Check for updates

*Original Article*

JCBFM

Journal of Cerebral Blood Flow & Metabolism
2024, Vol. 44(12) 1459–1471
© The Author(s) 2024
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/0271678X241270284
journals.sagepub.com/home/jcbfm
Sage

# Systemic administration of blood-derived exosomes induced by remote ischemic post-conditioning, by delivering a specific cluster of miRNAs, ameliorates ischemic damage and neurological function

**Ornella Cuomo[1], Serenella Anzilotti[2], Paola Brancaccio[1], Pasquale Cepparulo[1], Giovanna Lombardi[1], Viviana Viscardi[1,3], Antonio Vinciguerra[4], Lucio Annunziato[5] and Giuseppe Pignataro[1]**

## Abstract

MicroRNAs, contained in exosomes or freely circulating in the plasma, might play a pivotal role in the infarct-sparing effect exerted by **r**emote limb ischemic postconditioning (RLIP). The aims of the present study were: (1) To evaluate the effect of pure exosomes isolated from plasma of animals subjected to RLIP systemically administered to ischemic rats; (2) To finely dissect exosomes content in terms of miRNAs; (3) To select those regulatory miRNAs specifically expressed in protective exosomes and to identify molecular pathways involved in their neurobeneficial effects. Circulating exosomes were isolated from blood of animals exposed to RLIP and administered to animals exposed to tMCAO by intracerebroventricular, intraperitoneal or intranasal routes. Exosomal miRNA signature was evaluated by microarray and FISH analysis. Plasmatic exosomes isolated from plasma of RLIP rats attenuated cerebral ischemia reperfusion injury and improved neurological functions until 3 days after ischemia induction. Interestingly, miR-702-3p and miR-423-5p seem to be mainly involved in exosome protective action by modulating NOD1 and NLRP3, two key triggers of neuroinflammation and neuronal death. Collectively, the results of the present work demonstrated that plasma-released exosomes after RLIP may transfer a neuroprotective signal to the brain of ischemic animals, thus representing a potentially translatable therapeutic strategy in stroke.

## Keywords

Cerebral ischemia, conditioning, exosomes, miRNAs, neuroprotection

Received 30 March 2024; Revised 26 May 2024; Accepted 22 June 2024

## Introduction

Remote ischemic conditioning (RIC) represents an innovative and attractive neuroprotective approach in ischemia of brain and other organs.[1] The purpose of this strategy is to activate endogenous tolerance mechanisms by inducing a subliminal ischemic injury to the limbs, or to another "remote" region, leading to a protective systemic response against ischemic brain injury.[1] This evidence allows to hypothesize that remote limb ischemic postconditioning triggers a general genetic reprogramming at the brain level that can be mediated by circulating factors. Among the possible mediators,

[1]Division of Pharmacology, Department of Neuroscience, School of Medicine, University of Naples Federico II, Naples, Italy
[2]Department of Science and Technology, University of Sannio, Benevento, Italy
[3]International School of Advanced Studies, University of Camerino, Camerino, Italy
[4]Department of Biomedical Sciences and Public Healty, University "Politecnica delle Marche", Ancona, Italy
[5]IRCCS SYNLAB SDN S.p.A., Naples, Italy

**Corresponding author:**
Ornella Cuomo, Division of Pharmacology, Department of Neuroscience, School of Medicine, University of Naples Federico II, 80131 Naples, Italy.
Email: ornella.cuomo@unina.it

short non-coding RNAs have recently emerged as the most promising candidates. In particular, microRNAs (miRNAs) are non-protein coding short ribonucleic acid molecules (usually 18–25 nucleotides) found in eukaryotic cells that target messenger RNAs and favour their degradation or prevent their translation.[2,3] Originally, miRNAs have been thought to work within the cells in which they are generated; however, recently, miRNAs have been described in secreted exosomes or freely circulating in the plasma. Exosomes are complex "living" structures generated by many cell types containing a multitude of cell surface receptors,[4] encapsulating, among others, proteins, trophic factors, miRNAs, and other RNAs.[5–8] These bioactive vesicles, through their stored molecules, can mediate intercellular communication.[9–12] Indeed, exosomes are secreted by many different cell types through biological fluids, including synovial fluid, breast milk, blood, urine, saliva, amniotic liquid, and blood serum,[4] suggesting that they play a major role in intercellular communication and in triggering physiological and pathophysiological responses. External and internal environmental challenges, such as stress conditions or pathological disorders, may influence the composition, biogenesis, and secretion of exosomes.[4] Importantly, exosomal short non-coding RNAs are protected from the digestion of RNAase or trypsin[8] and several evidence show that exosomes transfer miRNAs to recipient cells,[8] thus modifying their features.[11,12] Taking into account these premises and considering that miRNA delivery represents one of the main limitations of the miRNA-based therapeutic strategies, exosomes appear as an ideal miRNA-cargo. The advantages of engineered exosomes as delivery systems are evident: they only contain biogenic substances and are readily transferred into target cells, as well as they have potentially wide utility for the brain delivery of nucleic acids, and possibly for selectively targeting different cell types.[13,14] Thus, systemic exosome administration may represent a promising strategy to deliver protective mediators to the CNS.

In light of this evidence, the main aim of the present paper was to evaluate whether exosomes isolated from the plasma of rats subjected to remote limb ischemic postconditioning may be used as a therapeutic approach in cerebral ischemia. Indeed, the specific objectives of the present paper were:

1. To evaluate "in vivo" the effect of the administration by different routes (i.e. icv, intranasal and intraperitoneal) of plasmatic exosomes released after remote postconditioning on stroke damage in rats subjected to transient occlusion of middle cerebral artery.
2. To characterize the peculiar miRNA signature of plasmatic exosomes extracted from animals subjected to remote post-conditioning in order to identify neurobeneficial miRNAs, as new potential modulators of druggable targets.
3. To identify the possible targets of the neuroprotective exosomal miRNAs.

## Materials and methods

### *In vivo studies*

*In vivo experimental groups.* Ninety Sprague–Dawley male rats (Charles River Laboratories, Calco, Varese, Italy) weighing 150 to 175 g were housed under diurnal lighting conditions (12-hour darkness/light). Twenty percent of the animals (15 rats) used has been excluded (Table S1) since they did not reach a cerebral blood flow reduction of at least 70% or because they died during the surgical procedures. Experiments were performed according to the international guidelines for animal research and Italian guidelines for animal use (DL 26/2014), and in compliance with the ARRIVE guidelines. The experimental protocol was approved by the Animal Care Committee of the "Federico II" University of Naples. Sample size was calculated using G power software. Animals were randomly assigned to the following four experimental groups: (1) Control, (2) Femoral Artery Occlusion (FAO), (3) transient Middle Cerebral Artery Occlusion (tMCAO), (4) tMCAO + FAO, indicated as Remote Limb Ischemic PostConditioning (RLIP). The blinding procedure for each experimental group was applied not only to the operator performing neurological assessments but also to the scientist performing tMCAO and FAO procedures. In particular, the operator performing tMCAO was different from the one performing FAO and from the researcher performing the vehicle or exosome administration groups.

### *Animal model of focal cerebral ischemia*

Transient focal ischemia was induced as previously described.[17] In brief, transient occlusion of the middle cerebral artery (tMCAO) was performed in male rats anesthetized using a mixture of oxygen and sevoflurane at 3.5% (Medical Oxygen Concentrator LFY-I-5A). A 5-O surgical monofilament nylon suture (Doccol, Sharon, MA) was inserted from the right external carotid artery into the right internal carotid artery and advanced into the circle of Willis up to the branching point of the MCA, thereby occluding the MCA. The filament was left in place for 100'. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow in the area of the right MCA through a

disposable microtip fiber optic probe (diameter 0.5 mm) connected through a Master Probe to a laser Doppler computerized main unit (PF5001; Perimed, Järfälla, Sweden) and analyzed using PSW Perisoft 2.519. Animals not showing a cerebral blood flow reduction of at least 70% were excluded from the experimental group, as well as animals that died after ischemia induction.[18] Rectal temperature was maintained at $37 \pm 0.5$°C with a thermostatically controlled heating pad and lamp. All surgical procedures were performed under an operating stereomicroscope.

### *Animal model of Remote Limb Ischemic Postconditioning*

Remote Limb Ischemic Postconditioning (RLIP) was induced by subjecting animals to two subsequent surgical procedures, tMCAO and FAO. In the first phase, animals were exposed to 100' of tMCAO, as above described. In the second phase, femoral artery occlusion (FAO) was performed 20' after tMCAO reperfusion. In particular, animals were re-anesthetized, the femoral artery was identified, isolated and occluded with two microserrafine clips (Fine Science Tools GmbH, Germany) to stop the blood flow for a period of 20' followed by reperfusion.[1] By contrast, the FAO experimental group was composed of animals subjected to the only 20' FAO followed by reperfusion without tMCAO procedure.

### *Blood sample collection and exosomes isolation and characterization*

One hour and half after FAO procedure, animals blood samples were collected from the tail vein and exosomes were extracted according to the procedure below described (Figure 1(a)). Total Exosome Isolation Kit (Invitrogen, Thermo Fisher Scientific, USA, Cat. No. 4484450) was used to isolate exosomes from rat plasma samples, following the manufacturer instructions.[15] Exosome's precipitate resulting from 200 µl of plasma was resuspended in 75 µl of sterile phosphate-buffered saline (PBS).

Size distribution and concentration of plasma EVs were determined by the nanoparticle tracking analyzer NanoSight NS300 (Malvern, UK), as previously described.[16] To confirm the exosomal nature of our preparations, western blot analysis was performed with antibodies against exosome proteins, such as Alix (Cell Signaling Technologies, Cat. No. 2171), CD63 (Abcam, Cat. No. ab134045), and CD9 (Abcam, Cat. No. ab92726). Briefly, after the last centrifugation to pull down the exosomes pellet, it was resuspended in lysis buffer (RIPA, Sigma-Aldrich) and homogenized and lysed in ice. Then, 30 µg of protein was mixed with Laemmli sample buffer (5% 2-mercaptoethanol) and heated at 95°C for 5 minutes, to be loaded on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to Polyvinylidene difluoride (PVDF) membrane by Trans-Blot Turbo System (Bio-Rad). Membranes were incubated with primary antibody overnight at 4°C, and with horseradish peroxidase (HRP)-conjugated secondary antibodies for one hour at room temperature. Both primary and secondary antibodies were diluted in 0.2% I-block solution (T2015, ThermoFisher Scientific, USA). Immunoreactive bands were detected using ECL (Amersham). The optical density of the bands was determined by Chemi-Doc Imaging System (Bio-Rad, Segrate, Italy).

### *Exosome administration in vivo*

Rats subjected to tMCAO were treated with fresh prepared exosomes or vehicle by intracerebroventricular (icv), intraperitoneal (ip) or intranasal (in) route. For icv route, each animal received 10 µL exosomes ($1 \times 10^{11}$ particles, comparable to 400 µg/Kg total exosome proteins, per rats) or vehicle twice, 24 hours before ischemia induction and 10 min after reperfusion. For intraperitoneal route, each animal received 0.5 ml PBS (vehicle) or exosomes ($1 \times 10^{11}$ particles, comparable to 400 µg/Kg total exosome proteins, per rats) dissolved into 0.5 ml phosphate-buffered solution (PBS, Thermo Fisher Scientific Inc., Grand Island, NY) administered twice, 100 min and 280 min after tMCAO. For the intranasal route, each animal received 10 µL exosomes (comparable to 400 µg/Kg total exosome proteins, per rats) or PBS as vehicle, twice, 100 min and 280 min after tMCAO.[19]

### *Evaluation of the infarct volume and of neurological deficit scores*

Animals subjected to ischemia and treated with exosomes or vehicle were undergo to 24 h after ischemia with isoflurane overdose. A subgroup of animals, intranasally administered with exosomes at the above indicated doses, were euthanized 3 days after ischemia induction. Brains were quickly removed, sectioned coronally at 1 mm intervals, and stained by immersion in the vital dye (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC). The infarct volume was calculated by summing the infarction areas of all sections and by multiplying the total by slice thickness. To avoid that edema could affect the infarct volume value, we expressed the infarct volume as percentage of the infarct, calculated by dividing the infarct volume by the total ipsilateral hemispheric volume.[18] Neurological scores were evaluated before euthanization according to the following two scales: a general

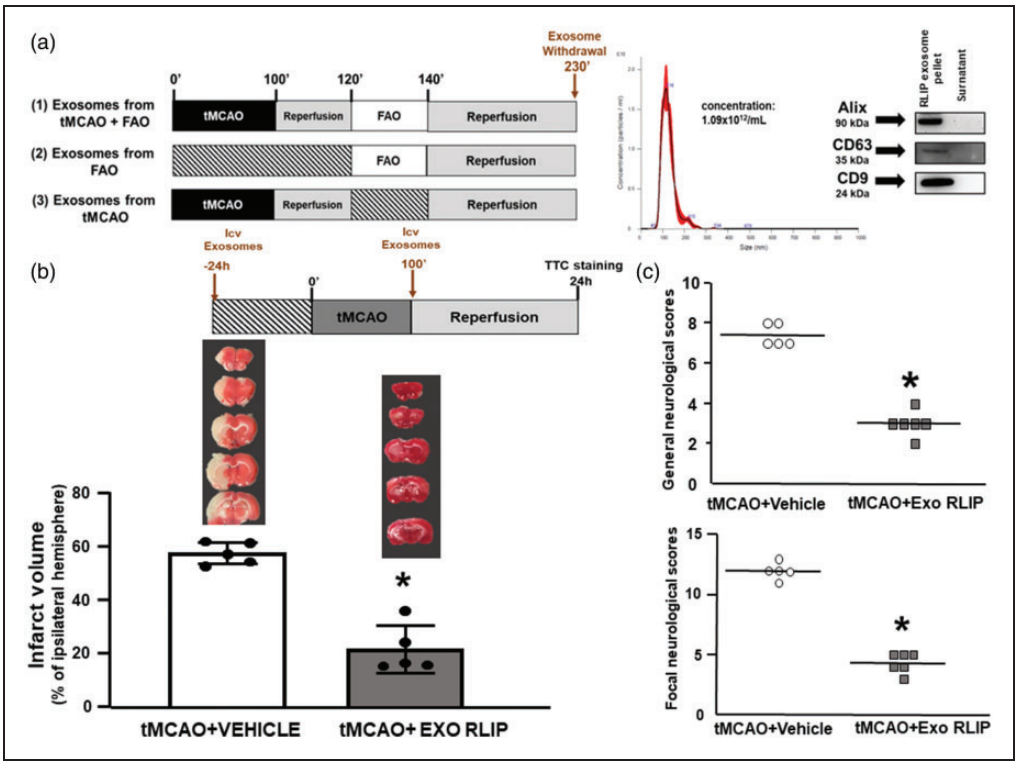



**Figure 1.** Exosome characterization and Evaluation of ischemic damage and neurological scores 24 hours after tMCAO in rats icv administered with plasmatic exosomes obtained by RLIP rats. (a) Left: Schematic representation of the protocols for blood withdrawal and exosome isolation; tMCAO: transient middle artery occlusion, FAO: femoral artery occlusion; middle panel: Determination of Exosome concentration by Nanoparticle tracking analysis, Right panel: Representative blots of exosome preparation obtained from RLIP animals showing the expression of specific exosomal membrane markers. (b) Evaluation of ischemic damage 24 hours after tMCAO in ischemic rats icv treated (24 h before ischemia and 10 min after reperfusion) with exosomes (400 μg/Kg) isolated from plasma of rats exposed to RLIP + 1.5 h reperfusion and (c) evaluation of general (up) and focal (bottom) neurological scores in the same groups. Neurological scores were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test. $p < 0.05$ versus ischemic rats treated with vehicle. $n = 5–6$ animals per group. Each column represents the mean ± SD.

neurological scale and a focal neurological scale. In the general score, the following six general neurological functions were evaluated: (i) hair conditions (0–2), (ii) position of ears (0–2), (iii) eyes conditions (0–4), (iv) posture (0–4), (v) spontaneous activity (0–4), and (vi) epileptic behavior (0–12). For each of the six general functions measured, animals received a score depending on the severity of the symptoms, higher is the score worse is the rat condition. The scores of investigated items were then summed to provide a total general score ranging from 0 to 28. In the focal score, the following seven areas were assessed: (i) body symmetry, (ii) gait, (iii) climbing, (iv) circling behavior, (v) front limb symmetry, (vi) compulsory circling, and (vii) whisker response. For each of these items, animals were rated between 0 and 4 depending on the severity. The seven items were then summed to give a total focal score ranging between 0 and 28. Infarct volumes and neurological scores were evaluated in a blinded manner by individuals who did not perform the surgical procedures.

### *miRNA profiling evaluation*

For the evaluation of miRNAs content of plasma derived exosomes, miRNA profiling was performed using TaqMan® Array MicroRNA Cards (Thermo Fisher Scientific, USA) on total RNA extracted from exosomes isolated from rat serum of animals subjected to RLIP, FAO or tMCAO.

### *Tissue processing, immunostaining, and confocal immunofluorescence*

Animals were anesthetized and transcardially perfused with saline solution containing 0.01 ml heparin, followed by 4% paraformaldehyde in 0.1 mol/l PBS saline solution. Brains were processed as previously described.[20–22] Briefly, brains were sectioned frozen on a sliding cryostat at 40 μm thickness, in rostrum-caudal direction. Afterwards, free floating serial sections were incubated with PB Triton X 0.3% and blocking solution (0.5% milk, 10% FBS, 1% BSA) for 1 h and 30 min. The sections were incubated overnight at +4°C with the

following primary antibodies: anti-NOD1 (#PA5-79747, Invitrogen, Thermo Fisher Scientific, USA), anti-NeuN (#ABN7,8 Merk Millipore, USA.) anti-NLRP3 (#PA5-79740, Invitrogen, Thermo Fisher Scientific, USA). The sections were then incubated with the corresponding fluorescent-labeled secondary antibodies, Alexa 488/ Alexa 594 conjugated antimouse/antirabbit IgG. Nuclei were counterstained with Hoechst. Images were observed using a Zeiss LSM700 META/laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

### *Fluorescent In situ hybridization (FISH) miRNA assay*

For in situ hybridization all the procedures were performed in autoclaved and RNAse free solutions. Rats were perfused with 1× phosphate-buffered saline (PBS) and 4% paraformaldehyde solution PBS. The brain tissues were fixed in 4% paraformaldehyde solution PBS overnight at 4°C and subsequently cryoprotected in 30% sucrose PBS overnight at 4°C and cryosectioned at 10 µm thickness.[23] Frozen tissue sections were prepared following the description of MicroRNA Protocol for In situ Hybridization on Frozen Sections (Exiqon, Denmark). Briefly, brain sections were submerged in Neutral buffered Formalin 10% for 15 min e then washed in PBS for 3 × 5 min. The sections were incubated in proteinase K buffer containing 1 M tris-HCl pH 7.4, 0.5 M EDTA, 5 M NaCl, proteinase K 15 µg/mL in RNase-free water, for 10 min at 37°C then the sections were washed 3 times × 3 min in PBS. Brain slices were then incubated in 3% H2O2 for 5 min to inhibit endogenous peroxidase activity, and then washed in PBS for 3× 3 min. Sections were sequentially hybridized for 1 h at 55° and 54°C for mir-423-5p (5'DIG and 3'DIG) and mir-702-3p (5'DIG and 3'DIG) respectively. The final concentration of probes was 20 nM. The sections were hybridized in hybridization buffer containing: 50% deionized formamide 0.3 M NaCl, 20 mM Tris HCL, pH 8.0, 5 mM EDTA, 10 mM NaPO4, pH 8.0, 10% Dextran Sulfate, 1× Denhardt's solution, 0.5 mg/mL yeast RNA and probes. Post-hybridization washes were performed sequentially 2× 5 min at hybridization temperature in 5× SSC buffer, 3× 5 min at hybridization temperature in 1× SSC buffer, 2× 5 min at hybridization temperature in 0,2× SSC and 1× 5 min at room temperature in 0,2× SSC buffer. Following the stringent washing, sections were incubated in blocking solution containing: 2% sheep serum, 1% BSA in PBS-0,1% Tween for 15 min at room temperature. Then, the sections were incubated for 60 min with Anti-Digoxigenin-POD, Fab fragments (Roche Diagnostics GmbH, Germany) diluted 1:400 in 1% sheep serum 1% BSA and PBS 0.05% tween. Then, the sections were washed in PBS for 3 × 5 min and incubated for 5 min in Cy2 conjugated Tyramide (TSATM Plus Fluorescein kit, PerkinElmer, USA) by diluting TSA stock solution 1:50 in 1× Amplification Diluent. After washing 3× 10 min with TBS, sections were incubated for 30 min in 3% H2O2 in TBS to quench peroxidase activity from the initial TSA reaction. After washing, sections were incubated HOECHST for 20 min and mounted onto slides using Fluoromount™ Aqueous Mounting Medium, (SIGMA, Germany) air-dried and stored in the dark room. As controls, the sections were incubated without the Anti-Digoxigenin-POD or without the TSATM Plus Fluorescein or primary antibodies and the immunoreactivity was completely abolished (data not shown).

To obtain a measure of fluorescence, the images were performed by NIH image software by measuring the intensity of fluorescent of mir-423-5p and mir-702-3p immunolabeling in $n = 3$ animals for each group and three slides for each group. The intensity of miRNAs immunoreactivity were expressed in arbitrary units. Digital images were taken with a × 40 objective, and identical laser power settings and exposure times were applied to all images from each experimental set. Images from the same areas of each brain region were compared.[18]

### *Statistical analysis*

Values are expressed as means ± SD. To assess data distribution, Shapiro-Wilk test was used as normality test. Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA), using ANOVA followed by Newman–Keuls or Bonferroni post-hoc test for more than two groups. To compare two groups, unpaired t-test was used. Data related to focal and general neurological deficits were analyzed using the non-parametric test of Kruskal-Wallis, followed by Dunn's multiple comparison test. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).

## Results

### *Determination of exosome diameter and concentration and characterization of specific exosomal membrane markers*

Nanoparticle tracking analysis showed that isolated EVs had a mean diameter of 126 ± 0.9 nm (Figure 1(a)), and that in our conditions of isolation the number of particles obtained was estimated around $1.09 \times 10^{12}$/mL. According to these values, the concentration of administered EVs (100 µg) corresponded to approximately $2 \times 10^{10}$ vesicles. Exosome markers, Alix, CD9 and CD63, were revealed by Western blot analysis on the final pellet resulting from the last centrifuge obtained from a sample of plasmatic exosomes of RLIP animals (Figure 1(a)).

### *Isolated exosomes obtained from plasma of remote post-conditioned rats icv administered to ischemic rats reduced brain damage and improved general and focal neurological deficits*

To evaluate the effects of exosome administration on ischemic brain damage, exosomes obtained from plasma of RLIP rats, were intracerebroventricularly administered 24 hours before ischemia induction and 10 min after reperfusion. The analysis of the ischemic volume by TTC staining showed a significant reduction (65%) in the ischemic volume of animals treated with exosomes obtained from plasma of remote post-conditioned rats compared with the vehicle treated group (Figure 1(b)). Accordingly, the reduction in ischemic volume was also accompanied by a parallel improvement of both general and focal neurological deficits (Figure 1(c)). By contrast, exosomes obtained from plasma of rats exposed to FAO or tMCAO failed to confer protection when intracerebroventricularly injected to ischemic rats (Supplementary Figure S1). To evaluate whether the protective effects of exosomes on ischemic brain damage was maintained also when systemically administered in a more realistic therapeutic time window, exosomes obtained from plasma of animals exposed to remote post-conditioning, were ip or in administered 100 minutes and 280 minutes after tMCAO. The analysis of the ischemic volume by TTC staining showed a significant reduction (65%) in the ischemic volume of animals treated with exosomes obtained from plasma of remote post-conditioned rats compared with the vehicle treated group (Figure 2(a)). Accordingly, the decrease in ischemic volume was also accompanied by a parallel improvement of both general and focal neurological deficits (Figure 2(c)). More interestingly, exosomes continued to confer protection also 3 days after ischemia induction. Indeed, a significant decrease in ischemic volume and a parallel improvement of both general and focal neurological deficits was observed in animals intranasally administered with exosomes, 100 minutes and 280 minutes after tMCAO, and evaluated 72 hours after ischemia induction (Figure 3).

### *miR702-3p and miR423-5p were the two miRNAs upregulated in protective exosomes obtained from RLIP as compared to exosomes obtained from FAO or tMCAO rats*

In order to identify some of the microRNAs responsible for the protection exerted by RLIP-induced exosomes, a miRNA expression profiling of circulating exosomes obtained after RLIP, FAO or tMCAO ischemic conditions was performed. The whole miRNome of rats was analyzed in order to discriminate, among miRNAs, those having an opposite trend of expression in the three RLIP, FAO or tMCAO experimental ischemic groups. In particular, 1218 miRNAs were assessed by microarray analysis in plasmatic exosomes isolated from rats subjected to the neuroprotection elicited by RLIP and were compared to all 1218 miRNAs of exosomes isolated from rats exposed to FAO or to tMCAO (Figure 4). Results showed that, when the RLIP-derived exosomes were compared to exosomes derived from FAO, 15 miRNAs were upregulated and 9 miRNAs were downregulated (Figure 4(a)). On the other hand, when the RLIP-derived exosomes were compared to exosomes derived from tMCAO, 8 miRNAs were upregulated and 22 miRNAs were downregulated (Figure 4(b)). Interestingly, miR-702-3p and miR-423-5p were the only two miRNAs significantly upregulated in RLIP-derived exosomes when compared both to FAO-derived exosomes and to tMCAO-derived exosomes. In particular, the fold change of the first miRNA, miR-702-3p, was 1.95 vs FAO and 1.92 vs tMCAO, and the fold change of the other upregulated miRNA, miR-423-5p, was 2.47 vs FAO and 1.92 vs tMCAO. These results suggest a possible involvement of these two miRNAs in the neuroprotective effect of RLIP-derived exosomes.

### *miR-702-3p and miR-423-5p are upregulated in temporoparietal cortex of rats exposed to tMCAO and administered with RLIP-derived protective exosomes*

In order to assess that the administration of RLIP-derived exosomes effectively increased the brain levels of the two selected miRNAs, miR-702-3p and miR-423-5p, both in control and in tMCAO rats intracerebroventricularly infused with vehicle or RLIP-derived exosomes, double fluorescence in situ hybridization was performed. In particular, in tissue slices from ipsilateral temporoparietal cortex, FISH staining revealed that, after RLIP-derived exosome treatment, both sham-operated and ischemic rats showed an increased in miR-423-5p (Figure 5(a)) and miR-702-3p (Figure 5 (b)) fluorescent signal.

### *NOD1 and NLRP3, two biomarkers of neuroinflammation and targets of miR-702-3p and miR-423-5p, were downregulated in temporoparietal cortex of rats exposed to tMCAO and administered with RLIP-derived protective exosomes*

In order to evaluate whether NOD1 and NLRP3, two key triggers of neuroinflammation and neuronal death and already known as targets of the two selected

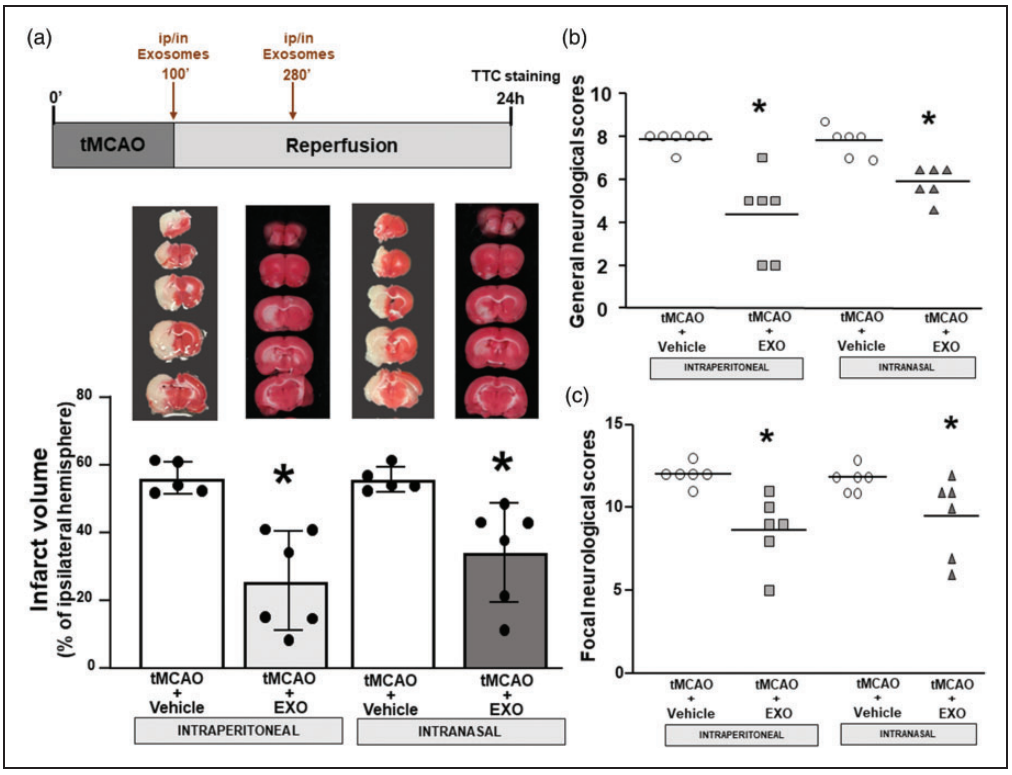



**Figure 2.** Evaluation of ischemic damage and neurological scores 24 hours after tMCAO in rats intraperitoneally and intranasally administered with plasmatic exosomes obtained by RLIP rats. (a) Evaluation of ischemic damage 24 hours after tMCAO in ischemic rats intraperitoneally and intranasally treated (twice, at reperfusion and 3 h later) with exosomes (400 μg/Kg) isolated from plasma of rats exposed to RLIP + 1.5 h reperfusion (b–c). Evaluation of general and focal neurological scores in the same groups. Neurological scores were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test. *$p < 0.05$ versus ischemic rats treated with vehicle. $n = 5$–6 animals per group. Each column represents the mean ± SD. *$p < 0.05$ versus ischemic rats treated with vehicle.

miRNAs, miR-702-3p and miR-423-5p, were downregulated in the brain after icv treatment with RLIP-derived exosomes, immunohistochemical analysis was performed on ipsilesional temporoparietal cortex of ischemic rats treated with vehicle or exosomes and compared to sham-operated animals. Confocal microscopy analysis showed that the upregulation of NOD1 and NLRP3 signal observed in ischemic rats treated with vehicle was prevented in animals administered with RLIP-derived exosomes (Figures 6 and 7). Interestingly, the downregulation of NOD1 and NLRP3 paralleled with the upregulation of the two corresponding miRNAs, miR-702-3p and miR-423-5p, recorded in the same cortical region after exosome administration.

## Discussion

The results of the present study demonstrated for the first time that exosomes spontaneously released in plasma of rats protected by remote postconditioning may transfer a neuroprotective humoral signal to the brain of ischemic animals, thus representing a potentially translatable and successful therapeutic strategy in a preclinical model reproducing cerebral ischemia.

Interestingly, only exosomes derived from remote postconditioned rats were protective. This may be explained by the fact that the cooperation of such neural and humoral pathways activated by the conditioning stimulus and the biochemical response activated in the ischemic brain, may induce a general cellular reprogramming which in turn may influence the total exosome content. The analysis of miRNA signature revealed a different content in RLIP-derived protective exosomes compared to FAO-derived and tMCAO-derived non-protective exosomes thus suggesting the possible involvement of miRNAs in transferring the neuroprotective signal. In particular, to finely dissect the protective role mediated by miRNAs, we focused our attention to those miRNAs resulting upregulated in RLIP-derived exosomes. Our findings suggest that some miRNAs might be loaded in exosomes after the

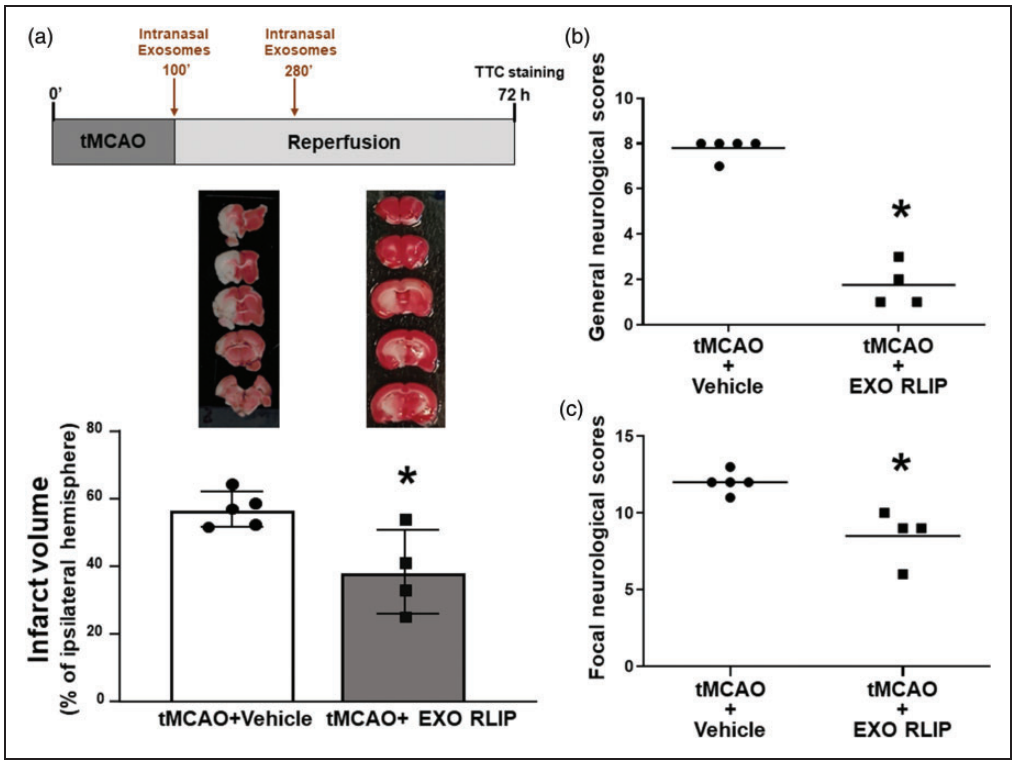
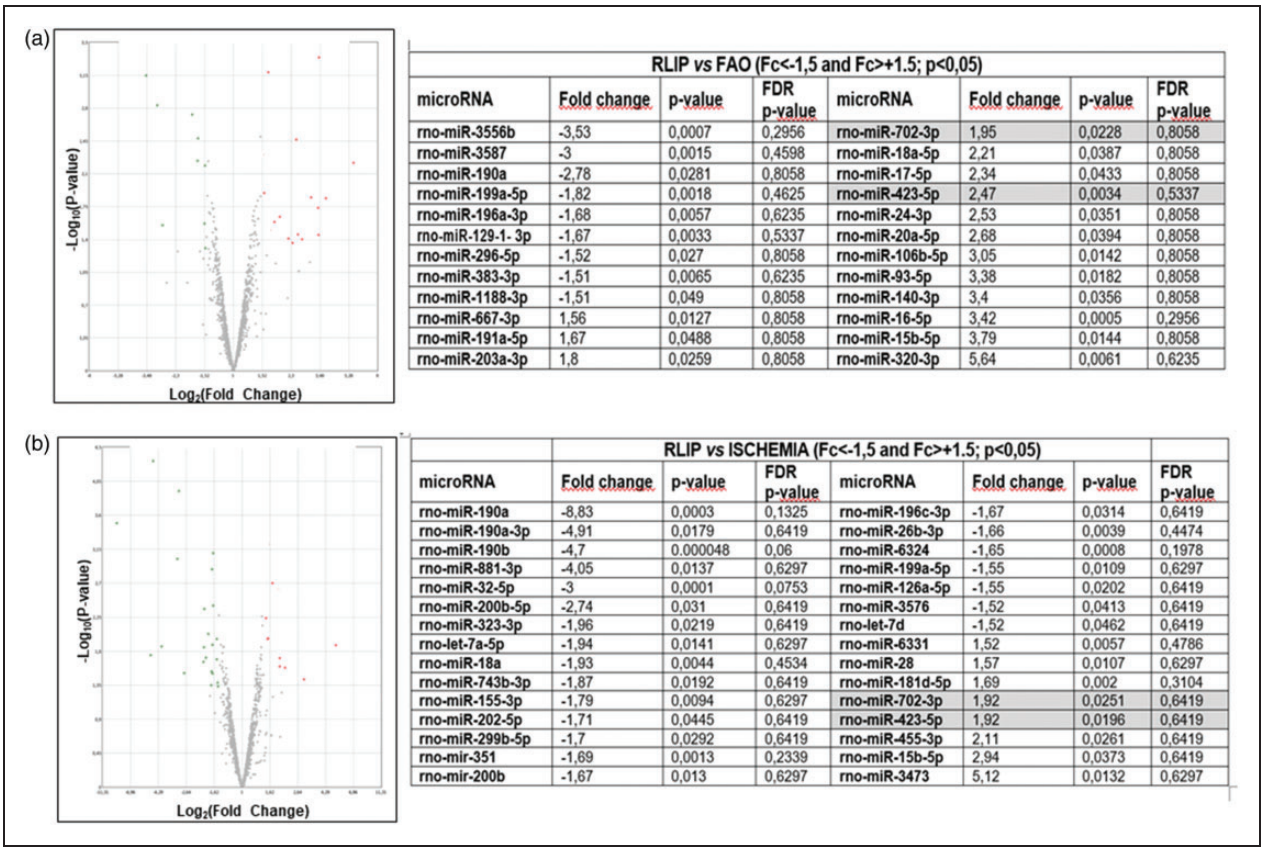



**Figure 3.** Evaluation of ischemic damage and neurological scores 3 days after tMCAO in rats intranasally treated with plasmatic exosomes obtained by RLIP rats. Evaluation of ischemic damage 3 days after tMCAO in ischemic rats intranasally treated (twice, at reperfusion and 3 h later) with exosomes (400 μg/Kg) isolated from plasma of rats exposed to RLIP + 1.5h reperfusion (b–c). Evaluation of general and focal neurological scores in the same groups. Neurological scores were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test. Each column represents the mean. *$p < 0.05$ versus ischemic rats treated with vehicle. n = 4–5 animals per group.

RLIP induction and may take part to the transfer of a protective message. Among these differentially expressed miRNAs, miR-702-3p and miR-423-5p resulted the only two microRNAs upregulated in RLIP-derived neurobeneficial exosomes compared to both FAO-derived and tMCAO-derived non-protective exosomes. Notably, the levels of these two specific miRNA were upregulated in the brain of animals exposed to exosomes derived from remote postconditioned animals, thus supporting our working hypothesis. Intriguingly, these two miRNAs are well known to mediate protection by modulating neuroinflammation-linked pathways. In particular, recent evidence demonstrated that miR-423-5p suppressed the activation of the inflammasome complex of NLR family pyrin domain containing 3 (NLRP3) by binding to the NLRP3 3′UTR and that upregulation of miR-423-5p could prevent microglia activation from lipopolysaccharide-induced cell damage by targeting NLRP3.[23] In this way, miR-423-5p may prevent the activation of cytoplasmic inflammasome complexes which represents an essential step in neuroinflammation and a key trigger for neuronal death. Interestingly, NLRP3 inflammasome in neurons has been found involved also in driving neuroinflammation in a model of acute ischemic stroke. Notably, early blockade of NLRP3 by miR-423-5p upregulation protects from ischemic injury and mitigates inflammation and stabilizes the blood–brain barrier.[23]

Similarly, up-regulation of miR-702-3p ameliorated inflammatory injury by targeting the nucleotide-binding oligomerization domain 1 (NOD1), a member of the NOD-like receptor (NLR) family,[24] an intracytoplasmic immune receptor driving the innate immune response and triggering a cascade of inflammatory signalling events upon sensing pathogens or tissue injury. It has recently been shown that NOD1 inhibition is protective in a model of hemorrhagic brain injury and that this protective effect may result from suppression of the microglia-induced inflammatory response. NOD1 is involved in the pathogenesis of hypoxic-ischemic (HI)-induced brain injury through modulation of autophagy-related proteins and inflammatory responses. In particular, HI injury significantly increases mRNA levels of NOD1 and autophagy-related genes. NOD1 inhibition following HI induction suppressed autophagy signaling as well as HI-induced proinflammatory cytokine production. Importantly, pharmacological NOD1




RLIP *vs* FAO (Fc<-1,5 and Fc>+1.5; p<0,05)

| microRNA | Fold change | p-value | FDR p-value | microRNA | Fold change | p-value | FDR p-value |
|---|---|---|---|---|---|---|---|
| rno-miR-3556b | -3,53 | 0,0007 | 0,2956 | rno-miR-702-3p | 1,95 | 0,0228 | 0,8058 |
| rno-miR-3587 | -3 | 0,0015 | 0,4598 | rno-miR-18a-5p | 2,21 | 0,0387 | 0,8058 |
| rno-miR-190a | -2,78 | 0,0281 | 0,8058 | rno-miR-17-5p | 2,34 | 0,0433 | 0,8058 |
| rno-miR-199a-5p | -1,82 | 0,0018 | 0,4625 | rno-miR-423-5p | 2,47 | 0,0034 | 0,5337 |
| rno-miR-196a-3p | -1,68 | 0,0057 | 0,6235 | rno-miR-24-3p | 2,53 | 0,0351 | 0,8058 |
| rno-miR-129-1- 3p | -1,67 | 0,0033 | 0,5337 | rno-miR-20a-5p | 2,68 | 0,0394 | 0,8058 |
| rno-miR-296-5p | -1,52 | 0,027 | 0,8058 | rno-miR-106b-5p | 3,05 | 0,0142 | 0,8058 |
| rno-miR-383-3p | -1,51 | 0,0065 | 0,6235 | rno-miR-93-5p | 3,38 | 0,0182 | 0,8058 |
| rno-miR-1188-3p | -1,51 | 0,049 | 0,8058 | rno-miR-140-3p | 3,4 | 0,0356 | 0,8058 |
| rno-miR-667-3p | 1,56 | 0,0127 | 0,8058 | rno-miR-16-5p | 3,42 | 0,0005 | 0,2956 |
| rno-miR-191a-5p | 1,67 | 0,0488 | 0,8058 | rno-miR-15b-5p | 3,79 | 0,0144 | 0,8058 |
| rno-miR-203a-3p | 1,8 | 0,0259 | 0,8058 | rno-miR-320-3p | 5,64 | 0,0061 | 0,6235 |

RLIP *vs* ISCHEMIA (Fc<-1,5 and Fc>+1.5; p<0,05)

| microRNA | Fold change | p-value | FDR p-value | microRNA | Fold change | p-value | FDR p-value |
|---|---|---|---|---|---|---|---|
| rno-miR-190a | -8,83 | 0,0003 | 0,1325 | rno-miR-196c-3p | -1,67 | 0,0314 | 0,6419 |
| rno-miR-190a-3p | -4,91 | 0,0179 | 0,6419 | rno-miR-26b-3p | -1,66 | 0,0039 | 0,4474 |
| rno-miR-190b | -4,7 | 0.000048 | 0,06 | rno-miR-6324 | -1,65 | 0,0008 | 0,1978 |
| rno-miR-881-3p | -4,05 | 0,0137 | 0,6297 | rno-miR-199a-5p | -1,55 | 0,0109 | 0,6297 |
| rno-miR-32-5p | -3 | 0,0001 | 0,0753 | rno-miR-126a-5p | -1,55 | 0,0202 | 0,6419 |
| rno-miR-200b-5p | -2,74 | 0,031 | 0,6419 | rno-miR-3576 | -1,52 | 0,0413 | 0,6419 |
| rno-miR-323-3p | -1,96 | 0,0219 | 0,6419 | rno-let-7d | -1,52 | 0,0462 | 0,6419 |
| rno-let-7a-5p | -1,94 | 0,0141 | 0,6297 | rno-miR-6331 | 1,52 | 0,0057 | 0,4786 |
| rno-miR-18a | -1,93 | 0,0044 | 0,4534 | rno-miR-28 | 1,57 | 0,0107 | 0,6297 |
| rno-miR-743b-3p | -1,87 | 0,0192 | 0,6419 | rno-miR-181d-5p | 1,69 | 0,002 | 0,3104 |
| rno-miR-155-3p | -1,79 | 0,0094 | 0,6297 | rno-miR-702-3p | 1,92 | 0,0251 | 0,6419 |
| rno-miR-202-5p | -1,71 | 0,0445 | 0,6419 | rno-miR-423-5p | 1,92 | 0,0196 | 0,6419 |
| rno-miR-299b-5p | -1,7 | 0,0292 | 0,6419 | rno-miR-455-3p | 2,11 | 0,0261 | 0,6419 |
| rno-mir-351 | -1,69 | 0,0013 | 0,2339 | rno-miR-15b-5p | 2,94 | 0,0373 | 0,6419 |
| rno-mir-200b | -1,67 | 0,013 | 0,6297 | rno-miR-3473 | 5,12 | 0,0132 | 0,6297 |

**Figure 4.** Microarray analysis of RLIP-derived protective exosomes vs FAO-derived or tMCAO-derived non-protective exosomes. Left panels: Volcano plot analysis showing the comparison of miRNA levels of RLIP-derived exosomes versus FAO-derived or tMCAO-derived exosomes. The y-axis corresponds to the mean expression value of log10 (p-value), and the x-axis displays the log2 fold change value. The blue dots represent the miRNA levels decreased in RLIP exosomes vs FAO-derived (a) or vs tMCAO-derived (b) exosomes and the red dots represent the miRNA upregulated in RLIP exosomes vs FAO or tMCAO. Right panels: List of differentially expressed microRNAs in plasma exosomes isolated from rats subjected to remote limb ischemic postconditioning versus plasma exosomes isolated from rats subjected to FAO (a) or tMCAO (b), with respective fold change, p-values and adjusted p-values (FDR p-values); n = 3 for each experimental group.

inhibition after HI improved long-term cognitive function, without impacting exploratory and locomotor activities.[24] Accordingly with the evidence supporting the involvement of these proteins in the evolution of brain ischemic damage, it should be underlined that, in our model, wherever we found an increase in miR-423-5p and of miR-702-3p levels in the ipsilesional temporoparietal cortex 24 hours after RLIP-derived exosomes administration, we also found a downregulation of NOD1 and NLRP3 protein levels in the same cortical region. Therefore, since after exosome administration miR-423-5p and of miR-702-3p levels significantly increase in brain it is possible to speculate that exosomes are effectively able to release protective miRNAs, and take part to neuroprotective effect induced by remote postconditioning by preventing the upregulation of neurotoxic pathways, such as NOD1 and NLRP3, occurring after ischemia. On the other hand, in support of our hypothesis that the different content in exosomal miRNA may contribute to the different effect of these vesicles on ischemic brain damage, it is important to underline that the results of the present study demonstrated that miR-let-7a-5p, that has been previously reported to be related to stroke progression,[25] was significantly downregulated in plasmatic RLIP-derived exosomes compared to tMCAO-derived exosomes. This finding is in accordance with our previous study showing that RLIP prevented the upregulation of miR-let-7a-5p levels in brain tissue induced by tMCAO, and that, more interestingly, exogenous miR-let-7a-5p administration prevented remote-induced neuroprotection.[22]

Although our findings support the hypothesis that exosomes may exert their protective effect by transferring miRNAs, we cannot exclude that, beyond miRNAs, other regulatory non-coding RNAs or exosomal proteins might participate to the neuroprotective action mediated by remote conditioning-derived exosomes. At the same time, we cannot rule out the

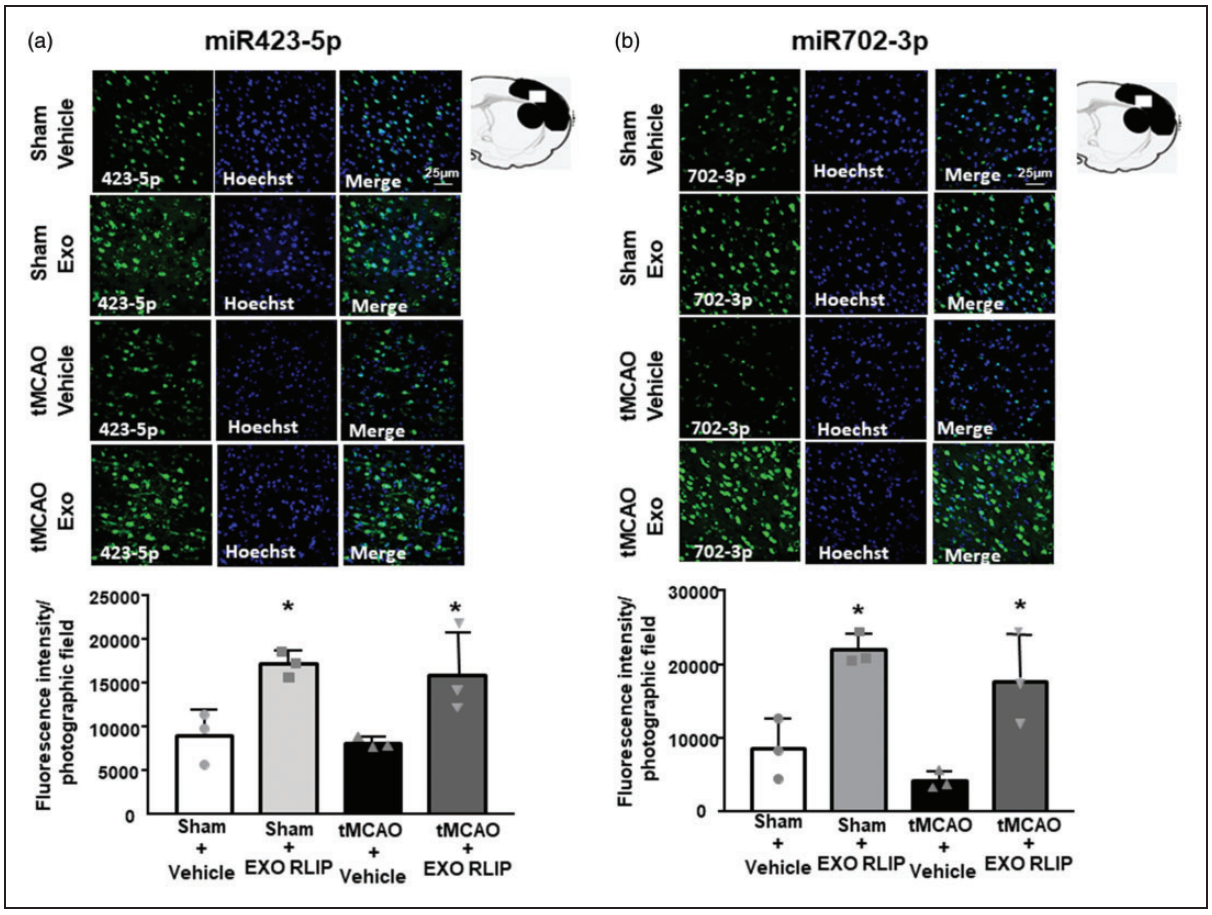



**Figure 5.** Effect of RLIP-derived protective exosomes administration on miR-423-5p and miR-702-3p expression in ipsilesional temporoparietal cortex. (a) Confocal microscopic images displaying miR-423-5p in green, Hoechst in blue and Merge in yellow in the ipsilateral temporoparietal cortex of rats subjected to sham-operation or tMCAO induction and treated with vehicle or exosomes examined after 24 hours reperfusion. A representative brain slice cartoon indicating the area of interest is on the right of the figure. Scale bars 25 μm. Fluorescent intensity of miR-423-5p labeling was performed by NIH image software. The intensity of miR-423-5p signal was expressed in arbitrary units. n = 3 animals per group. Each column represents the mean ± SD. *$p < 0.05$ versus sham operated rats treated with vehicle and (b) confocal microscopic images displaying miR-702-3p in green, Hoechst in blue and Merge in yellow in the ipsilateral temporoparietal cortex of rats subjected to sham-operation or tMCAO induction and treated with vehicle or exosomes examined after 24 hours reperfusion. A representative brain slice cartoon indicating the area of interest is on the right of the figure. Scale bars 25 μm. Fluorescent intensity of miR-702-3p labeling was performed by NIH image software. The intensity of miR-702-3p signal was expressed in arbitrary units. n = 3 animals per group. Each column represents the mean ± SD. *$p < 0.05$ versus sham operated rats treated with vehicle.

hypothesis that other targets of the two selected miRNAs and different from NOD1 and NLRP3 are also involved in exosome-mediated neuroprotection. However, the evidence that exosomes spontaneously released after a conditioning stimulus are able to transfer neuroprotection confers relevance to the present paper, more than the identification of a specific pathway involved in the protection, also because exosomes may act on multiple pathways, and this aspect renders difficult to explain their effect through just one of their single components. Our hypothesis is in line with recent studies carried out in in vitro and in vivo models of cardiac ischemic injury, where it has been demonstrated that circulating exosomes may transfer a protective message through the release of miRNAs.[26,27] Furthermore, a growing body of experimental evidence demonstrated that exosomes derived from either stem cells or other cells exert protective and restorative effects in in vivo models of brain ischemia.[28] Interestingly, it should be underlined that also in pathological conditions different from ischemia, such as diabetic peripheral neuropathy or chemotherapy-induced peripheral neuropathy, systemic exosome administration has been successfully used as therapeutic strategy.[29,30] On the other hand, recent evidence support the idea that protective treatments for stroke, such as physical exercise, could also induce a change in exosome content.[31]

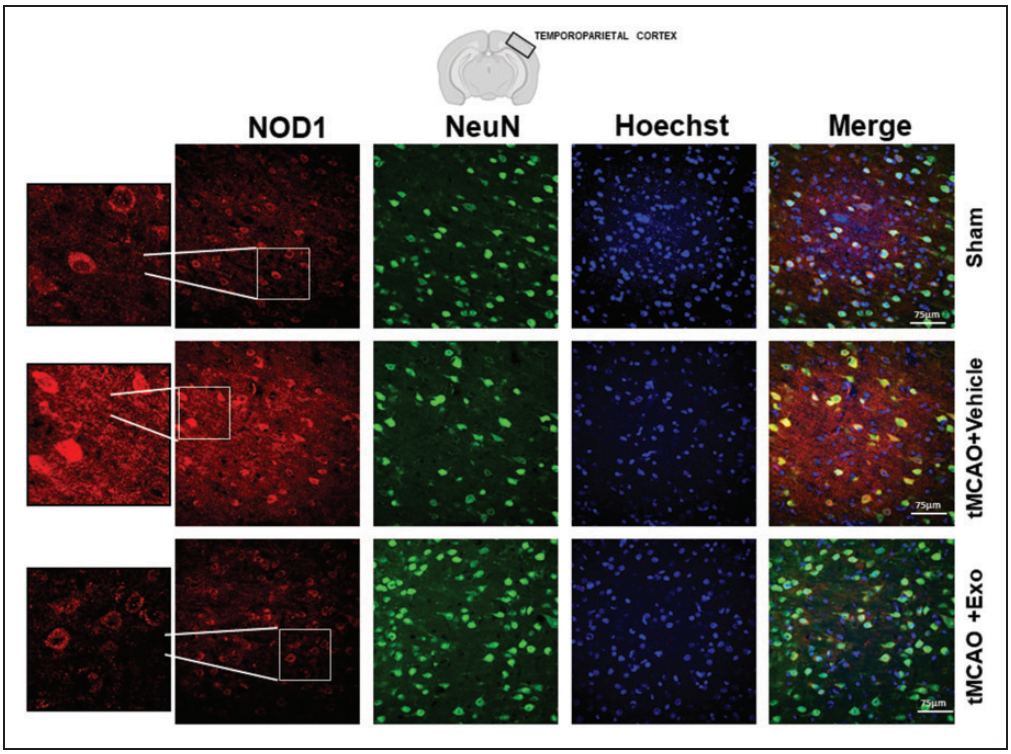



**Figure 6.** Effect of RLIP-derived protective exosomes administration on NOD1 and NLRP3 immunosignal in ipsilesional temporoparietal cortex. Confocal double labeling experiments displaying the co-expression of NOD1 (red) with the neuronal marker NeuN (green) and hoechst (blue) in the ipsilateral temporoparietal cortex of sham-operated animals and ischemic animals treated with vehicle or RLIP-derived protective exosomes. Scale bars 75 μm. High magnification of NOD1 expression was on the left.

Noticeably, the exosome neuroprotective action in our animal models of brain ischemia displayed some other relevant pharmacological aspects that ought to be emphasized for their potential clinical perspectives. First of all, its effectiveness through a systemic route of administration, which reinforces the possibility of its clinical applicability. Intriguingly, the idea to start from spontaneously plasma-released exosomes in response to a conditioning stimulus as a therapeutic strategy, represents a useful innovation in the context of miRNA-based therapy and gives relevance to the present paper. Increasing evidence has showed that exosomes may include advantages on both synthetic lipid nanoparticles and cell-mediated drug delivery, avoiding the rapid clearance and toxicity associated with synthetic vesicles, as well as the complexity in utilizing cell-mediated therapeutics delivery systems in clinic. Interestingly, it should be underlined that RLIP-derived exosomes continued to confer protection also 3 days after ischemia induction, as revealed by the amelioration of neurological scores and by the reduction of the ischemic damage observed after 72 hours, thus supporting the idea that the protective effect was persistent. The rationale for the long term evaluation of exosome effect through intranasal route resides in the fact that this route may be considered, among the examined routes, the less invasive and the most translatable for clinical application.

Collectively, the results of the present study revealed exosomes as a possible promising tool for a successful therapeutic intervention during cerebral ischemia and as an attractive option to use them as a delivery vehicle of neuroprotection for stroke treatment.

## Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by grant CN00000041 "National Center for Gene Therapy and Drugs based on RNA Technology" (concession number 1035 of 17 June 2022-PNRR MUR – M4C2 – Investment 1.4 Call "National Centers", financed by EU- NextGenerationEU), code project (CUP) E63C22000940007 to G.P. and O.C. We also acknowledge Fondi FRA 2020 LINEA A EXOREMOTE – CUOMO, to OC and PE Mnesys Spoke 7 SDN IRCCS, to LA.

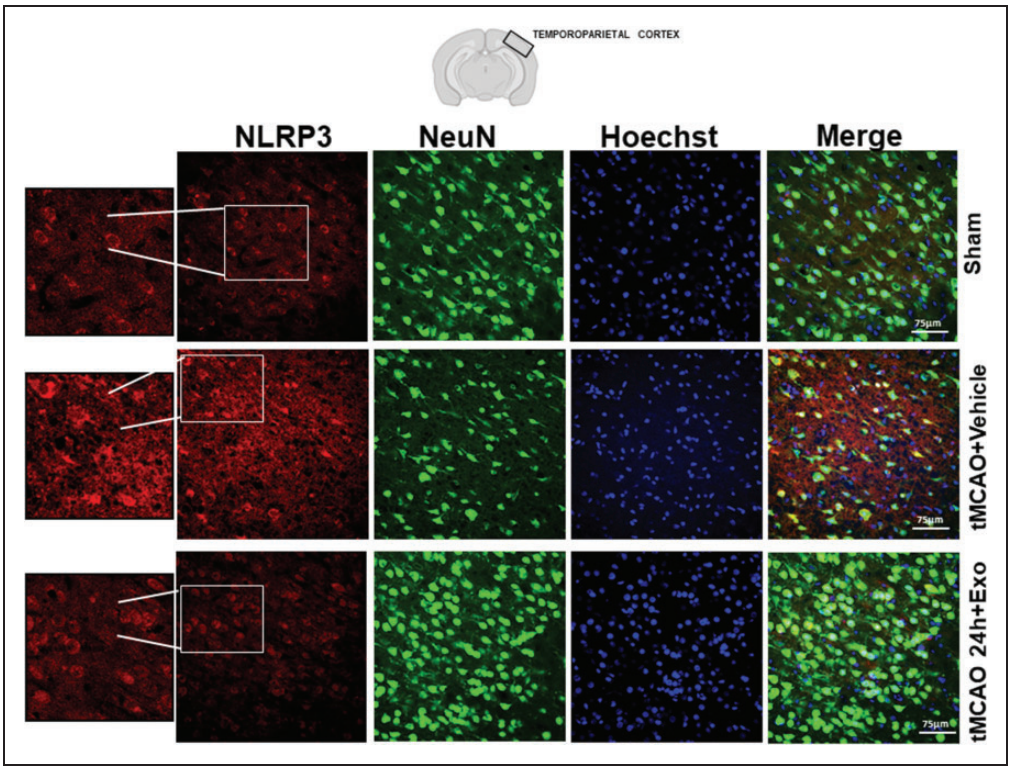



**Figure 7.** Effect of RLIP-derived protective exosomes administration on NLRP3 immunosignal in ipsilesional temporoparietal cortex. Confocal double labeling experiments displaying the co-expression of NLRP3 (red) with the neuronal marker NeuN (green) and hoechst (blue) in the ipsilateral temporoparietal cortex of sham-operated animals and ischemic animals treated with vehicle or RLIP-derived protective exosomes. Scale bars 75 μm. High magnification of NLRP3 expression was on the left.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Authors' contributions

Conceptualization: Cuomo, Pignataro; Methodology: Cuomo, Brancaccio, Anzilotti, Lombardi; Viscardi; Formal analysis and investigation: Cuomo, Cepparulo, Vinciguerra; Formisano Writing – original draft preparation: Cuomo, Pignataro, Annunziato; Funding acquisition: Cuomo, Pignataro, Annunziato; Supervision: Cuomo, Pignataro, Annunziato.

## Supplementary material

Supplemental material for this article is available online.

## ORCID iD

Pignataro Giuseppe https://orcid.org/0000-0002-7290-4397

## References

1. Pignataro G, Esposito E, Sirabella R, et al. nNOS and p-ERK involvement in the neuroprotection exerted by remote postconditioning in rats subjected to transient middle cerebral artery occlusion. *Neurobiol Dis* 2013; 54: 105–114.
2. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; 116: 281–297.
3. Bartel DP. MicroRNAs:target recognition and regulatory functions. *Cell* 2009; 136: 215–233.
4. Shen B, Wu N, Yang JM, et al. Protein target into exosomes/microvesicles by plasmamembrane anchors. *J Biol Chem* 2011a; 286: 14383–14395.
5. Koh W, Sheng CT, Tan B, et al. Analysis of deep sequencing microRNA expression profile from human embryonic stem cells derived mesenchymal stem cells reveals possible role of let-7 microRNA family in downstream targeting of hepatic nuclear factor 4alpha. *BMC Genomics* 2010; 11 Suppl 1: S6.
6. Yeo RW, Lai RC, Zhang B, et al. Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv Drug Deliv Rev* 2013; 65: 336–341.
7. Yu L, Yang F, Jiang L, et al. Exosomes with membrane-associated TGF-beta1 from gene-modified dendritic cells inhibit murine EAE independently of MHC restriction. *Eur J Immunol* 2013; 43: 2461–2472.
8. Xin H, Li Y, Buller B, et al. Exosome-mediated transfer ofmiR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 2012; 30: 1556–1564.
9. Zhang HG and Grizzle WE. Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *Am J Pathol* 2014; 184: 28–41.

10. Zhang L and Wrana JL. The emerging role of exosomes in wnt secretion and transport. *Curr Opin Genet Dev* 2014; 27: 14–19.
11. Wang J, Hendrix A, Hernot S, et al. Bone marrow stromal cell-derived exosomes as communicators in drug resistance in multiple myeloma cells. *Blood* 2014; 124: 555–566.
12. Lv H, Zhang S, Wang B, et al. Toxicity of cationic lipids and cationic polymers in gene delivery. *J Control Release* 2006; 114: 100–109.
13. Clayton A, Turkes A, Dewitt S, et al. Adhesion and signaling by B cell-derived exosomes:the role of integrins. *Faseb J* 2004; 18: 977–979.
14. Vinciguerra A, Formisano L, Cerullo P, et al. MICRORNA-103-1 selectively downregulates brain NCX1 and its inhibition by antimirna ameliorates stroke damage and neurological deficits. *Mol Ther* 2014; 22: 1829–1838.
15. Tang YT, Huang YY, Zheng L, et al. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. *Int J Mol Med* 2017; 40: 834–844.
16. Wang X, Zhou Y, Gao Q, et al. The role of exosomal microRNAs and oxidative stress in neurodegenerative diseases. *Oxid Med Cell Longev* 2020; 2020: 3232869.
17. Cuomo O, Pignataro G, Sirabella R, et al. Sumoylation of LYS590 of NCX3 f-Loop by SUMO1 partecipates in brain neuroprotection induced by ischemic preconditioning. *Stroke* 2016; 47: 1085–1093.
18. Cuomo O, Cepparulo P, Anzilotti S, et al. Anti-miR-223-5p ameliorates ischemic damage and improves neurological function by preventing NCKX2 downregulation after ischemia in rats. *Mol Ther Nucleic Acids* 2019; 18: 1063–1071.
19. Xin H, Li Y, Liu Z, et al. MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 2013; 31: 2737–2746.
20. Guida N, Laudati G, Mascolo L, et al. MC1568 inhibits Thimerosal-Induced apoptotic cell death by preventing HDAC4 up-regulation in neuronal cells and in rat prefrontal cortex. *Toxicol Sci* 2016; 154: 227–240.
21. Anzilotti S, Brancaccio P, Simeone G, et al. Preconditioning, induced by sub-toxic dose of the neurotoxin L-BMAA, delays ALS progression in mice and prevents na+/Ca2+ exchanger 3 downregulation. *Cell Death Dis* 2018; 9: 206.
22. Vinciguerra A, Cepparulo P, Anzilotti S, et al. Remote postconditioning ameliorates stroke damage by preventing let-7a and miR-143 up-regulation. *Theranostics* 2020; 10: 12174–12188.
23. Cheng J, Hao J, Jiang X, et al. Ameliorative effects of miR-423-5p against polarization of microglial cells of the M1 phenotype by targeting a NLRP3 inflammasome signaling pathway. *Int Immunopharmacol* 2021; 99: 108006.
24. Liu X, Ma H, Fei L, et al. HPV-mediated downregulation of NOD1 inhibits apoptosis in cervical cancer. *Infect Agent Cancer* 2020; 15: 6.
25. Wang Z, Liu F, Wang Y, et al. Let-7a gene knockdown protects against cerebral ischemia/reperfusion injury. *Neural Regen Res* 2016; 11: 262–269.
26. Minghua W, Zhijian G, Chahua H, et al. Plasma exosomes induced by remote ischaemic preconditioning attenuate myocardial ischaemia/reperfusion injury by transferring miR-24. *Cell Death Dis* 2018; 9: 320.
27. Lassen TR, Just J, Hjortbak MV, et al. Cardioprotection by remote ischemic conditioning is transferable by plasma and mediated by extracellular vesicles. *Basic Res Cardiol* 2021; 116: 16.
28. Yongfang L, Yaohui T and Guo-Yuan Y. Therapeutic application of exosomes in ischaemic stroke. *Stroke Vasc Neurol* 2021; 6: 483–495.
29. Wang L, Chopp M, Szalad A, et al. Exosomes derived From schwann cells ameliorate peripheral neuropathy in type 2 diabetic mice. *Diabetes* 2020; 69: 749–759.
30. Zhang Y, Li C, Qin Y, et al. Small extracellular vesicles ameliorate peripheral neuropathy and enhance chemotherapy of oxaliplatin on ovarian cancer. *J Extracell Vesicles* 2021; 10: e12073.
31. Wang J, Liu H, Chen S, et al. Moderate exercise has beneficial effects on mouse ischemic stroke by enhancing the functions of circulating endothelial progenitor cell-derived exosomes. *Exp Neurol* 2020; 330: 113325.