

Niosome-Encapsulated 10–23 DNzyme Targeting NF- κ B p65 Attenuates Secondary Cerebral Ischemia–Reperfusion Injury after Transient MCAO in Rats

Solmaz Nasser Maleki

Iran University of Medical Sciences

Shayan Roshdi Dizaji

Iran University of Medical Sciences

Maryam Naseroleslami

Iran University of Medical Sciences

Nahid Aboutaleb

Iran University of Medical Sciences

Mahmoud Yousefifard

yousefifard20@gmail.com

Iran University of Medical Sciences

Article

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Abstract

Background: Reperfusion after ischemic stroke initiates secondary inflammatory and oxidative cascades, in part driven by NF- κ B p65. We investigated whether a 10–23 DNAzyme targeting rat NF- κ B p65, delivered by nanoniosome, can mitigate post-reperfusion brain injury.

Methods: Male Wistar rats underwent transient middle cerebral artery occlusion (60 min). Immediately at the onset of reperfusion, animals received a single intraventricular dose of free DNAzyme, niosome-encapsulated DNAzyme, empty niosomes ($n = 16$). Neurological deficit scores were assessed at 24 h, 7 days, and 14 days. At 14 days post-reperfusion, infarct volume (edema-corrected TTC), brain water content, histology (H&E/Nissl), NF- κ B p65 activation, TNF- α /IL-1 β , MMP-2/9, and oxidative stress markers (MDA, SOD, CAT) were quantified in randomly selected animals ($n = 4$ /group).

Results: Niosomal DNAzyme designed to NF- κ B p65 significantly reduced total NF- κ B p65 ($p = 0.0001$) and phosphorylated NF- κ B ($p < 0.0003$) after focal cerebral ischemia causing suppressing inflammatory mediators (TNF- α , IL-1 β) and matrix metalloproteinases (MMP-2/9), accompanied by attenuation of oxidative stress, infarct volume and cerebral edema. Despite these tissue-level benefits, neurological deficit scores did not differ significantly from those of untreated MCAO controls at 24 h, 7 days, or 14 days.

Conclusion: Targeted inhibition of NF- κ B p65 using a niosome-delivered DNAzyme dampens secondary inflammatory/oxidative injury and reduces structural brain damage after cerebral ischemia–reperfusion, supporting further optimization to translate these effects into functional recovery.

Introduction

Stroke is the second leading cause of mortality and morbidity worldwide, imposing a considerable burden on individuals and society, particularly in low and middle-income countries (1). Acute ischemic stroke accounts for 87% of all stroke cases, with large vessel artery occlusion representing up to 46% of acute ischemic strokes (2, 3). Currently, timely revascularization utilizing intravenous thrombolytics or mechanical clot retrieval by interventional thrombectomy stands as the mainstay of ischemic stroke management.

Although these interventions have revolutionized the care of ischemic stroke patients, their restricted time windows and stringent eligibility criteria have left many patients without any benefits from these reperfusion strategies (1, 2). Even in the case of prompt revascularization, the reestablishment of cerebral blood flow may paradoxically entail exacerbating neurological damage through aggravation of oxidative stress, infiltration of leukocytes, release of proinflammatory cytokines, chemokines, as well as propagating blood-brain barrier (BBB) disintegration, collectively known as cerebral ischemia/reperfusion (I/R) injury (3, 4).

Over the past decades, significant research efforts have been directed towards identifying therapeutic targets to mitigate the neurological impairments resulting from cerebral I/R injury. In this line, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) has been identified as a critical signaling pathway involved in cerebral I/R pathophysiology, functioning as a central regulatory hub in the control of neuroinflammatory and neurodegenerative processes (5, 6). NF- κ B is composed of a versatile family of transcription factors that orchestrates both physiological and pathological neural responses through the differential activation of its constituent subunit dimers (7-9). In the context of ischemic insult, a mounting body of evidence has demonstrated the implication of the p65 (RelA) subunit in mediating neuronal injury (10-12). Upon receiving signals from diverse extracellular stimuli such as damage-associated molecular patterns (DAMPs) and pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6), p65 becomes activated. This activation occurs through phosphorylation of P65. As a result, the p65-p50 heterodimer translocated into nucleus. This nuclear translocation initiates the transcription of target genes involved in inflammatory responses, immune regulation, and apoptotic pathways (13).

Given the extensive spectrum of its activators and the multifaceted downstream signaling pathways it governs, targeting the p65 subunit represents a compelling therapeutic strategy to mitigate neuronal damage associated with cerebral I/R injury. To this end, various molecular agents, including antisense oligonucleotides, small interfering RNAs, microRNAs, ribozymes, and DNAzymes, can be employed to inhibit gene expression (14, 15). Among these, DNAzymes are particularly promising due to their high target specificity, structural versatility, cost-effectiveness, self-catalytic nature, and high turnover rate, making them an attractive tool for gene silencing in neurotherapeutic interventions (16-19).

DNAzymes are a group of single-stranded deoxyribonucleotides with catalytic activity that do not naturally occur in living microorganisms (20). Each DNAzyme consists of a catalytic core and two recognition flanking arms that bind to specific complementary sequences on a target RNA, enabling site-specific cleavage of phosphodiester bonds within the RNA substrate (21). DNAzymes initially drew interest from researchers as a promising treatment for a wide range of diseases, including viral hepatitis, cardiovascular diseases, and cancers (22-24). However, the enthusiasm surrounding DNAzyme applications was followed by a period of stagnation due to hurdles in their in vivo application. Major issues included dependence on endogenous metal ion cofactors, poor cellular penetration and endosomal escape, and vulnerability to nucleolytic degradation, all of which limited its therapeutic translation (21, 25, 26). However, recent advances in drug delivery systems have provided practical solutions through enhancing DNAzyme stability, enabling sustained release, and improving delivery to target tissues (17, 23).

Niosomes are a group of lamellar vesicular nanocarriers with structural similarities to liposomes, capable of encapsulating and delivering both hydrophilic and hydrophobic payload formulations (27). In contrast to liposomes, which are primarily composed of phospholipids, niosomes are formed from non-ionic surfactants combined with cholesterol, conferring improved biocompatibility, biodegradability, and physicochemical stability. Additionally, the ease of manufacturing, lower costs, and enhanced storage

stability of niosomes compared to conventional liposomal formulations underscore their promise as a flexible and efficient platform for drug delivery (28).

Despite encouraging results from various targeted therapies in preclinical models of stroke, clinical translation has remained elusive, underscoring the urgent need for innovative therapeutic strategies. To date, no study has assessed the therapeutic potential of DNAzyme in the context of stroke. Therefore, the present study was designed to investigate the neuroprotective efficacy of a niosome-encapsulated DNAzyme targeting the NF- κ B p65 subunit in a rat model of transient middle cerebral artery occlusion (MCAO). We demonstrated that delivery of DNAzyme in niosomes would potentiate its bioavailability and efficacy if administered immediately after reperfusion in MCAO rats. Furthermore, suppression of the NF- κ B pathway employing target-specific DNAzyme was associated with reduced pro-inflammatory cytokine levels, matrix metalloproteinase activity, and ameliorated oxidative stress injury, thereby mitigating neuronal cell death, smaller infarction volume, and diminished brain edema following ischemia-reperfusion in MCAO rats.

Methods

Design of the 10–23 DNAzyme Targeting NF- κ B p65

approach incorporating specificity screening, secondary-structure analysis, and transcript sequence evaluation was employed to develop the rat NF- κ B p65–specific 10–23 DNAzyme.

The final DNAzyme, 5'-TCAAAGTCATAGGCTAGCTACAACGAACAGCCATC-3', was created by assembling this catalytic sequence with the improved binding arms (Table 1). Metabion (Planegg, Germany) created the final oligonucleotide using a typical phosphodiester backbone.

Niosome preparation

Niosomes were prepared using the thin-film hydration method with Span 60 as the non-ionic surfactant, as described previously (29). Briefly, 58 mg Span 60 and 42 mg cholesterol were dissolved in 6 mL chloroform and 3 mL methanol in a 50 mL round-bottom flask connected to a rotary evaporator (Heidolph Instruments, Schwalbach, Germany). The organic solvent was evaporated under reduced pressure at 60 °C and 120 rpm for 30 minutes, forming a dried thin film on the inner wall of the flask. To load the hydrophilic DNAzyme, an aqueous solution of DNAzyme, containing 1 mL of the oligo dissolved in 4 mL of PBS, was added to the dried film, and the mixture was then subjected to rotary evaporation at 120 rpm for an additional 30 minutes at 60 °C. Afterward, the film was hydrated with 10 mL of PBS. The resulting suspension was probe-sonicated for 2 minutes (Hielscher UP50H ultrasonic processor, Germany) to produce niosomes with a homogeneous size distribution.

Physicochemical characterization of niosomal formulations

Fourier-transform infrared (FTIR) spectroscopy was employed to investigate the chemical composition and potential interactions between niosome and DNAzyme. Samples were mixed thoroughly with

potassium bromide (KBr), and pellets were prepared using a hydraulic press. At room temperature, spectra were captured using an FTIR spectrometer (Spectrum Two, PerkinElmer, Waltham, MA, USA) with a scanning range of 4000-400 cm⁻¹. UV Visible absorption spectra were obtained using a UV Visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan).

For surface morphology analysis, a drop (5-10 µL) of 0.1% (w/v) niosome dispersion was dropwise deposited onto glass slides, air-dried, and sputter-coated with a thin layer of gold. Imaging was performed using a field emission scanning electron microscope (Shimadzu, Kyoto, Japan).

The hydrodynamic diameter, polydispersity index (PDI), and surface charge (zeta potential) of niosome and niosomal DNAzyme formulations were measured by dynamic light scattering (DLS) using a Zeta-sizer Nano S90 instrument equipped with a 633 nm laser at 25 °C (Malvern Panalytical Ltd., Malvern, UK).

Release kinetics profile

The release profile of DNAzyme from niosomal formulations was characterized using a dialysis (dialysis bag) method. Briefly, 1 mL of the niosomal DNAzyme suspension and 1 mL of free DNAzyme were separately enclosed in cellulose-derived semipermeable dialysis tubing with a molecular weight cut-off of 12 kDa. Each bag was sealed and submerged in 50 mL of PBS (pH 7.4), maintained at 25°C and 37°C for 48h with constant magnetic stirring. At predetermined intervals, aliquots of the external medium were collected for analysis and replaced with an equal volume of pre-warmed PBS to preserve volume and the concentration gradient (30). DNAzyme concentrations in the collected samples were quantified spectrophotometrically by measuring absorbance at 210 nm using a UV–Visible spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Kyoto, Japan). The zero-order model was used to quantitatively analyse DNAzyme release dynamics in the sample.

Study design and timeline

Transient focal cerebral ischemia was induced by MCAO for 60 min followed by reperfusion. Treatments were administered immediately at the onset of reperfusion (t = 0; immediately after filament withdrawal). Neurological deficit scores were evaluated at 24 h, 7 days, and 14 days after reperfusion. Tissue-level outcomes were assessed at day 14 in randomly selected animals. All behavioral and outcome assessments were performed by investigators blinded to group allocation.

Animals

Healthy adult male Wistar rats weighing 250-280 grams were procured from the Animal Laboratory Center of Iran University of Medical Sciences. Animals were housed in groups of five in polycarbonate cages under a 12-hour light/dark cycle, controlled temperature (25 ± 1 °C), and 60%-70% humidity with access to food and water ad libitum. The animals were randomly assigned to five experimental groups: (1) sham-operated controls (Sham), (2) middle cerebral artery occlusion for 60 minutes (MCAO), (3) MCAO + empty niosomes (Niosome), (4) MCAO + free DNAzyme (DNAzyme), and (5) MCAO + DNAzyme

encapsulated in niosomes (Niosomal DNAzyme). Overall, 113 rats underwent MCAO surgery attempts; 33 rats (29.2%) died before recovery from anaesthesia (prior to treatment administration) and were excluded. Surgery was continued until 80 rats survived to reperfusion and recovery and were enrolled/randomized for follow-up (final analysed n = 80).

The Institutional Animal Care and Use Ethics Committee Iran University of Medical Sciences approved the study (IR.IUMS.AES.1402.109). The National Institutes of Health's standards for the Care and Use of Laboratory Animals, 8th edition, were followed in all animal studies used in this investigation.

MCAO procedure

According to Longa et al., the intraluminal filament approach was used to create transient focal cerebral ischemia (31). Rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg, i.p.) and positioned supine, with respiration monitored throughout the procedure. A midline cervical incision was made to expose the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). The ICA was momentarily clamped, and the right CCA and ECA were ligated. Through a small arteriotomy in the CCA, a standard monofilament (Doccol, Philadelphia, PA, USA) was introduced and advanced 18–20 mm into the ICA until mild resistance was felt, indicating occlusion of the middle cerebral artery (MCA) origin. After 60 minutes of occlusion, the filament was withdrawn to initiate reperfusion. A heating pad was used to maintain core body temperature at $37 \pm 0.5^{\circ}\text{C}$. The identical procedure was performed on sham-operated animals except for filament insertion or vascular ligation. Postoperative care included temperature control, hydration, and routine monitoring.

Treatments

immediately after filament withdrawal, animals received a single intracerebroventricular injection of empty niosomes, free DNAzyme, or niosome-encapsulated DNAzyme. Animals were positioned in a stereotaxic frame under anaesthesia. Following a midline scalp incision, a burr hole was drilled at coordinates corresponding to the right lateral ventricle (0.6 mm posterior, 2.0 mm lateral, and 3.8 mm ventral relative to Bregma), based on the Paxinos and Watson rat brain atlas. A 50- μL Hamilton syringe was used to deliver a 5- μL volume of treatment solution at 1 $\mu\text{L}/\text{min}$ via a micro infusion pump. Treatment formulations consisted of either free DNAzyme (5 $\mu\text{g}/\text{kg}$) or niosomal DNAzyme (5 $\mu\text{g}/\text{kg}$), each co-administered with 10 μg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in approximately 5 μL of sterile PBS, or 4 $\mu\text{g}/\text{kg}$ of empty niosomes for the control group. To minimize backflow, the needle was maintained in place for 2 minutes post-infusion before gradual withdrawal and the incision was sutured.

Neurological deficit score

At 24 hours, 7 days, and 14 days following reperfusion, all animals (n = 16 per group) were evaluated for motor neurological deficit using the five-score scale test as previously described by Longa et al. (31). All assessments were performed by two experienced examiners who were blinded to group allocation. Briefly, each rat was placed on a flat surface and allowed to walk freely for 2 minutes. Afterward, it was

gently suspended by the tail approximately 30 cm above the table. Neurological deficits were scored on a scale from 0 to 4 according to the following criteria: 0: no observable neurological deficit; 1: inability to fully extend the contralateral (paralyzed) forepaw; 2: consistent circling toward the contralateral (paralyzed) side when walking; 3: tendency to fall to the contralateral (paralyzed) side when walking; 4: absence of spontaneous motor activity and loss of consciousness.

Brain infarction volume

To quantify brain infarct volume, four rats per group were euthanized by CO₂ chamber asphyxiation and decapitated at 14 days post-reperfusion. Brains were rapidly extracted, cleared of meninges and connective tissue, and immersed in cold saline (4 °C) for 5 minutes. Using a brain matrix (Zivic Instruments, Pittsburgh, PA, USA), coronal sections (2 mm thick) were obtained from the frontal pole to the cerebellum. Slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, Taufkirchen, Germany; Cat. No. 108383) in PBS at 37 °C for 15 minutes, resulting in viable tissue stained red, while infarcted areas remained pale. Digital images were acquired, and infarct areas were delineated using ImageJ software (NIH, Bethesda, MD, USA) by an examiner blinded to treatment groups. Infarct volume was calculated using the edema-corrected formula:

Infarct volume (%) = [volume of contralateral hemisphere – (volume of non-ischemic ipsilateral hemisphere)] / volume of contralateral hemisphere × 100.

Brain edema

At 14 days post-reperfusion, rats (4 animals per group) were euthanized, and brains were quickly removed, with the brain stem and cerebellum dissected. The infarcted hemispheres were separated and weighed immediately to determine the wet weight. Samples were then dried in an oven at 90 °C for 24 hours until a constant weight was achieved. Specimens were then reweighed to determine their dry weight. Brain water content (BWC, %) was calculated as:

BWC (%) = [(wet weight - dry weight) / wet weight] × 100

Western blot

At 14 days post-reperfusion, four rats per experimental group were deeply anesthetized, decapitated, and their brains were promptly excised. The prefrontal motor cortex of the ischemic hemisphere was dissected on ice, flash-frozen in liquid nitrogen, and stored at -80 °C until further analysis. Tissue samples were homogenized in lysis buffer consisting of 200 µL of RIPA buffer supplemented with 0.05% protease inhibitor cocktail (Cat No: DB9719, DNAbiotech, Iran). Homogenates were centrifuged at 10000 rpm for 20 minutes at 4 °C (Model 5424 R, Eppendorf, Hamburg, Germany), and the supernatants were collected. Total protein concentration was determined using the BCA assay kit (Cat No: DB9684, DNAbiotech, Iran) according to the manufacturer's instructions. Equal amounts of protein (35 µg) were prepared using Laemmli Sample Buffer (2X) (Cat. No. 1610737, Bio-Rad, Hercules, CA, USA) and heated at 95 °C for 5 min before electrophoresis.

Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to 0.2 μ m PVDF membranes (Cat. No. 1704156, Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 hours at room temperature, followed by overnight incubation at 4 °C with following primary antibodies: Rabbit TNF- α (1:1000; Cat No: ab307164, Abcam, USA), Rabbit IL1- β (1:1000; Cat No: ab254360, Abcam, USA), Rabbit NF-kB p65 (0.5 μ g/mL; Cat No: ab16502, Abcam, USA), Rabbit pNF-kB p65 (1:1000; Cat No: ab76302, Abcam, USA), Rabbit MMP9 (1:1000; Cat No: ab283575, Abcam, USA) Rabbit GAPDH (1:2500; Cat No: ab8245, Abcam, USA), and Rabbit MMP2 (1:2500; Cat No: ab92536, Abcam, USA) antibodies. After washing, membranes were incubated with horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (H&L) (1/10000; Cat No: ab6721, Abcam, USA) for 2 hours at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence substrate (ECL; Cat No: B111421, PARS CO, Mashhad, Iran). Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD, USA), and protein expression levels were normalized to GAPDH and reported as fold-changes relative to control.

Oxidative stress evaluation

Oxidative stress markers were quantified 14 days after reperfusion using motor cortex tissue from four randomly selected rats per group. Freshly isolated tissues were homogenized on ice in cold RIPA buffer (100:1, buffer volume: tissue weight) and centrifuged at 4000 rpm for 10 minutes at 4 °C. The resulting supernatants were collected and used to measure malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, and catalase (CAT) activity using the MDA Assay Kit (ZB-MDA-96A; Zellbio, Germany), SOD Activity Assay Kit (ZB-SOD-96A; Zellbio, Germany), and CAT Activity Assay Kit (ZB-CAT-96A; Zellbio, Germany), respectively, following the manufacturers' instructions. Absorbance was measured at 532 nm for MDA, 450 nm for SOD, and 405 nm for CAT using a microplate reader, and concentrations were determined from respective standard curves. All results were normalized to total protein content and expressed as nmol/mg protein for MDA and U/mg protein for SOD and CAT.

Histological examination

Hematoxylin and eosin (H&E) and Nissl staining were performed on brain tissue sections collected 14 days post-reperfusion to assess general histopathological changes and neuronal viability, respectively. Four rats per group were randomly selected, anesthetized with 10% chloral hydrate (400 mg/kg, i.p), and transcardially perfused with 150–200 mL of ice-cold 0.9% saline, followed by 150–200 mL of 4% paraformaldehyde in PBS over 15 minutes. Then, extracted whole brains were post-fixed in 4% paraformaldehyde for 72 hours, after which the cerebrum was sectioned into 6–7 coronal slices (1.5 mm thick) using a razor blade. The slices were embedded in paraffin and coronally sectioned at 4 μ m thickness using a rotary microtome. For staining, sections were deparaffinized, rehydrated through a graded ethanol series, dewaxed in xylene, and stained with ~0.1% H&E or 2% cresyl violet (Merck, Darmstadt, Germany). Histological examination was performed using a light microscope (Olympus BX51, Tokyo, Japan) equipped with a digital camera (Olympus DP71). For each specimen, five randomly

selected fields within the prefrontal cortex were analyzed at 20× and 40× magnification. The number of Nissl-positive neurons was quantified per field, and the mean of five fields was reported for each rat. All histological assessments were conducted by a board-certified veterinary anatomical pathologist blinded to group allocation.

Statistical analysis

Data were analysed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA) and are presented as mean $\pm$ standard error of mean (SEM). Neurological deficit scores (24 h, 7 days, and 14 days) were analysed using two-way repeated-measures ANOVA (group $\times$ time) followed by Bonferroni's multiple comparisons test. Molecular and histological data were analysed using one-way ANOVA followed by Bonferroni multiple comparisons post hoc test. A p-value < 0.05 was considered statistically significant.

Results

FTIR spectroscopy

The possible interactions between DNAzyme and niosome carriers were investigated using FTIR spectroscopy. As illustrated in Figure 1, the FTIR spectrum of the niosomal DNAzyme exhibited characteristic absorption bands at approximately 3420 cm^{-1} (O–H stretching), 2920 cm^{-1} (C–H stretching), 1735 cm^{-1} (C=O stretching), and 1100–1050 cm^{-1} (C–O and P=O stretching), which were also observed in the spectra of the free DNAzyme and empty niosomes. However, slight shifts in the position and intensity of certain characteristic peaks were observed in the niosomal DNAzyme spectrum relative to the individual components, suggesting weak noncovalent interactions, such as hydrogen bonding or electrostatic attraction, between the DNAzyme and the niosomal matrix. No new peaks were observed, indicating that the DNAzyme and niosome compositions remained unchanged.

Physicochemical properties

The surface characteristics of unloaded niosomes and niosomes containing DNAzyme were examined utilizing FESEM, which elucidated a uniform, regular spherical morphology for both niosomes (Figure 2a) and niosomal DNAzyme (Figure 2b).

The unloaded niosomes displayed a mean diameter of 80 nm, as determined by DLS. The encapsulation of DNAzyme within the niosomes led to an increased size to an average particle size of 126 nm. Moreover, niosomes (PDI 0.176) and niosomal DNAzyme (PDI 0.158) exhibited a consistent particle size distribution, Figure 2 (c-f). The surface charge of empty niosomes was determined to be -50 mV, within the desirable range (< -30 mV) for electrostatic stability as a drug delivery system. The loading of niosomes with DNAzyme also exhibited a zeta potential of -54 mV, indicating a conserved energy state and low aggregation propensity, Figure 2g.

In-Vitro Drug Release

As illustrated in Figure 3, encapsulation of DNAzyme within bilayer niosomes produced a sustained, progressive release profile, in compare to the free DNAzyme at both temperatures examined (pH 7.4, 25°C and 37°C).

Both free and encapsulated DNAzyme demonstrated an initial rapid release phase within the first 8 hours; however, periodic diffusion analyses revealed a pronounced burst release of free DNAzyme (98%) during this timeframe, in contrast to the niosomal DNAzyme, which released only 60% of its incorporated DNAzyme in the same duration. Over the 48 hours of the study, the niosomes released 80-85% of the loaded DNAzyme.

The modestly higher release observed at 37°C compared to 25°C is consistent with thermally enhanced bilayer permeability under physiological temperature conditions. these findings confirm that the niosomal formulation provides effective prolonged DNAzyme release, supporting its suitability as a controlled delivery vehicle for subsequent in vivo application.

Neurological deficit

Figure 4 demonstrates that the sham group differed significantly from all surgically treated groups at 24 hours post-reperfusion ($F(8, 150) = 8.139$; $p < 0.0001$), with this significant separation persisting through days 7 and 14 ($p < 0.0001$). These consistent differences confirm the model's reliability and the successful induction of cerebral ischemia in the animals. In contrast, the differences observed between the treatment groups (DNAzyme and niosomal DNAzyme) and the MCAO group were not statistically significant at any of the assessed time points.

Brain infarct volume and cerebral edema

Compared to the sham group, TTC staining at 14 days post-reperfusion illustrated that all groups subjected to MCAO demonstrated pronounced brain infarct injury, characterized by pale lesions in the right hemispheric cortex and striatum, confirming the successful modeling of cerebral I/R injury [$F(3, 12) = 32.76$, $p < 0.0001$, Figure 5a-b]. As shown in Figure 5b, the administration of either free DNAzyme (14.0 ± 0.91) and niosomal DNAzyme (7.0 ± 1.35) resulted in a reduction in brain infarct volume compared to the MCAO (22.75 ± 2.01 , $p = 0.0131$ and $p = 0.0001$, respectively) and niosome groups (28.50 ± 2.06 , $p = 0.0002$ and $p < 0.0001$, respectively). Notably, treatment with niosomal DNAzyme was associated with a more significant reduction in infarct volume compared to free DNAzyme ($p = 0.048$), indicating the augmented neuroprotective effects associated with employing niosomal carriers for DNAzyme delivery.

In line with these results, Figure 5c demonstrates MCAO induction was associated with significant brain edema in relation to the intact brain of the sham group [$F(4, 15) = 26.5$, $p < 0.0001$]. Both DNAzyme (77.95 ± 0.22 %) and niosomal DNAzyme (77.79 ± 0.06 %) resulted in a notable reduction of brain edema compared to the MCAO group (78.7 ± 0.1 %, $p = 0.122$ and $p = 0.0024$, respectively) and the niosome group (78.76 ± 0.14 %, $p = 0.006$ and $p = 0.0012$, respectively). Unlike the effect observed on brain

infarction volume, no significant difference was noted between DNAzyme and niosomal DNAzyme concerning decreasing brain edema ($p = 0.922$).

Oxidative stress

Oxidative stress induced by cerebral I/R was evident in MCAO rats, as indicated by a significant increase in MDA levels, a marker of lipid peroxidation [$F(4,15) = 27.13$; $p < 0.0001$], accompanied by reduced activity of the antioxidant enzymes SOD [$F(4,15) = 61.77$; $p < 0.0001$] and CAT [$F(4,15) = 38.79$; $p < 0.0001$] at 14 days post-reperfusion (Figure 6). Treatment with DNAzyme or niosomal DNAzyme significantly attenuated oxidative stress, as reflected by lower MDA levels ($p = 0.014$ and $p = 0.0014$, respectively, vs. MCAO) and increased SOD ($p = 0.025$ and $p < 0.0001$, respectively) and CAT activity ($p = 0.001$ and $p < 0.0001$, respectively). While DNAzyme and niosomal DNAzyme groups exerted comparable effects on MDA ($p > 0.999$), niosomal DNAzyme produced a significant enhancement of SOD ($p = 0.03$) and CAT ($p = 0.041$) activity compared with free DNAzyme.

Histological examinations

Nissl staining demonstrated dense, well-organized cortical neurons in the sham group, whereas MCAO rats displayed a significant reduction in Nissl bodies and neuronal disarray (Figure 7a). Treatment with DNAzyme, particularly in its niosomal formulation, markedly increased the number and staining intensity of Nissl-positive neurons. Quantitative analysis corroborated these histological findings, revealing a significant decrease in Nissl-positive neurons following MCAO relative to the intact brains of the sham group [$F(4,13) = 113.3$; $p < 0.0001$; Figure 7c).

Treatment with the anti-p65 DNAzyme significantly increased the number of surviving neurons, both when administered in free form (41.4 ± 2.7 $p < 0.0001$ vs. MCAO) and when delivered via nanoniosome (56.0 ± 3.2 $p < 0.0001$ vs. MCAO). There was a significant difference in neuronal survival between the DNAzyme-treated and DNAzyme+nano groups ($p = 0.0115$).

H&E staining of the motor cortex in MCAO rats revealed pronounced neuronal loss, characterized by reduced cell density, shrunken neurons with triangular, pyknotic nuclei, and vacuolation within the surrounding neuropil. Conversely, animals treated with DNAzyme or niosomal DNAzyme exhibited attenuated histopathological damage, with preservation of neuronal morphology, clearer delineation of cellular boundaries, and diminished nuclear condensation (Figure 7b).

NF- κ B activation

Western blot analysis revealed a substantial upregulation of NF- κ B p65 following the cerebral ischemia induction. All groups that underwent MCAO surgery had significantly higher levels of total NF- κ B p65 [$F(4,15) = 15.69$; $p = 0.0001$] and phosphorylated NF- κ B p65 [$F(4,15) = 10.78$; $p < 0.0003$] than sham animals. Compared with the MCAO group, administration of niosomes alone did not produce a statistically significant changes in total and phosphorylated NF- κ B p65 (both $p > 0.1$) (Figure 8a). In contrast, DNAzyme and niosomal DNAzyme treatments significantly reduced total ($p = 0.0045$ vs MCAO

and $p = 0.0002$ vs Noisome) and phosphorylated NF- κ B p65 expression ($p < 0.0001$ vs MCAO and Niosome). Importantly, phosphorylated NF- κ B p65 levels were significantly lower after treatment with niosomal DNAzyme than after free DNAzyme ($p = 0.01$), highlighting the increased therapeutic potential provided by the niosomal delivery technology (Figure 8b).

Expression of proinflammatory cytokines

The I/R injury was associated with a significant upregulation of proinflammatory cytokines, including TNF- α [$F(4,15) = 35.2$; $p < 0.0001$] and IL-1 β [$F(4,15) = 18.5$; $p < 0.0001$], compared to sham controls at 14 days post-reperfusion (Figure 9a-b). Rats treated with niosome alone exhibited cytokine levels comparable to those observed in the MCAO group ($p > 0.999$). In contrast, administration of DNAzyme ($p = 0.0033$) and niosomal DNAzyme ($p < 0.0001$) significantly reduced TNF- α expression relative to the MCAO group (Figure 9a). Similarly, both DNAzyme ($p = 0.0124$) and niosomal DNAzyme ($p < 0.001$) treatments resulted in a significant decrease in IL-1 β levels compared to MCAO controls (Figure 9b). No statistically significant difference was observed between DNAzyme and niosomal DNAzyme treatments in the suppression of TNF- α ($p = 0.3788$) but not in IL-1 β ($p > 0.999$) expression.

Matrix metalloproteinases

The degradation of the extracellular matrix in brains subjected to I/R insult was observed by a significant elevation in the expression levels of MMP-2 [$F(4,15) = 54$; $p < 0.0001$] and MMP-9 [$F(4,15) = 44.59$; $p < 0.0001$] in rats after MCAO relative to the sham-operated controls. Administration of niosomes alone did not significantly alter the expression of MMP-2 ($p > 0.999$) and MMP-9 ($p > 0.999$). In contrast, treatment with either DNAzyme ($p < 0.001$) and niosomal DNAzyme ($p < 0.0001$) resulted in a marked reduction in the expression of these matrix metalloproteinases compared to the MCAO group. Notably, niosomal delivery of DNAzyme demonstrated a significant enhancement in suppressing MMP-2 expression relative to the free DNAzyme group ($p = 0.0387$), whereas the effect on MMP-9 expression did not show statistical significance ($p = 0.1608$) (Figure 9c-d).

Discussion

This study offers pioneering evidence supporting the therapeutic potential of a rationally engineered DNAzyme targeting the NF- κ B p65 subunit in mitigating cerebral I/R injury in a rat model of tMCAO. Our results demonstrated that the transient silencing of NF- κ B by DNAzyme markedly attenuated multiple pathogenic processes associated with I/R, including NF- κ B-driven neuroinflammation, oxidative stress, and extracellular matrix degradation. Moreover, DNAzyme encapsulation within niosomal nanocarriers appears to enhance its neuroprotective efficacy, potentially by improving its stability, bioavailability, and sustained release at the ischemic region. These synergistic and augmented effects contributed to the preservation of the BBB integrity, a reduction in cerebral edema and infarct volume, and the promotion of neuronal survival within the ischemic cortex.

Our findings build upon extensive evidence highlighting the pivotal role of the NF- κ B pathway, particularly the p65/p50 heterodimer, as a key regulator of I/R injury. Aberrant activation of NF- κ B induces a series of deleterious events, including the upregulation of pro-apoptotic mediators, augmenting ROS generation, expression and release of BBB-disrupting enzymes, and the inflammatory response, with the latter serving as a principal contributor to tissue damage (10, 13). Consistently, DNAzyme-mediated NF- κ B suppression reduced TNF- α and IL-1 β expression, two pleiotropic cytokines implicated in perpetuating ischemic injury by triggering neural apoptosis, exacerbating oxidative damage, amplifying inflammatory cytokines and chemokines release, promoting leukocyte infiltration, and impairing microvascular function (32-36).

Microglia, activated within minutes of noxious ischemia, constitute a primary source of TNF- α and IL-1 β in both rodent models and human stroke cases (37, 38). Previous research has underscored the key role of the p65 subunit of NF- κ B in governing microglial activation and polarization post-ischemia (39-42). Although microglial activation was not directly measured in the current study, the observed pro-inflammatory cytokine reduction likely reflects upstream inhibition of NF- κ B-dependent microglial signalling. Notably, Ganbold et al. provided the most direct evidence supporting this mechanism employing siRNA-mediated silencing of p65 via nanocarrier delivery to microglia/macrophages, which induced a phenotypic shift from a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype with a corresponding decrease in TNF- α , IL-1 β , inducible nitric oxide synthase (iNOS), and ROS while upregulating IL-10, IL-4, and TGF- β , ultimately leading to improved neurobehavioral outcomes (43).

Intertwined with inflammation, oxidative stress represents another key mechanism of I/R injury, arising from excessive production of reactive oxygen and nitrogen species that overwhelm the endogenous antioxidant defence system (44, 45). Our results indicated that DNAzyme-mediated NF- κ B inhibition mitigated oxidative stress, evidenced by enhanced activities of enzymatic scavengers and decreased lipid peroxidation. Activation of NF- κ B has been shown to amplify ROS production by inducing pro-oxidant enzymes such as NADPH oxidase and iNOS, which are implicated in stroke-related oxidative damage (46-48). It can also suppress antioxidant defence via crosstalk with the Nrf2-ARE pathway, the master regulator of redox homeostasis that controls expression of antioxidant enzymes like CAT, SOD, HO-1, and NQO1 (49). It has been shown that under stress conditions, NF- κ B activation antagonizes Nrf2 signalling through sequestration of transcriptional coactivators like CREB binding protein and recruitment of histone deacetylase 3, leading to diminished antioxidant gene expression (50). Conversely, NF- κ B suppression has been shown to potentiate Nrf2-ARE activity, leading to upregulation of NQO1 and HO-1 and attenuation of oxidative damage in myocardial I/R models (51).

Notably, ROS and NF- κ B form a self-perpetuating feedback loop, with NF- κ B enhancing ROS production, while ROS, in turn, activates NF- κ B. In this line, Song et al. demonstrated that overexpression of SOD in transgenic mice with MCAO curtailed ROS accumulation and prevented prolonged NF- κ B activation, underscoring this reciprocal link (52). Hence, DNAzyme-mediated NF- κ B inhibition likely disrupts this vicious cycle, concurrently reducing pro-oxidant enzyme expression and restoring antioxidant capacity to re-establish redox equilibrium.

Beyond its established roles in inflammation and oxidative stress, NF- κ B activation also drives post-ischemic structural injury by inducing ECM-modifying enzymes, most notably MMPs (53-55). These zinc-dependent endopeptidases, produced predominantly by microglia, infiltrating leukocytes, and endothelial cells, degrade basement membrane and tight-junction proteins such as occludin, claudin-5, and collagen IV, thereby compromising BBB integrity (56). In our study, silencing NF- κ B with DNAzyme reduced MMP-2 and MMP-9 expression in the ischemic cortex. MMP-2 is primarily implicated in the early, transient opening of the BBB during reperfusion through tight-junction degradation, whereas MMP-9 contributes to progressive basal lamina fragmentation and endothelial disruption at later stages of injury, establishing both as potential targets for neurovascular protection (57-59). In concert, the inflammatory and oxidative cascades discussed earlier likely act synergistically in aggravating BBB disruption, as ROS-induced junctional protein modification, leukocyte adhesion, and endothelial apoptosis further destabilize the vascular barrier (60, 61). The resultant BBB disruption facilitates extravasation of plasma proteins, cytokines, and immune cells, triggering vasogenic edema and predisposing to haemorrhagic transformation (62-65). Supporting this interplay, NF- κ B inhibition by DNAzyme reduced brain water content, indicating preserved BBB integrity and alleviated edema.

Neuronal injury after cerebral I/R arises from the convergence of multiple pathological mechanisms, including neuroinflammation, oxidative stress, excitotoxicity, mitochondrial dysfunction, apoptosis, and BBB disruption (66, 67). By mitigating the intertwined cascades of inflammation, oxidative stress, and neurovascular injury, NF- κ B inhibition ultimately translated into pronounced neuroprotection at the tissue level with markedly reduced infarct volume, consistent with prior reports showing smaller infarcts following NF- κ B inhibition (68, 69). Histologically, this protection was reflected by increased Nissl-positive neurons and preserved cortical cytoarchitecture, with fewer pyknotic nuclei and diminished vacuolation in the peri-infarct cortex.

Despite these pronounced structural benefits, no significant improvement in neurological function was observed on Longa's five-point scale at 14 days post-reperfusion. Our results contrast with the similar studies on specific and selected inhibition of NF- κ B employing siRNA (14), the NEMO (IKK γ)-binding domain peptide (70), which yielded a significant improvement in neurological deficits on functional and behavioural tests. While differences in stroke model, treatment timing, and administration route may partly account for this discrepancy, other explanations should be considered. Functional restitution after ischemia is inherently gradual, relying on neuroplastic remodelling, such as axonal sprouting, synaptogenesis, and circuit reorganization, that unfolds over weeks rather than days (71, 72). Thus, early histological preservation may not immediately translate into measurable behavioural improvement within a short follow-up. Furthermore, the Longa scale detects only gross motor deficits and lacks sensitivity for subtle functional gains, which could be captured by more refined behavioural assessments (73). Therefore, although NF- κ B inhibition achieved robust histopathological neuroprotection, extended follow-up and comprehensive, quantitative behavioural evaluations are required to delineate its full functional and translational potential.

We also acknowledge some limitations associated with our study. The current study involves the use of unmodified 10–23 DNAzyme, which, although catalytically active, is susceptible to nuclease degradation. Incorporating chemical modifications such as 3'-inverted dT, 2'-O-methylation, or locked-nucleic-acid substitutions could improve stability and half-life, but their impact on catalytic efficiency requires further evaluation (17, 18). Additionally, intracerebroventricular administration ensured local bioavailability yet limits clinical translation and may itself exacerbate focal injury. Considering that niosomes can traverse the BBB, future research should explore ligand-functionalized or targeted formulations amenable to systemic administration (74).

Further in-vitro studies could delineate the temporal kinetics of p65 mRNA and protein silencing, assess DNAzyme degradation and turnover rates, and examine cell-type-specific responses in neurons, astrocytes, and microglia. Likewise, further investigations are warranted to define the optimal dosage, timing, and duration of treatment, evaluate long-term behavioural and cognitive outcomes, and examine their integration with tissue plasminogen activators to appraise their synergistic effect. Furthermore, preclinical studies in higher mammalian species are warranted to establish comprehensive safety profiles, immune responses, and pharmacokinetic parameters essential for clinical translation. Such refinements will enhance understanding of its mechanistic scope and strengthen the translational potential of DNAzyme as a novel therapeutic avenue for ischemic stroke.

Conclusion

This study concludes by showing that targeted silencing of NF- κ B p65 by DNAzyme provides strong neuroprotection after cerebral I/R injury by coordinating the reduction of oxidative, inflammatory, and MMP-mediated BBB damage.

The therapeutic potency was greatly increased by encapsulation within niosomal nanocarriers, highlighting its potential as a reliable and effective gene-silencing platform. The mechanistic and structural advantages offer strong support for NF- κ B suppression as a therapeutic target, even though histological preservation did not result in quantifiable functional recovery during the brief observation period. To determine the translational viability of DNAzyme based NF- κ B suppression in ischemic stroke, further studies should enhance systemic distribution, improve DNAzyme chemistry, and expand behavioral and safety assessments.

Limitations

Despite these results are encouraging, there are a few limitations that should be taken into account. Although the MCAO rat model is the most widely validated focal cerebral ischemia model and accurately reproduces key aspects of human ischemic stroke, the inherent differences between rodent and human cerebrovascular disease cautious clinical extrapolation remains warranted.

Furthermore, conclusive findings about long-term functional recovery and the stability of the identified molecular changes across the subacute and chronic damage phases are not possible due to the short

follow-up period, even though it is suitable for capturing acute neuroinflammatory responses. In addition, the chosen extracellular matrix and inflammatory markers under investigation are well-known mediators of ischemia-reperfusion pathophysiology however the role of multi molecular cascades, such as apoptotic pathways, cannot be excluded. and deserves more research.

Collectively, the current findings offer a solid mechanistic basis for relevant future translational and clinical research.

Declarations

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Conflicts of interest

The authors affirm no conflict of interest.

Availability of data and materials

All data supporting the finding of this study are available from the corresponding authors upon reasonable request.

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Tables

Table 1. Sequence characteristics of the DNAzyme recognition arms

Parameter	Left Arm	Right Arm	Notes/Method
Sequence (5'→3')	TCAAAGTCATACAGCCATC	CTACCGACATATGAAACT	Complementary to nucleotides flanking positions 1017–1037
Length (nt)	9	10	Calculated using Oligo 7
GC Content (%)	30	55	Determined via sequence analysis
T _m (°C)	26	28	Nearest-neighbour model (Oligo 7)
Predicted ΔG (kcal/mol)	0	-0.7	Standard conditions (1 M NaCl)
Secondary Structure Concerns	No	No	Hairpin/dimer formation minimized
Rationale for Selection	Structurally exposed region; stable hybridization	Supports catalytic alignment	Ensures optimal cleavage geometry

Figures

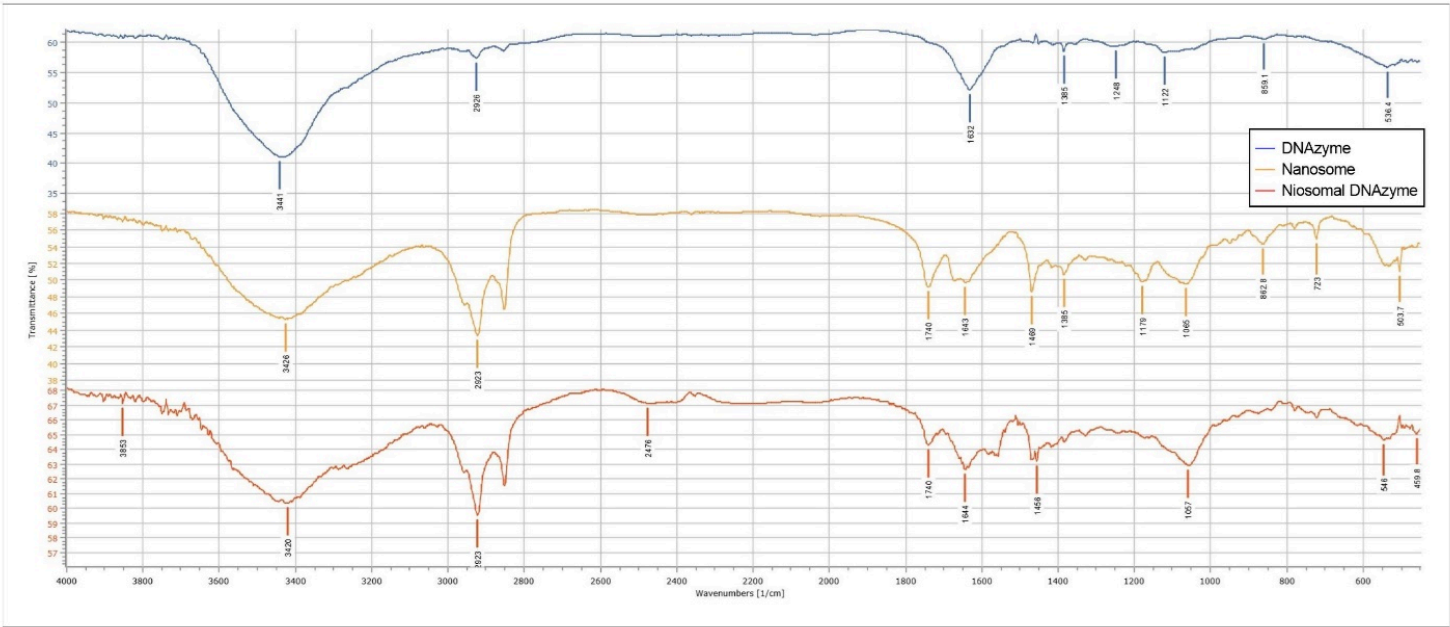


Figure 1

FTIR spectra of free DNAzyme, empty niosomes, and niosomal DNAzyme. Characteristic bands (O–H, C–H, C=O, and C–O/P=O) are preserved across all samples, with slight peak shifts in the niosomal

DNAzyme indicating weak non-covalent interactions and absence of chemical modification during encapsulation.

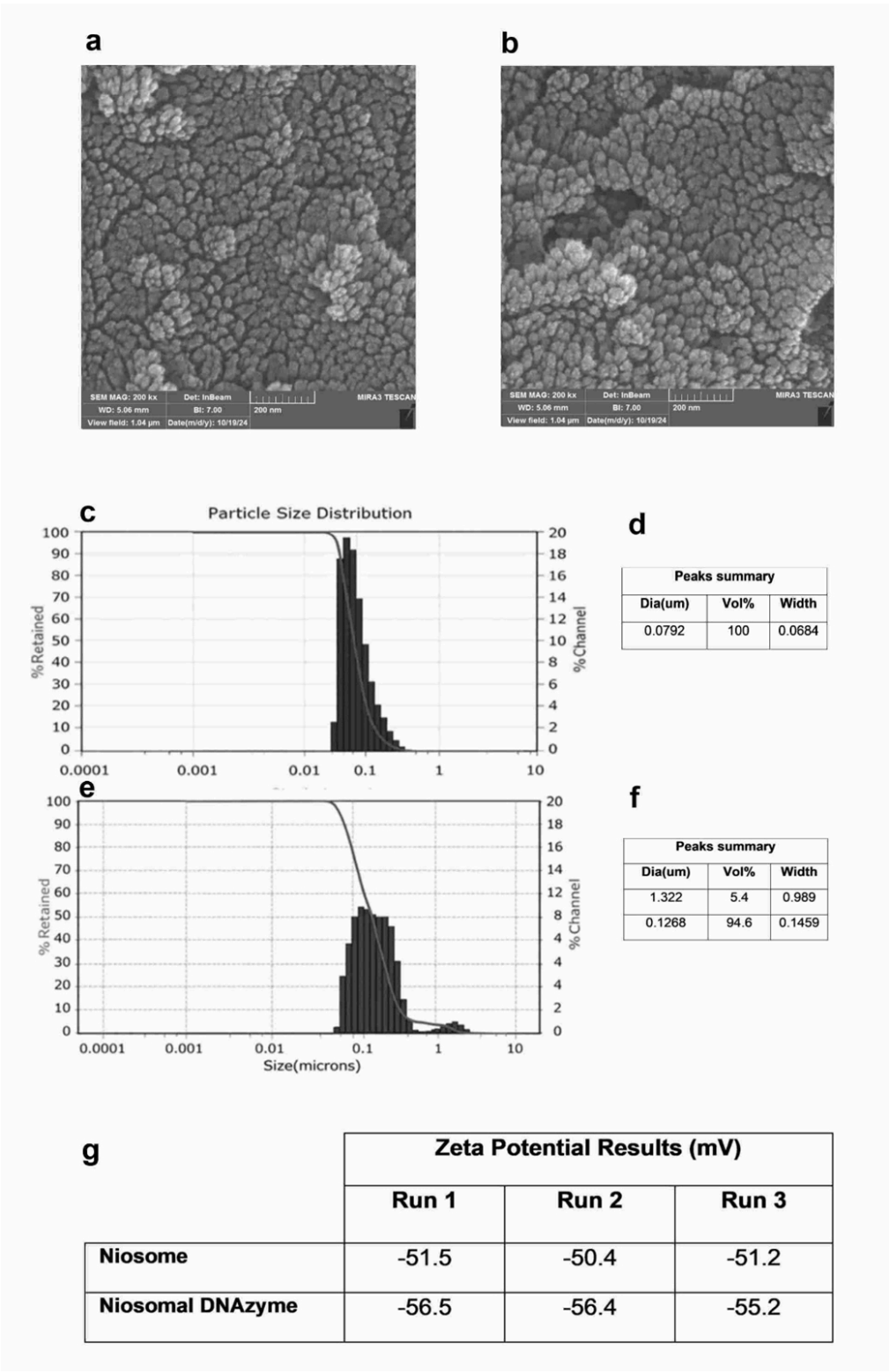


Figure 2

Surfacemorphology of (a) niosomes and (b) DNAzyme-loaded niosomes was examined by FESEM, Particle size distribution of (c, d) niosomes and (e, f) DNAzyme-loaded niosomes was measured by DLS

and (g) zeta potential showing an increased size range after DNAzyme encapsulation.

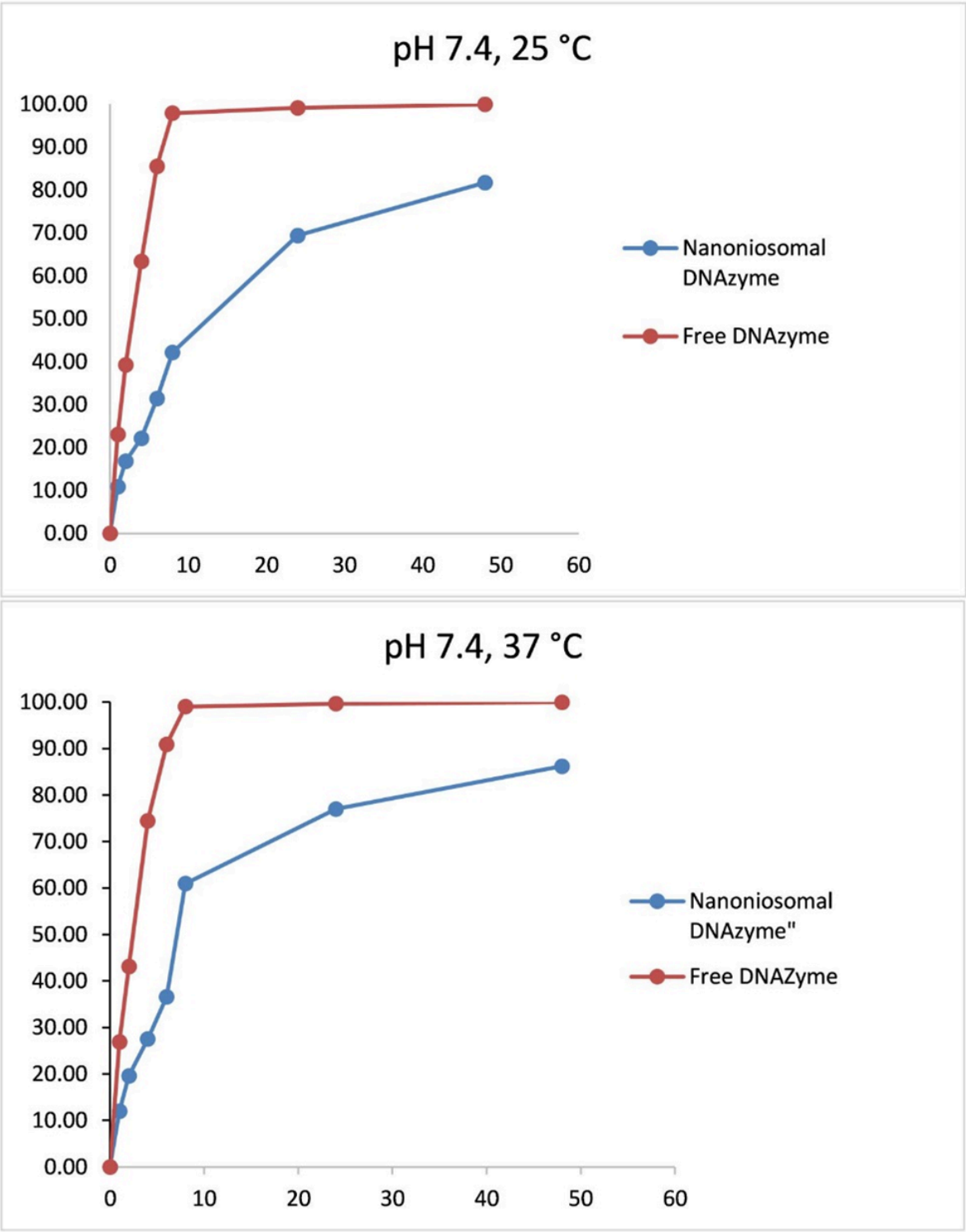


Figure 3

In vitro drug release profile of niosomal DNAzyme compared to free DNAzyme at physiological pH 7.4, assessed at temperatures of 25 °C (a) and 37 °C (b).

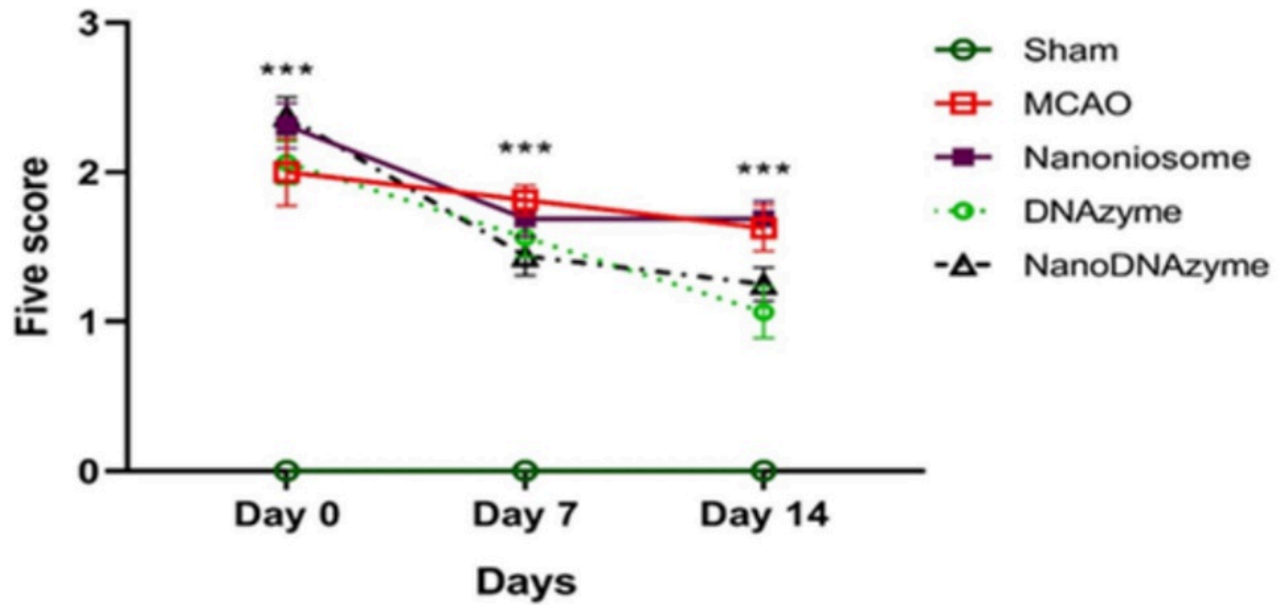


Figure 4

The effect of DNAzyme on neurological deficit after transient MCAO. Neurological deficit was evaluated using the five-point neurological deficit score, which showed no significant difference at 24 h, 7 days, or 14 days post-reperfusion ($n = 16$ per group). No significant recovery was detected in the DNAzyme-treated groups compared with the MCAO group. Data are represented as mean $\pm$ SEM. Statistical significance was determined using two-way repeated-measures ANOVA (group $\times$ time) followed by Bonferroni's multiple comparisons test.

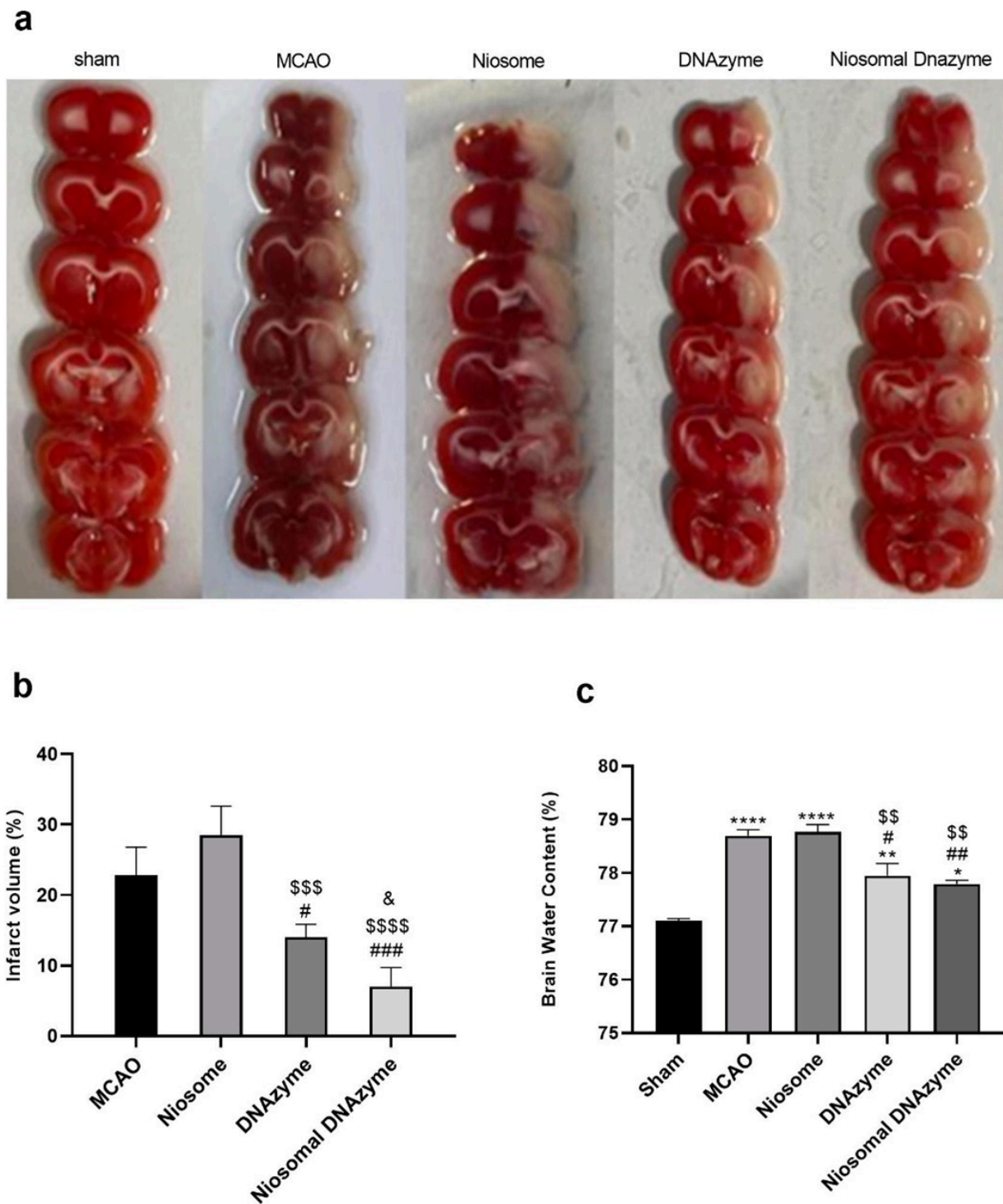


Figure 5

Intraventricular administration of DNazyme and niosomal DNazyme reduced brain infarct volume and edema in rats at 14 days after transient MCAO. (a) Representative images of TTC-stained brain slices. (b) Quantified infarct volume and (c) brain water content demonstrated that free DNazyme and niosomal DNazyme significantly decreased infarction volume and brain edema (n = 4 for each group). Data are represented as mean $\pm$ SEM. Statistical significance was determined using one-way analysis of variance

(ANOVA) followed by a Bonferroni post hoc test. *: $p < 0.05$, **: $p < 0.01$, ****: $p < 0.0001$ vs. Sham group; #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ vs. MCAO group;

: $p < 0.01$,

§: $p < 0.001$,

: $p < 0.0001$ vs. niosome group; &: $p < 0.05$ vs DNAzyme group. MCAO: Middle cerebral artery occlusion; TTC: 2,3,5-triphenyltetrazolium chloride.

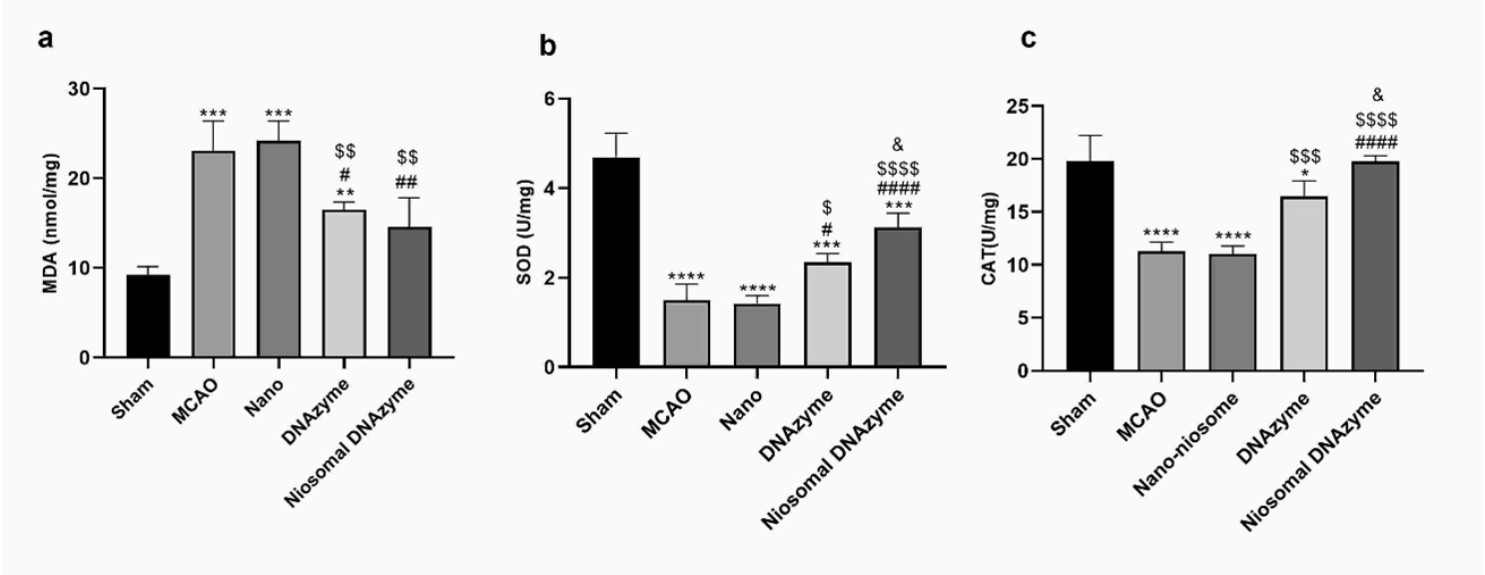


Figure 6

Attenuation of oxidative stress by DNAzyme and niosomal DNAzyme at 14 days post-reperfusion. Treatment with DNAzyme or niosomal DNAzyme significantly reduced (a) malondialdehyde (MDA) and increased (b) superoxide dismutase (SOD) and (c) catalase (CAT). All measurements were normalized to total protein concentration and are expressed as mean ± SEM (n = 4 per group). Statistical significance was determined using one-way ANOVA followed by a Bonferroni post hoc test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ vs. Sham group; #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ vs. MCAO group; §: $p < 0.05$,

: $p < 0.01$,

§: $p < 0.001$,

: $p < 0.0001$ vs. niosome group; &: $p < 0.05$ vs. DNAzyme group.

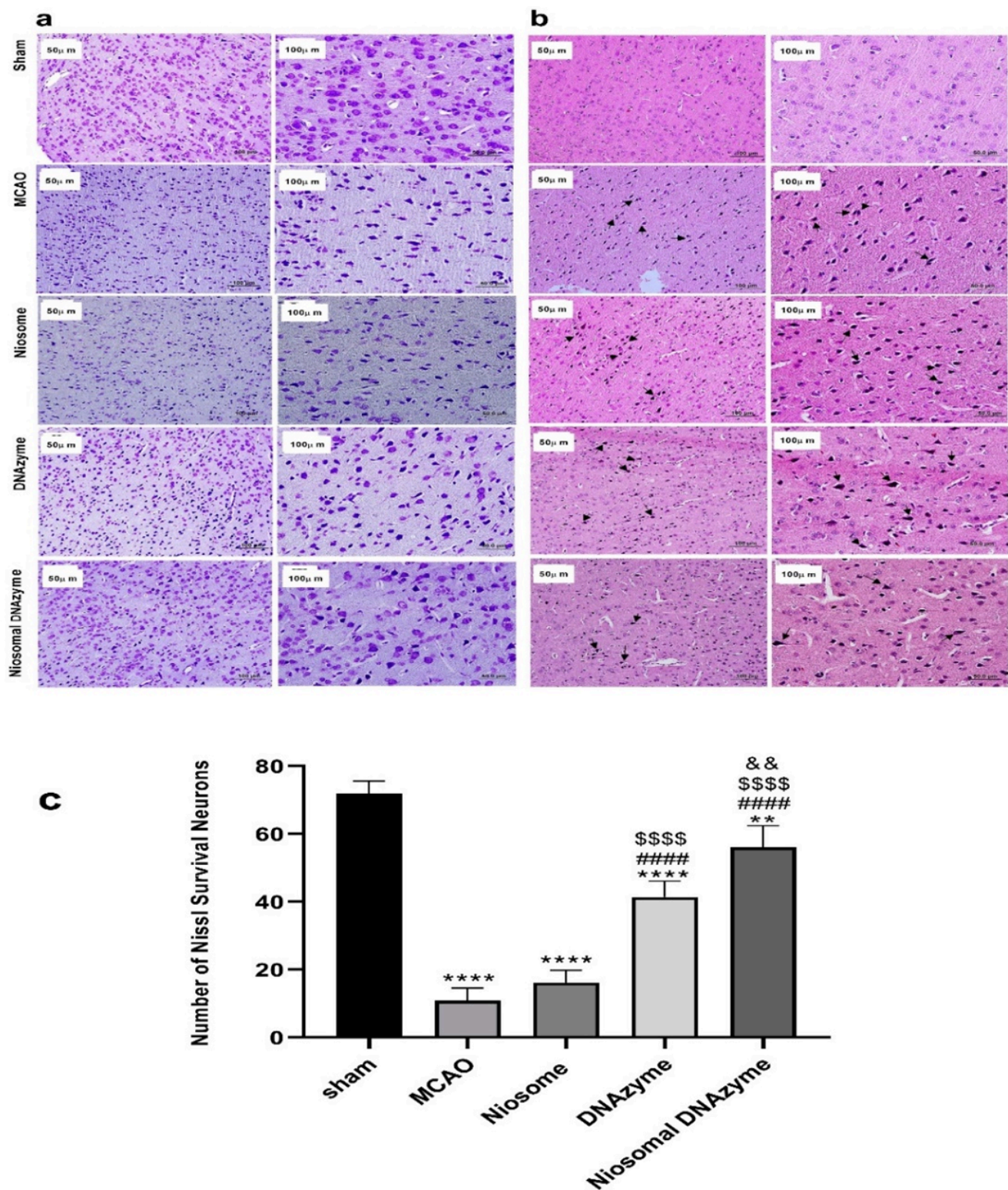


Figure 7

Intraventricular injection of DNAzyme and niosomal DNAzyme could preserve neuronal structure and morphology and reduce neuronal loss 14 days after MCAO. (a) Representative images of Nissl staining in the motor cortex showing marked neuronal loss following MCAO, with an enhanced number of viable Nissl-positive neurons in DNAzyme and niosomal DNAzyme groups. (b) Representative images of H&E staining of the right prefrontal showing histopathological changes resulting from MCAO, including

reduced cell density and shrunken neurons with condensed nuclei (Scale bar = 100µm, 50 µm). (c) Quantitative analysis of Nissl-positive neurons. Data represented as Mean ± SEM (n = 3 for MCAO and Sham groups; (n = 4 per other groups). Statistical significance was determined using one-way ANOVA followed by Bonferroni post hoc test. **: p<0.01, ****: p<0.0001 vs. Sham group; ####: p<0.0001 vs. MCAO group; : p<0.0001 vs. Nanoniosome group; &: p<0.05 vs. DNAzyme group. H&E: haematoxylin and eosin; MCAO: middle cerebral artery occlusion.

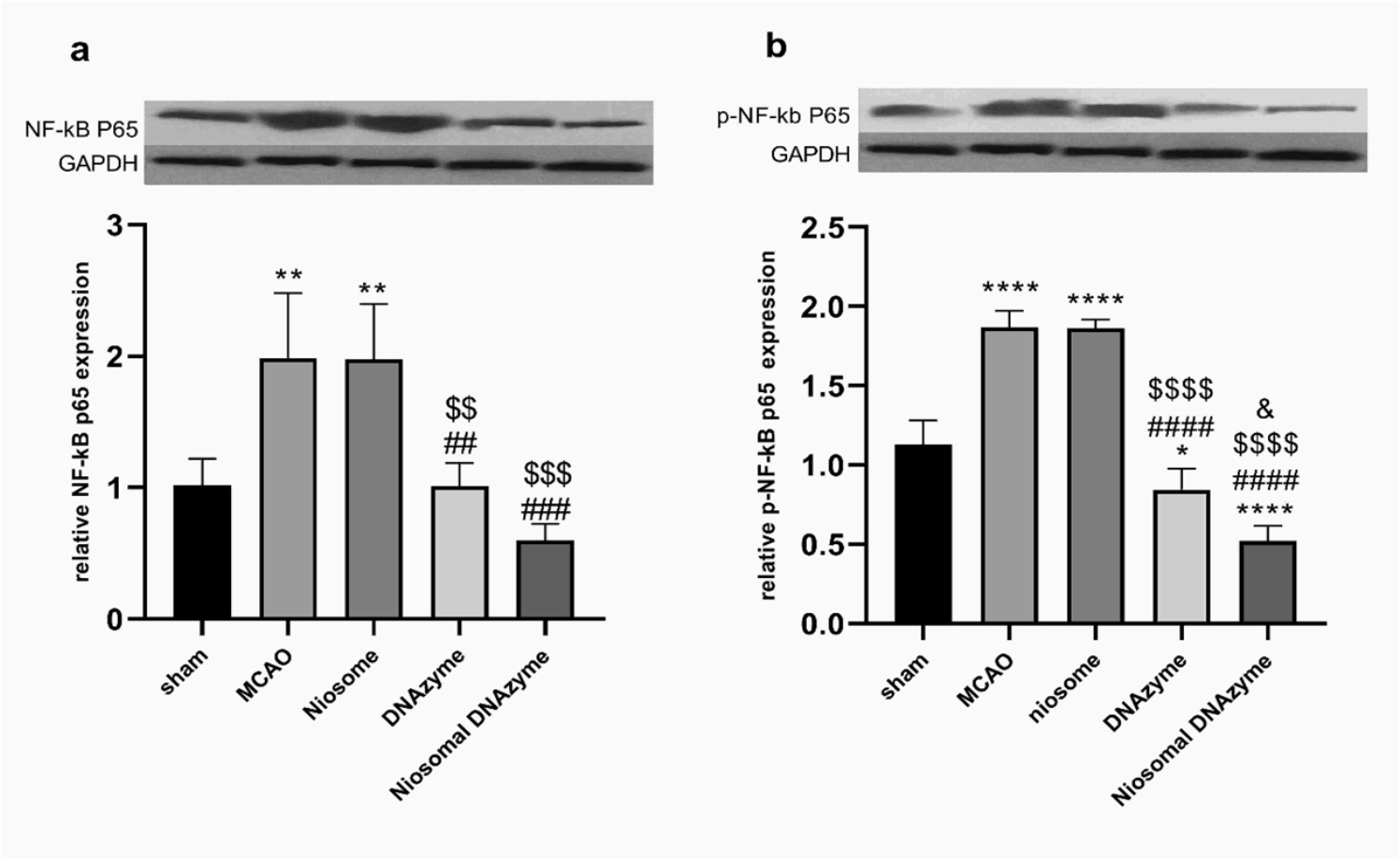


Figure 8

Effects of DNAzyme and niosomal DNAzyme treatment on NF-κB p65 activation following cerebral ischemia/reperfusion. (a) Relative protein expression levels of total NF-κB p65 and (b) NF-κB p65 phosphorylated. Densitometric data are normalized to GAPDH and presented as mean ± SEM (n = 4 per group). Statistical significance was determined using one-way ANOVA followed by Bonferroni post hoc test. *: p < 0.05, **: p < 0.01, ****: p < 0.0001 vs. Sham group; #: p < 0.01, ####: p < 0.0001 vs. MCAO group;

: $p < 0.01$,

\$\$: $p < 0.0001$ vs. niosome group; &: $p < 0.05$ vs. DNAzyme group. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NF- κ B: Nuclear Factor kappa B; TNF- α : Tumor necrosis factor alpha.

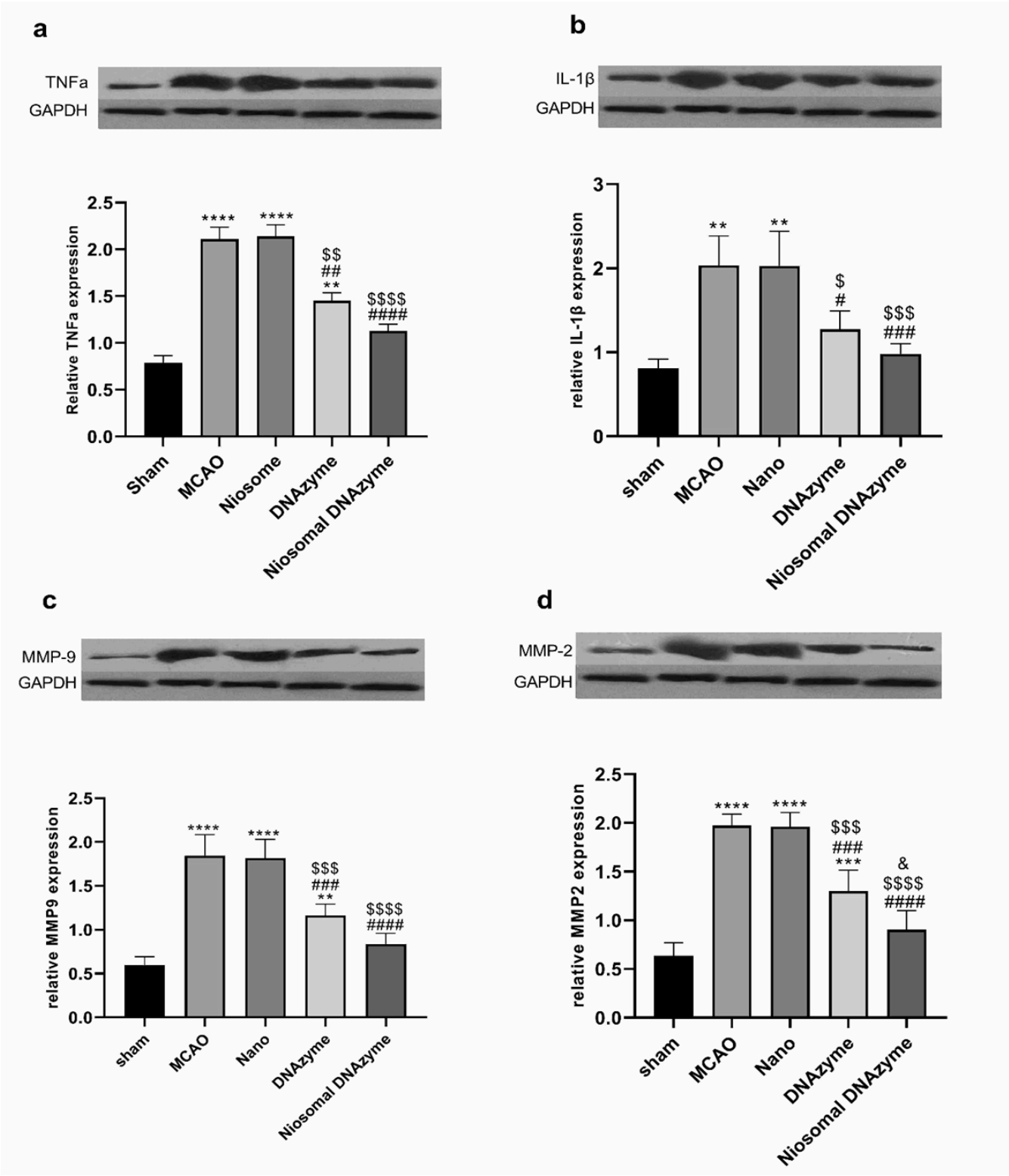


Figure 9

Modulation of NF- κ B activity by DNAzyme resulting in decreased expression of proinflammatory cytokines TNF- α and IL-1 β , as well as matrix metalloproteinases MMP-2 and MMP-9. Relative protein

expression levels of proinflammatory cytokines TNF- α (a) and IL-1 β (b), as well as MMP-9 (c) and MMP-2 (d). Densitometric data are normalized to GAPDH and presented as mean $\pm$ SEM (n = 4 per group). Statistical significance was determined using one-way ANOVA followed by Bonferroni post hoc test. **: p < 0.01, ***: p < 0.001, ****: p < 0.0001 vs. Sham group; ###: p < 0.001, ####: p < 0.0001 vs. MCAO group;

\$: $p < 0.001$,

\$\$: p < 0.0001 vs. niosome group; &: p < 0.05 vs. DNazyme group. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL-1 β : Interleukin-1 beta; MMP: Matrix metalloproteinase; NF- κ B: Nuclear Factor kappa B; TNF- α : Tumor necrosis factor alpha.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigures.pdf](#)