, vol. 13, no. 1, p. 79, 2012.

[13] G. Erbil, S. Ozbal, U. Sonmez et al., "Neuroprotective effects of selenium and ginkgo biloba extract (EGb761) against ischemia and reperfusion injury in rat brain," *Neurosciences*, vol. 13, no. 3, pp. 233–238, 2008.

[14] B. V. Lakshmi, M. Sudhakar, and K. S. Prakash, "Protective effect of selenium against aluminum chloride-induced Alzheimer's disease: behavioral and biochemical alterations in rats," *Biological Trace Element Research*, vol. 165, no. 1, pp. 67–74, 2015.

[15] A. Ahmad, M. M. Khan, T. Ishrat et al., "Synergistic effect of selenium and melatonin on neuroprotection in cerebral ischemia in rats," *Biological Trace Element Research*, vol. 139, no. 1, pp. 81–96, 2011.

[16] S. H. Kim, V. J. Johnson, T. Y. Shin, and R. P. Sharma, "Selenium attenuates lipopolysaccharide-induced oxidative stress responses through modulation of p38 MAPK and NF-κB signaling pathways," *Experimental Biology and Medicine*, vol. 229, no. 2, pp. 203–213, 2004.

[17] N. D. Solovyev, "Importance of selenium and selenoprotein for brain function: from antioxidant protection to neuronal signalling," *Journal of Inorganic Biochemistry*, vol. 153, pp. 1–12, 2015.

[18] P. Aguilar-Alonso, D. Martinez-Fong, N. G. Pazos-Salazar et al., "The increase in zinc levels and upregulation of zinc transporters are mediated by nitric oxide in the cerebral cortex after transient ischemia in the rat," *Brain Research*, vol. 1200, pp. 89–98, 2008.

[19] C. Tomas-Sanchez, V. M. Blanco-Alvarez, J. A. Gonzalez-Barrios et al., "Prophylactic chronic zinc administration increases neuroinflammation in a hypoxia-ischemia model," *Journal of Immunology Research*, vol. 2016, Article ID 4039837, 15 pages, 2016.

[20] T. G. Dzhandzhgava and R. R. Shakarishvili, "Effect of alpha-tocopherol and selenium on the activity of antioxidant enzymes and level of lipid peroxidation products in erythrocytes of patients with cerebral ischemia," *Voprosy Meditsinskoĭ Khimii*, vol. 37, no. 5, pp. 79–82, 1991.

[21] J. A. Gonzalez-Barrios, B. Escalante, J. Valdes, B. A. Leon-Chavez, and D. Martinez-Fong, "Nitric oxide and nitric oxide synthases in the fetal cerebral cortex of rats following transient uteroplacental ischemia," *Brain Research*, vol. 945, no. 1, pp. 114–122, 2002.

[22] D. Gerard-Monnier, I. Erdelmeier, K. Regnard, N. Moze-Henry, J. C. Yadan, and J. Chaudiere, "Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation," *Chemical Research in Toxicology*, vol. 11, no. 10, pp. 1176–1183, 1998.

[23] J. J. Sedmak and S. E. Grossberg, "A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250," *Analytical Biochemistry*, vol. 79, no. 1-2, pp. 544–552, 1977.

[24] R. Morris, "Developments of a water-maze procedure for studying spatial learning in the rat," *Journal of Neuroscience Methods*, vol. 11, no. 1, pp. 47–60, 1984.

[25] J. M. Gidday, "Cerebral preconditioning and ischaemic tolerance," *Nature Reviews. Neuroscience*, vol. 7, no. 6, pp. 437–448, 2006.

[26] J. M. Lee, J. M. Lee, K. R. Kim, H. Im, and Y. H. Kim, "Zinc preconditioning protects against neuronal apoptosis through the mitogen-activated protein kinase-mediated induction of heat shock protein 70," *Biochemical and Biophysical Research Communications*, vol. 459, no. 2, pp. 220–226, 2015.

[27] S. K. Kansal, U. Jyoti, S. Sharma, A. Kaura, R. Deshmukh, and S. Goyal, "Effect of zinc supplements in the attenuated cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 388, no. 6, pp. 635–641, 2015.

[28] V. Narayan, K. C. Ravindra, C. Liao, N. Kaushal, B. A. Carlson, and K. S. Prabhu, "Epigenetic regulation of inflammatory gene expression in macrophages by selenium," *The Journal of Nutritional Biochemistry*, vol. 26, no. 2, pp. 138–145, 2015.

[29] Y. D. Hu, W. Pang, C. C. He et al., "The cognitive impairment induced by zinc deficiency in rats aged 0 ~ 2 months related to BDNF DNA methylation changes in the hippocampus," *Nutritional Neuroscience*, vol. 20, no. 9, pp. 519–525, 2017.

[30] B. Speckmann, S. Schulz, F. Hiller et al., "Selenium increases hepatic DNA methylation and modulates one-carbon metabolism in the liver of mice," *The Journal of Nutritional Biochemistry*, vol. 48, pp. 112–119, 2017.

[31] E. Jablonska and E. Reszka, "Selenium and epigenetics in cancer: focus on DNA methylation," *Advances in Cancer Research*, vol. 136, pp. 193–234, 2017.

[32] R. Greco, C. Demartini, A. M. Zanaboni, F. Blandini, D. Amantea, and C. Tassorelli, "Endothelial nitric oxide synthase inhibition triggers inflammatory responses in the brain of male rats exposed to ischemia-reperfusion injury," *Journal of Neuroscience Research*, vol. 96, no. 1, pp. 151–159, 2017.

[33] S. Wei, J. Huang, Y. Li et al., "Novel zinc finger transcription factor ZFP580 promotes differentiation of bone marrow-derived endothelial progenitor cells into endothelial cells via eNOS/NO pathway," *Journal of Molecular and Cellular Cardiology*, vol. 87, pp. 17–26, 2015.

[34] M. H. Zou, C. Shi, and R. A. Cohen, "Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite," *The Journal of Clinical Investigation*, vol. 109, no. 6, pp. 817–826, 2002.

[35] A. V. Santhanam, L. V. d'Uscio, L. A. Smith, and Z. S. Katusic, "Uncoupling of eNOS causes superoxide anion production and impairs NO signaling in the cerebral microvessels of hph-1 mice," *Journal of Neurochemistry*, vol. 122, no. 6, pp. 1211–1218, 2012.

[36] H. Ren, J. Mu, J. Ma et al., "Selenium inhibits homocysteine-induced endothelial dysfunction and apoptosis via activation of AKT," *Cellular Physiology and Biochemistry*, vol. 38, no. 3, pp. 871–882, 2016.

[37] G. W. Kim, T. Kondo, N. Noshita, and P. H. Chan, "Manganese superoxide dismutase deficiency exacerbates cerebral infarction after focal cerebral ischemia/reperfusion in mice: implications for the production and role of superoxide radicals," *Stroke*, vol. 33, no. 3, pp. 809–815, 2002.

[38] T. Matsui, A. Ohta, and H. Takagi, "Morphological studies on Mn-SOD, NOS and calcium binding proteins in the rat hippocampus," *Osaka City Medical Journal*, vol. 42, no. 1, pp. 1–13, 1996.

[39] F. Yang, B. Li, X. Dong, W. Cui, and P. Luo, "The beneficial effects of zinc on diabetes-induced kidney damage in murine rodent model of type 1 diabetes mellitus," *Journal of Trace Elements in Medicine and Biology*, vol. 42, pp. 1–10, 2017.

[40] S. Nedd, R. L. Redler, E. A. Proctor, N. V. Dokholyan, and A. N. Alexandrova, "Cu, Zn-superoxide dismutase without Zn is folded but catalytically inactive," *Journal of Molecular Biology*, vol. 426, no. 24, pp. 4112–24, 2014.

[41] M. S. Farias, P. Budni, C. M. Ribeiro et al., "Antioxidant supplementation attenuates oxidative stress in chronic hepatitis C patients," *Gastroenterología y Hepatología*, vol. 35, no. 6, pp. 386–394, 2012.

[42] H. Sheng, R. D. Bart, T. D. Oury, R. D. Pearlstein, J. D. Crapo, and D. S. Warner, "Mice overexpressing extracellular superoxide dismutase have increased resistance to focal cerebral ischemia," *Neuroscience*, vol. 88, no. 1, pp. 185–191, 1999.

[43] H. Sheng, M. Kudo, G. B. Mackensen, R. D. Pearlstein, J. D. Crapo, and D. S. Warner, "Mice overexpressing extracellular superoxide dismutase have increased resistance to global cerebral ischemia," *Experimental Neurology*, vol. 163, no. 2, pp. 392–398, 2000.

[44] K. Sampei, A. S. Mandir, Y. Asano et al., "Stroke outcome in double-mutant antioxidant transgenic mice," *Stroke*, vol. 31, no. 11, pp. 2685–2691, 2000.

[45] J. W. Francis, J. Ren, L. Warren, R. H. Brown Jr., and S. P. Finklestein, "Postischemic infusion of Cu/Zn superoxide dismutase or SOD:Tet 451 reduces cerebral infarction following focal ischemia/reperfusion in rats," *Experimental Neurology*, vol. 146, no. 2, pp. 435–443, 1997.

[46] G. W. Kim, A. Lewen, J. Copin, B. D. Watson, and P. H. Chan, "The cytosolic antioxidant, copper/zinc superoxide dismutase, attenuates blood–brain barrier disruption and oxidative cellular injury after photothrombotic cortical ischemia in mice," *Neuroscience*, vol. 105, no. 4, pp. 1007–1018, 2001.

[47] C. Y. Huang, M. Fujimura, N. Noshita, Y. Y. Chang, and P. H. Chan, "SOD1 down-regulates NF-κB and c-Myc expression in mice after transient focal cerebral ischemia," *Journal of Cerebral Blood Flow & Metabolism*, vol. 21, no. 2, pp. 163–173, 2001.

[48] T. Kondo, A. G. Reaume, T. T. Huang et al., "Reduction of CuZn-superoxide dismutase activity exacerbates neuronal cell injury and edema formation after transient focal cerebral ischemia," *The Journal of Neuroscience*, vol. 17, no. 11, pp. 4180–4189, 1997.

[49] H. Sheng, T. C. Brady, R. D. Pearlstein, J. D. Crapo, and D. S. Warner, "Extracellular superoxide dismutase deficiency worsens outcome from focal cerebral ischemia in the mouse," *Neuroscience Letters*, vol. 267, no. 1, pp. 13–16, 1999.

[50] M. Fujimura, Y. Morita-Fujimura, M. Kawase et al., "Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome C and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice," *The Journal of Neuroscience*, vol. 19, no. 9, pp. 3414–3422, 1999.

[51] H. J. Bidmon, K. Kato, A. Schleicher, O. W. Witte, and K. Zilles, "Transient increase of manganese-superoxide dismutase in remote brain areas after focal photothrombotic cortical lesion," *Stroke*, vol. 29, no. 1, pp. 203–211, 1998.

[52] P. H. Chan, H. Kamii, G. Yang et al., "Brain infarction is not reduced in SOD-1 transgenic mice after a permanent focal cerebral ischemia," *Neuroreport*, vol. 5, no. 3, pp. 293–296, 1993.

[53] K. Murakami, T. Kondo, M. Kawase et al., "Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency," *The Journal of Neuroscience*, vol. 18, no. 1, pp. 205–213, 1998.

[54] J. P. Thomas, P. G. Geiger, M. Maiorino, F. Ursini, and A. W. Girotti, "Enzymatic reduction of phospholipid and cholesterol hydroperoxides in artificial bilayers and lipoproteins," *Biochimica et Biophysica Acta*, vol. 1045, no. 3, pp. 252–260, 1990.

[55] N. Ishibashi, O. Prokopenko, M. Weisbrot-Lefkowitz, K. R. Reuhl, and O. Mirochnitchenko, "Glutathione peroxidase inhibits cell death and glial activation following experimental stroke," *Brain Research. Molecular Brain Research*, vol. 109, no. 1-2, pp. 34–44, 2002.

[56] A. Seiler, M. Schneider, H. Forster et al., "Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death," *Cell Metabolism*, vol. 8, no. 3, pp. 237–248, 2008.

[57] A. M. Mannes, A. Seiler, V. Bosello, M. Maiorino, and M. Conrad, "Cysteine mutant of mammalian GPx4 rescues cell death induced by disruption of the wild-type selenoenzyme," *The FASEB Journal*, vol. 25, no. 7, pp. 2135–2144, 2011.

[58] P. J. Crack, J. M. Taylor, N. J. Flentjar et al., "Increased infarct size and exacerbated apoptosis in the glutathione peroxidase-1 (Gpx-1) knockout mouse brain in response to ischemia/reperfusion injury," *Journal of Neurochemistry*, vol. 78, no. 6, pp. 1389–1399, 2001.

[59] H. Balaban, M. Naziroglu, K. Demirci, and I. S. Ovey, "The protective role of selenium on scopolamine-induced memory impairment, oxidative stress, and apoptosis in aged rats: the involvement of TRPM2 and TRPV1 channels," *Molecular Neurobiology*, vol. 54, no. 4, pp. 2852–2868, 2016.

[60] N. G. Gourmala, M. Buttini, S. Limonta, A. Sauter, and H. W. Boddeke, "Differential and time-dependent expression of monocyte chemoattractant protein-1 mRNA by astrocytes and macrophages in rat brain: effects of ischemia and peripheral lipopolysaccharide administration," *Journal of Neuroimmunology*, vol. 74, no. 1-2, pp. 35–44, 1997.

[61] X. Che, W. Ye, L. Panga, D. C. Wu, and G. Y. Yang, "Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice," *Brain Research*, vol. 902, no. 2, pp. 171–177, 2001.

[62] L. S. Arabanian, F. A. Fierro, F. Stolzel et al., "MicroRNA-23a mediates post-transcriptional regulation of CXCL12 in bone marrow stromal cells," *Haematologica*, vol. 99, no. 6, pp. 997–1005, 2014.

[63] J. Chen, R. Ning, A. Zacharek et al., "MiR-126 contributes to human umbilical cord blood cell-induced neurorestorative effects after stroke in type-2 diabetic mice," *Stem Cells*, vol. 34, no. 1, pp. 102–113, 2016.

[64] L. D. Bennett, J. M. Fox, and N. Signoret, "Mechanisms regulating chemokine receptor activity," *Immunology*, vol. 134, no. 3, pp. 246–256, 2011.

[65] Y. Kumai, H. Ooboshi, J. Takada et al., "Anti—monocyte chemoattractant protein-1 gene therapy protects against focal brain ischemia in hypertensive rats," *Journal of Cerebral Blood Flow & Metabolism*, vol. 24, no. 12, pp. 1359–1368, 2004.

[66] D. Widera, W. Holtkamp, F. Entschladen et al., "MCP-1 induces migration of adult neural stem cells," *European Journal of Cell Biology*, vol. 83, no. 8, pp. 381–387, 2004.

[67] P. Liu, J. W. Xiang, and S. X. Jin, "Serum CXCL12 levels are associated with stroke severity and lesion volumes in stroke patients," *Neurological Research*, vol. 37, no. 10, pp. 853–858, 2015.

[68] M. C. Kowarik, S. Cepok, J. Sellner et al., "CXCL13 is the major determinant for B cell recruitment to the CSF during neuroinflammation," *Journal of Neuroinflammation*, vol. 9, no. 1, p. 93, 2012.

[69] N. Weiss, C. Deboux, N. Chaverot et al., "IL8 and CXCL13 are potent chemokines for the recruitment of human neural precursor cells across brain endothelial cells," *Journal of Neuroimmunology*, vol. 223, no. 1-2, pp. 131–134, 2010.

[70] F. Del Grosso, S. Coco, P. Scaruffi et al., "Role of CXCL13-CXCR5 crosstalk between malignant neuroblastoma cells and Schwannian stromal cells in neuroblastic tumors," *Molecular Cancer Research*, vol. 9, no. 7, pp. 815–823, 2011.

[71] A. Guyon, "CXCL12 chemokine and its receptors as major players in the interactions between immune and nervous systems," *Frontiers in Cellular Neuroscience*, vol. 8, p. 65, 2014.

[72] A. M. Robin, Z. G. Zhang, L. Wang et al., "Stromal cell-derived factor 1α mediates neural progenitor cell motility after focal cerebral ischemia," *Journal of Cerebral Blood Flow & Metabolism*, vol. 26, no. 1, pp. 125–134, 2006.

[73] J. T. Miller, J. H. Bartley, H. J. Wimborne et al., "The neuroblast and angioblast chemotaxic factor SDF-1 (CXCL12) expression is briefly up regulated by reactive astrocytes in brain following neonatal hypoxic-ischemic injury," *BMC Neuroscience*, vol. 6, no. 1, p. 63, 2005.

[74] P. Thored, A. Arvidsson, E. Cacci et al., "Persistent production of neurons from adult brain stem cells during recovery after stroke," *Stem Cells*, vol. 24, no. 3, pp. 739–747, 2006.

[75] T. Ishrat, K. Parveen, M. M. Khan et al., "Selenium prevents cognitive decline and oxidative damage in rat model of streptozotocin-induced experimental dementia of Alzheimer's type," *Brain Research*, vol. 1281, pp. 117–127, 2009.

[76] A. Piechal, K. Blecharz-Klin, J. Pyrzanowska, and E. Widy-Tyszkiewicz, "Maternal zinc supplementation improves spatial memory in rat pups," *Biological Trace Element Research*, vol. 147, no. 1–3, pp. 299–308, 2012.