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Research Article

A Decrease of Brain MicroRNA-122 Level Is an Early Marker of Cerebrovascular Disease in the Stroke-Prone Spontaneously Hypertensive Rat

Rosita Stanzione,[1] Franca Bianchi,[1] Maria Cotugno,[1] Simona Marchitti,[1] Maurizio Forte,[1] Carla Busceti,[1] Larisa Ryskalin,[2] Francesco Fornai,[1,2] Massimo Volpe,[1,3] and Speranza Rubattu[1,3]

[1]*IRCCS Neuromed, Pozzilli, Isernia, Italy*
[2]*Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy*
[3]*Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University of Rome, S. Andrea Hospital, Rome, Italy*

Correspondence should be addressed to Speranza Rubattu; rubattu.speranza@neuromed.it

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Based on preliminary evidence that highlights microRNA-122 as a contributing factor to stroke pathogenesis, we aimed at assessing its expression level, along with the presence of early signs of cerebrovascular disease, in the brain of stroke-prone spontaneously hypertensive rat (SHRSP), a suitable model of human disease that accelerates stroke occurrence under a high sodium/low potassium (Japanese-style) diet (JD). After one month of JD, before stroke occurrence, brain microRNA-122 level was significantly decreased in SHRSP as compared to the stroke-resistant SHR (SHRSR). At this time, levels of markers of oxidative stress and inflammation, as well as of endothelial integrity and function, apoptosis and necrosis were differently modulated in the brains of JD-fed SHRSP as compared to SHRSR, pointing to a significant activation of all deleterious mechanisms underlying subsequent stroke development in SHRSP. We also showed that miR-122 improved survival of rat endothelial cerebral cells upon stress stimuli (excess NaCl, hydrogen peroxide). Our data suggest that a decrease of brain microRNA-122 level is deleterious and can be considered as an early marker of stroke in the SHRSP. Understanding the mechanisms by which microRNA-122 protects vascular cells from stress stimuli may provide a useful approach to improve preventive and treatment strategies against stroke.

1. Introduction

Stroke, a leading cause of death and disability worldwide, represents a multifactorial disease resulting from the complex interaction of multiple genetic and environmental factors [1, 2]. Hypertension is a major risk factor for stroke [3]. Interestingly, the contributory role of an epigenetic modulation into stroke predisposition has been recently underscored [4]. In particular, microRNAs, a family of important posttranscriptional regulators of gene expression acting on multiple epigenetic levels [5], are expressed in acute ischemic stroke where they appear to play a key contributory role as a main consequence of their vascular effects [6]. Thus, the expression level of few microRNAs can be differently modulated in both experimental models and ischemic stroke patients [6–11]. As a consequence, the potential role of microRNAs as circulating biomarkers for the detection of stroke has been implied [12]. Among others, the microRNA-122 (miR-122) level was significantly reduced in the blood of ischemic rats [8] and in both blood and brain tissues of the middle cerebral artery occlusion (MCAO) rat model [9]. In the latter model, intravenous administration of exogenous miR-122 led to inhibition of proteins involved in inflammation such as intercellular adhesion molecule 1

(ICAM-1) and, most importantly, to reduced brain injury and neurological deficits [9]. Notably, a decrease of miR-122 level was also detected in blood cells of acute ischemic stroke patients [7, 10, 11]. Herein, the involvement of miR-122 on the regulation of several genes and pathways associated with ischemic stroke, including leukocytes activation and thrombus formation, was demonstrated [10].

The stroke-prone spontaneously hypertensive rat (SHRSP) is a suitable model for studies of human disease. The exposure to a high sodium/low potassium Japanese-style diet (JD) accelerates the predisposition to spontaneous stroke leading to 100% phenotype occurrence by the 7th week of treatment [13, 14]. At the end of one month of JD feeding, the molecular mechanisms predisposing to cerebrovascular disease, such as mitochondrial dysfunction, increased oxidative stress and inflammation, and endothelial dysfunction, are already activated [15–17].

Based on the previous evidence linking epigenetics with stroke occurrence, and in order to reinforce the involvement of miR-122 in this pathological condition, we presently tested the hypothesis that a derangement of brain miR-122 expression level could be an early marker of cerebrovascular disease in our animal model. For this purpose, the brain miR-122 level was assessed at the end of one month of JD feeding in SHRSP, before the stroke phenotype occurrence.

2. Materials and Methods

2.1. Animal Study Design. Male inbred SHRSP and stroke-resistant spontaneously hypertensive rats (SHRSR) were used for these studies. Animals were kept at a constant temperature with a 12 hrs day–night cycle, with free access to regular rat chow and water. According to our standardized experimental protocol [13–17], 8 SHRSP and 8 SHRSR were shifted at 6 weeks of age to a stroke permissive diet (JD, Laboratory Dottori Piccioni, Milan, Italy) and 1% NaCl supplemented in the drinking water for 4 weeks. A second group of 8 SHRSP and 8 SHRSR was fed with regular diet (RD) and used as control. At the end of 4 weeks of either treatment, rats were sacrificed by cervical dislocation and the brains were removed for total RNA and protein extraction, as well as for histological analysis. The personnel who performed the laboratory analyses was unaware of the experimental group allocation. All experimental procedures were performed in accordance with our institutional guidelines (DLGS 26/2014), and they were approved by the Neuromed institutional committee for experimental work (OPBA = organization for the animal welfare).

2.2. Brain Molecular Studies

2.2.1. Quantitative RT-PCR of MicroRNA-122 (TaqMan Methodology). In order to assess the brain miR-122 expression level in SHRSP and SHRSR under both JD and RD, tissue total RNA was obtained using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), subjected to DNAse I treatment (Qiagen, Venlo, Netherlands) and subsequently purified using miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA integrity was assessed by denaturing agarose gel electrophoresis and its concentration was verified by using NanoDrop2000c UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA). Then, RT-PCR of miR-122 was performed through the use of a specific TaqMan microRNA assay (Applied Biosystems, Foster City, USA) using miRU87 as control. For this purpose, 1 μg of purified RNA was mixed with 3 μl of NCode VILO miRNA cDNA Synthesis Kit (Life Technologies) and 1x RT-primer (Life Technologies) in a total reaction volume of 20 μl. Reaction was incubated at 16°C for 30 min., 42°C for 30 min, and 85°C for 5 min in a T-100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Then, 2 μl of the RT reaction was added to 1 μl of a specific TaqMan microRNA assay 20x and 10 μl of TaqMan Universal PCR Master Mix II with UNG (Life Technologies) in a 20 μl final volume. RT-PCR was performed using the ViiA 7 Real-Time PCR System with cycling conditions of 95°C for 10 min and followed by 95°C for 15 s and 60°C for 60 s for a total of 40 cycles. Measurements were performed in triplicate. Results were expressed as relative levels of miR-122 under the different experimental conditions.

2.3. Protein Expression Analysis by Western Blot of gp91-phox, NF-kB, ICAM-1, vWF, CD31, eNOS, Caspase-3 (Cleaved Caspase-3), and c-Jun. For this purpose, total proteins were extracted from the brains of SHRSP and SHRSR at the end of either RD or JD treatment. Tissues were weighted and homogenized at 4°C in Triton-X lysis buffer 3 (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM β-glycerophosphate, all reagents purchased from Sigma-Aldrich, Milan, Italy). After 20 min of incubation on ice, samples were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants containing soluble proteins were collected. Protein concentrations were determined by the Bradford method [18]. 60 μg of total proteins were separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham, Piscataway, New Jersey, USA). Nonspecific binding sites were blocked with 5% nonfat dried milk for 2 hrs at room temperature. Membranes were then incubated overnight with the following primary antibodies: anti-gp91-phox (1 : 200 goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-NF-κB p65 (nuclear factor kappa B; 1 : 200 mouse monoclonal antibody; Santa Cruz Biotechnology); anti-eNOS (endothelial nitric oxide synthase; 1 : 200 mouse monoclonal antibody; Cell Signaling Technology, MA, USA); anti-phospho-eNOS (Ser1177) (1 : 200 rabbit polyclonal antibody; Cell Signaling Technology); anti-vWF (von Willebrand Factor); (1 : 200 goat polyclonal antibody; Santa Cruz Biotechnology); anti-ICAM-1 (1 : 200 mouse monoclonal antibody; Santa Cruz Biotechnology); anti CD31 (1 : 200 mouse monoclonal antibody; BD Pharmigen, 2350 CA, USA); anti-c-Jun (1 : 200 mouse monoclonal antibody; Cell Signaling Technology); anti-Caspase-3 (1 : 200 rabbit monoclonal antibody; Cell Signaling Technology); anti-β-actin (1 : 5000 mouse monoclonal antibody; Sigma Aldrich).

Secondary antibodies were goat anti-rabbit IgG-HRP (1 : 5000; Santa Cruz Biotechnology) and goat anti-mouse IgG-HRP (1 : 5000; Santa Cruz Biotechnology). After three washes of 10 min in Tween TBS, signals were revealed with an enhanced chemiluminescence detection system (Luminata Crescendo, Millipore, Darmstadt, Germany) and the immunoreactivity of bands was visualized on a high-performance chemiluminescence apparatus (ChemiDoc MP System, Bio-Rad). Protein bands were scanned and quantified densitometrically. They were finally normalized using β-actin levels.

2.4. Brain Histology. The brains of SHRSP and SHRSR, obtained at the end of either RD or JD treatment, were dissected out and fixed in a solution composed of ethanol (60%), acetic acid (10%), and chloroform (30%) (Carnoy fixative) and embedded in paraffin. For histological analysis, coronal sections (10 μm) were used for staining with hematoxylin/eosin (H/E). Deparaffinized sections were stained with hemotoxylin (Diapath, BG, Italy) for 4 min and then with eosin (Diapath) for 2.5 min. Cerebral blood vessel integrity was assessed under light microscopy using six coronal sections from each brain. H/E stained sections were photographed with a microscope (Zeiss, Jena, Germany) fitted with a digital camera. Analysis of cortical and striatal blood vessels was performed in randomly chosen 63x microscopic fields.

2.5. In Vitro Effects of Mimic miRNA-122 on Rat Brain Microvascular Endothelial Cells Viability. In order to support the in vivo findings and the hypothesis that miR-122 may be involved in cerebrovascular disease pathogenesis, we tested in vitro the ability of miR-122 to maintain cell survival upon two different stress stimuli. For this purpose, 2×10^5 cells/well rat brain microvascular endothelial cells (MEC, Innoprot Bizkaia, Spain) were seeded in precoated fibronectin 6 multiwell plates and cultured in endothelial cell medium (ECM) supplemented with 10% fetal bovine serum, endothelial cell growth supplements, and antibiotics, to reach a 70–80% confluence. Then, different sets of dishes received either medium supplemented with 20 mM NaCl (high-NaCl medium) for 72 hrs or with 200 μM H_2O_2 for 24 hrs. The same stimuli were applied to cell dishes in the presence of exogenous mimic miR-122 overexpression. For the latter purpose, a specific miR-122 mimic (miRvana miRNA mimic, Thermo Fisher, Waltham, USA) was incubated in OPTIMEM (Thermo Fisher) reduced serum medium with a nucleic acid transferring agent (lipofectamine RNAiMAX reagent, Invitrogen, Carlsbad USA) in a final volume of 2 ml/well each for 20 min, following the manufacturer's instructions. Five hours later, the complex was replaced with complete ECM, containing the specific treatment (either 20 mM NaCl or 200 μM H_2O_2). Cells transfected with RNAiMax lipofectamine complex were used as negative control. At the end of each stress exposure, performed both in the absence or in the presence of exogenous mimic miR-122, we assessed the effect of miR-122 on cell viability, apoptosis, and necrosis by fluorescent-activated cell sorting (FACS) (Accuri C6 flow cytometer, BD Biosciences, San Jose, CA, USA), following previously reported procedures (16).

In order to assess the efficiency of mimic miR-122 transfection, levels of miR-122 were measured through RT-PCR, as reported above.

2.6. Statistical Analysis. All values are shown as means ± S.E.M. Statistical analysis of RT-PCR results was performed by one-way ANOVA followed by Bonferroni post hoc test. In the Western blot analyses, the densitometric data comparisons between 2 groups were performed using Student t-test followed by post hoc Mann–Whitney test. For FACS analysis, two-way ANOVA followed by Bonferroni post hoc test was performed. The correlation between miR-122 levels and protein expression levels was conducted by using the Pearson correlation test. Statistical significance was stated at the $P < 0.05$ level. GraphPad Prism (Ver 5.01 GraphPad Software, Inc. La Jolla, CA, USA) statistical software was used for the statistical analysis.

3. Results

3.1. Brain Expression of MicroRNA-122 in SHRSP and SHRSR. At the end of 4 weeks of dietary treatment, miR-122 expression level was significantly increased in the brains of JD- versus RD-fed SHRSR, whereas it was significantly decreased in the brains of JD- versus RD-fed SHRSP (Figure 1(a)), thus reproducing previous findings in other animal models and in humans [7–11].

3.2. Protein Expression of gp91-phox and NF-kB. Next, to verify and confirm that mechanisms of cerebrovascular damage have been already activated at the end of 4 weeks of JD treatment in SHRSP, before stroke occurrence, we evaluated the brain expression level of proteins involved in the oxidative stress and inflammatory processes. In fact, whereas level of gp91-phox was unchanged and that of NF-kB was significantly decreased in the brains of JD- versus RD-fed SHRSR (Figure 1(b)), a significant increase of both gp91-phox and NF-kB was detected in the brains of JD- versus RD-fed SHRSP (Figure 1(c)), confirming previous evidence [16].

3.3. Protein Expression of vWF, ICAM-1, CD31, and eNOS. In parallel, the protein expression level of all markers of endothelial integrity and function was differentially regulated in the brain of the two strains. Notably, vWF and ICAM-1 were previously reported to be modulated in association with miR-122 changes in animal models of ischemic stroke [9]. Our results highlighted a significant increase of both vWF and ICAM-1 in the brains of JD- versus RD-fed SHRSP, whereas levels of vWF were significantly decreased and those of ICAM-1 were unchanged in JD- versus RD-fed SHRSR (Figures 2(a) and 2(b)). Levels of CD31 were also significantly upregulated only in the brain of JD-fed SHRSP (Figure 2(c)). A significant increase of brain expression level of eNOS was observed in JD-fed as compared to RD-fed SHRSR, whereas eNOS failed to increase in JD- versus RD-fed SHRSP, as a hallmark of endothelial dysfunction [19] (Figure 2(d)).

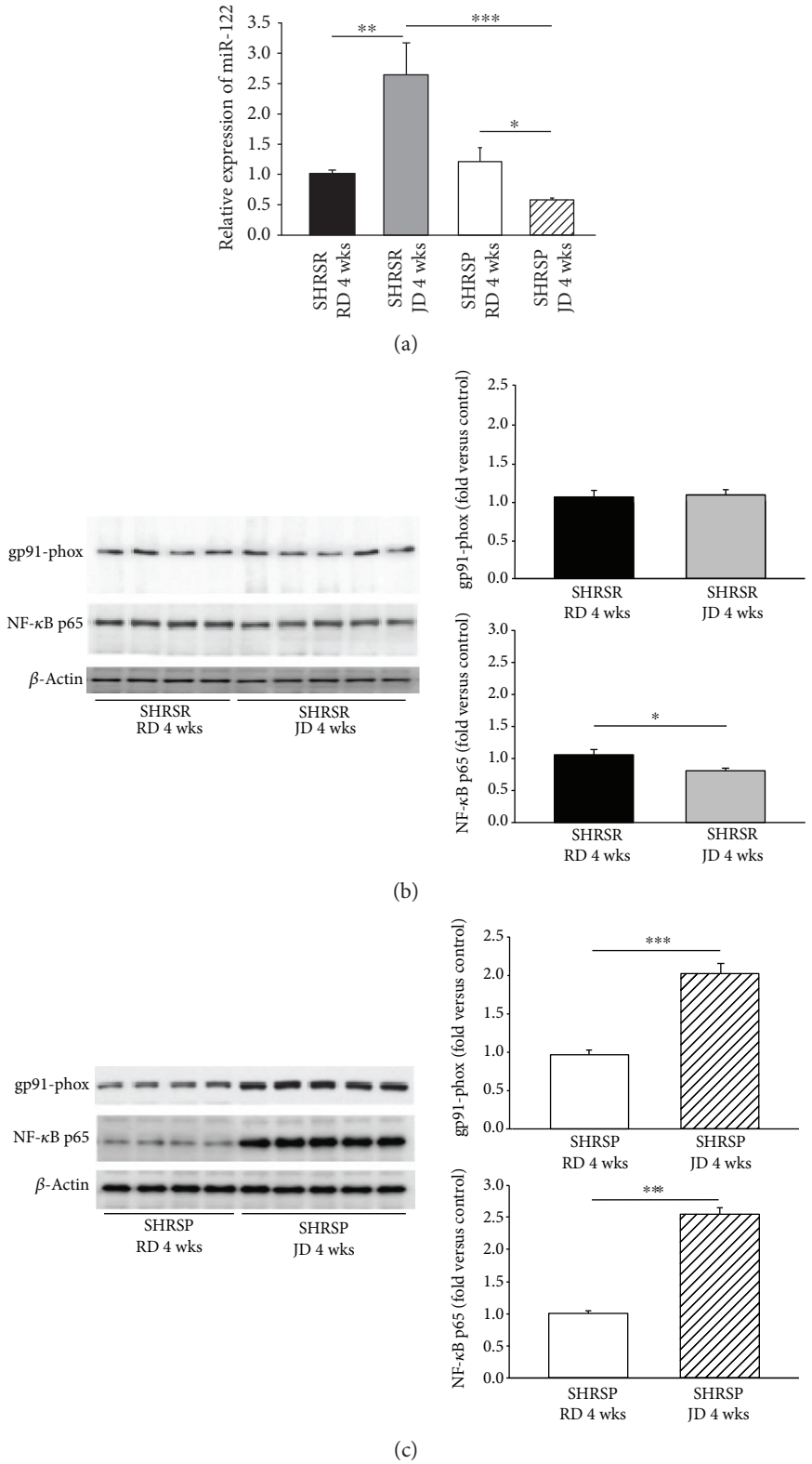
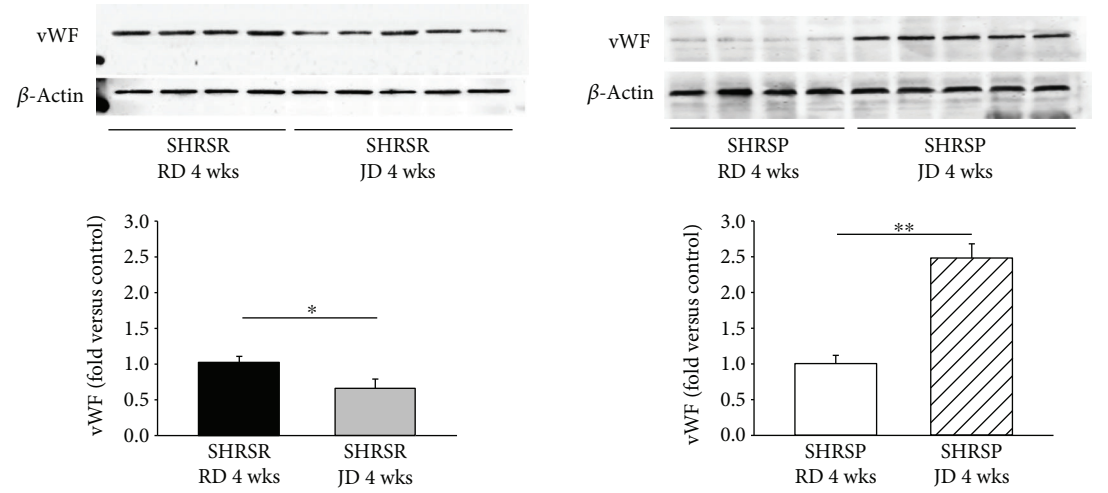
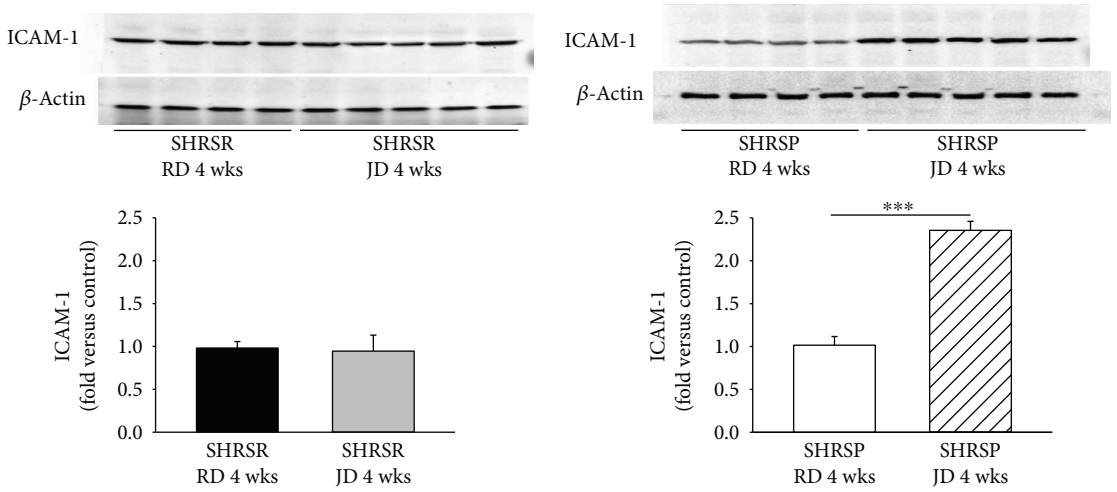


Figure 1: Characterization of brain expression level of microRNA-122, gp 91-phox, and NF-kB protein expression levels in JD-fed versus RD-fed SHRSR and SHRSP strains. (a) miR-122 expression level, as detected by RT-PCR, in the brains of RD- and JD-fed SHRSR and SHRSP; $^{*}p < 0.05$ JD- versus RD-fed SHRSP; $^{**}p < 0.001$ JD- versus RD-fed SHRSR; $^{***}p < 0.0001$ JD-fed SHRSR versus JD-fed SHRSP. (b) Protein expression level of gp91-phox and of NF-kB in SHRSR upon the two diets; $^{*}p < 0.05$ JD- versus RD-fed SHRSR; (c) protein expression level of gp91-phox and of NF-kB in SHRSP upon the two diets. $^{***}p < 0.0001$ JD- versus RD-fed SHRSP. Representative Western blots and corresponding densitometric analysis are shown in panels (a) and (b). JD = high-salt Japanese style diet; RD = regular diet. $N = 8$ for each experimental group.



(a)



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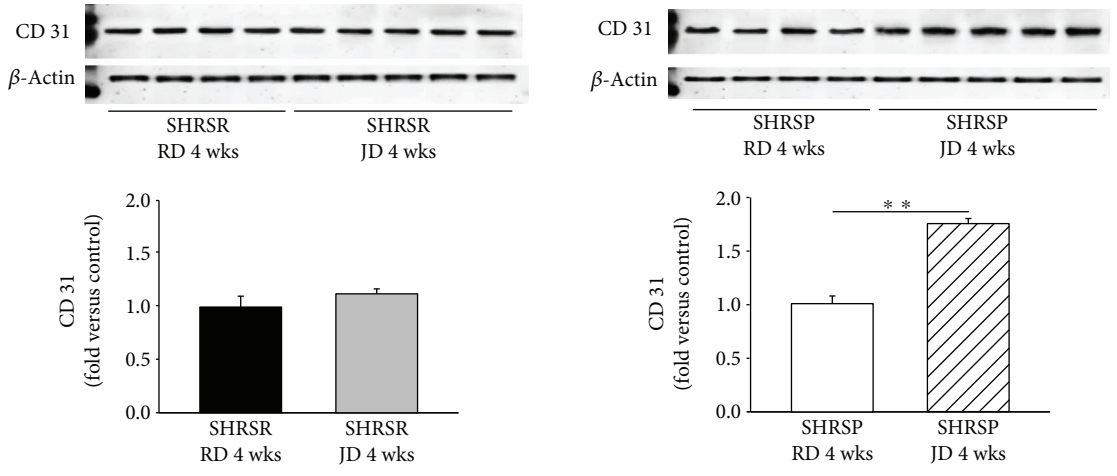
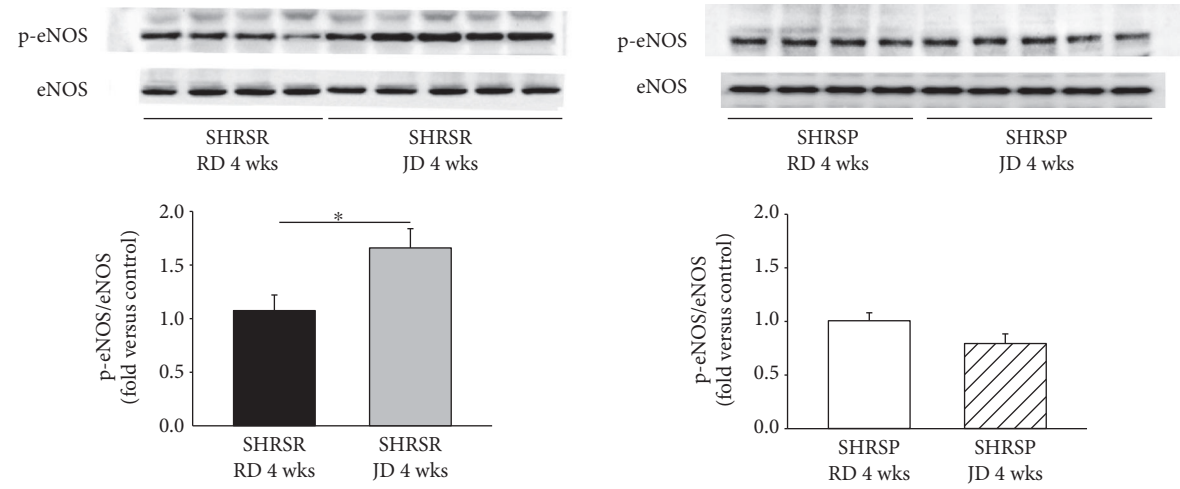


(c)

Figure 2: Continued.



(d)

Figure 2: Characterization of protein expression level of vWF, ICAM-1, CD31, and eNOS in the brains of JD- versus RD-fed SHRSR and SHRSP strains. Representative Western blots with corresponding densitometric analysis are shown for the four indicated proteins in the two strains (SHRSR, left side; SHRSP, right side). (a) vWF expression level; $^{*}p < 0.05$ JD- versus RD-fed SHRSR; $^{**}p < 0.001$ JD- versus RD-fed SHRSP. (b) ICAM-1 expression level; $^{***}p < 0.0001$ JD- versus RD-fed SHRSP. (c) CD31 expression level; $^{**}p < 0.001$ JD- versus RD-fed SHRSP. (d) eNOS and phospho-eNOS expression level; $^{*}p < 0.05$ JD- versus RD-fed SHRSR. JD = high-salt Japanese style diet; RD = regular diet. $N = 8$ for each experimental group.

3.4. Protein Expression of Cleaved Caspase-3 and c-Jun. A significant increase of both cleaved caspase-3 and c-Jun proteins expressions was observed in the brain of JD-fed SHRSP but not in JD-fed SHRSR (Figures 3(a) and 3(b)), pointing to a clear evidence that apoptosis and necrosis were already detectable at this early stage of cerebrovascular disease [13, 14].

3.5. Correlation Analysis between miR-122 and Protein Expression Levels in the Two Strains. The results of the correlation analysis between miR-122 and protein expression levels are shown in Figure 4 (SHRSP strain) and in Figure 5 (SHRSR strain). By performing this statistical test, we were able to obtain a significant linear inverse relationship between miR-122 level and levels of Gp91phox, Nf-kB, vWF, ICAM1, CD31, c-Jun, and caspase 3 in the SHRSP (Figure 4). A significant direct relationship was detected only with levels of p-eNOS in this strain (Figure 4).

On the other hand, levels of miR-122 in the SHRSR positively correlated with CD31 and p-eNOS levels, whereas they negatively correlated with the levels of NF-kB, caspase 3, and vWF (Figure 5).

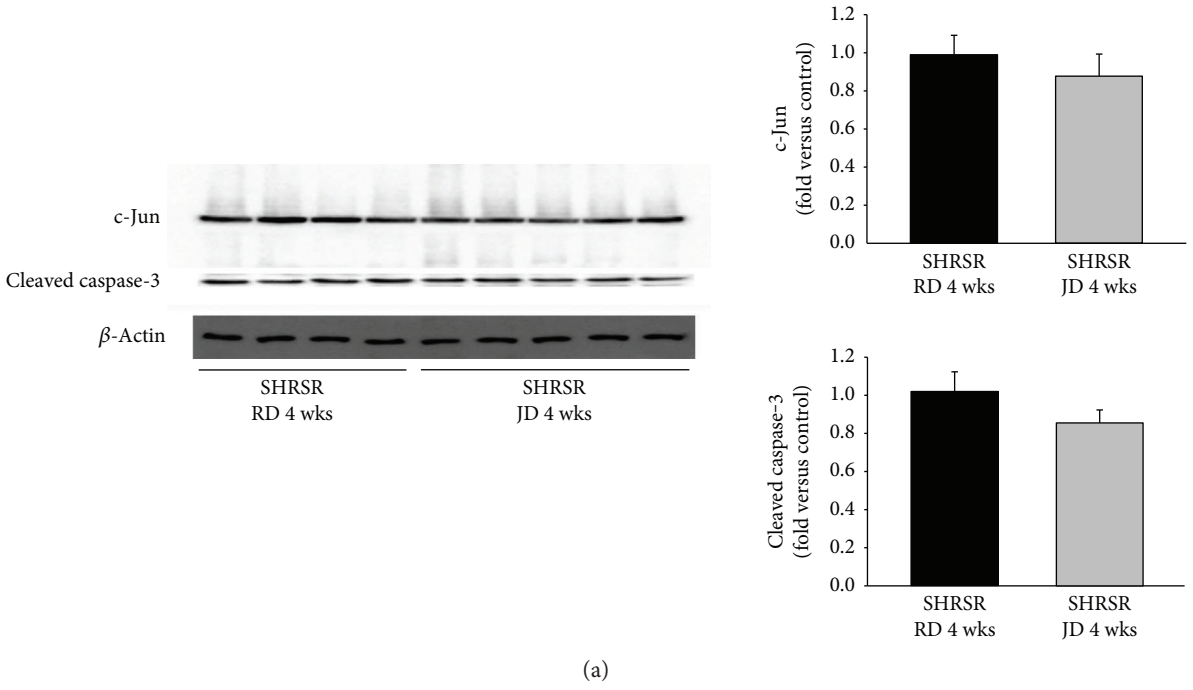
3.6. Brain Histology. Histological analysis of cortical and striatal blood vessels of either strain at the end of 4 weeks of high-salt dietary treatment did not provide evidence of vascular damage. In fact, we detected the presence of intact cerebral blood vessels, without signs of vascular alterations, in all examined experimental groups (Figure 6). These data demonstrate that the considered temporal window (4 weeks of high-sodium/low-potassium Japanese-style dietary treatment) is not characterized by the presence of vascular damage in either strain, confirming previous evidence [13].

3.7. Impact of miRNA122 on Cell Viability, Apoptosis, and Necrosis in MECs. The efficiency of miR-122 mimic transfection was validated by RT-PCR. Levels of miR-122 increased in cells transfected with mimic miR-122 (5.32 ± 0.05 fold changes versus nontransfected cells at 24 hrs; 5.84 ± 0.032 fold changes versus nontransfected cells at 72 hrs).

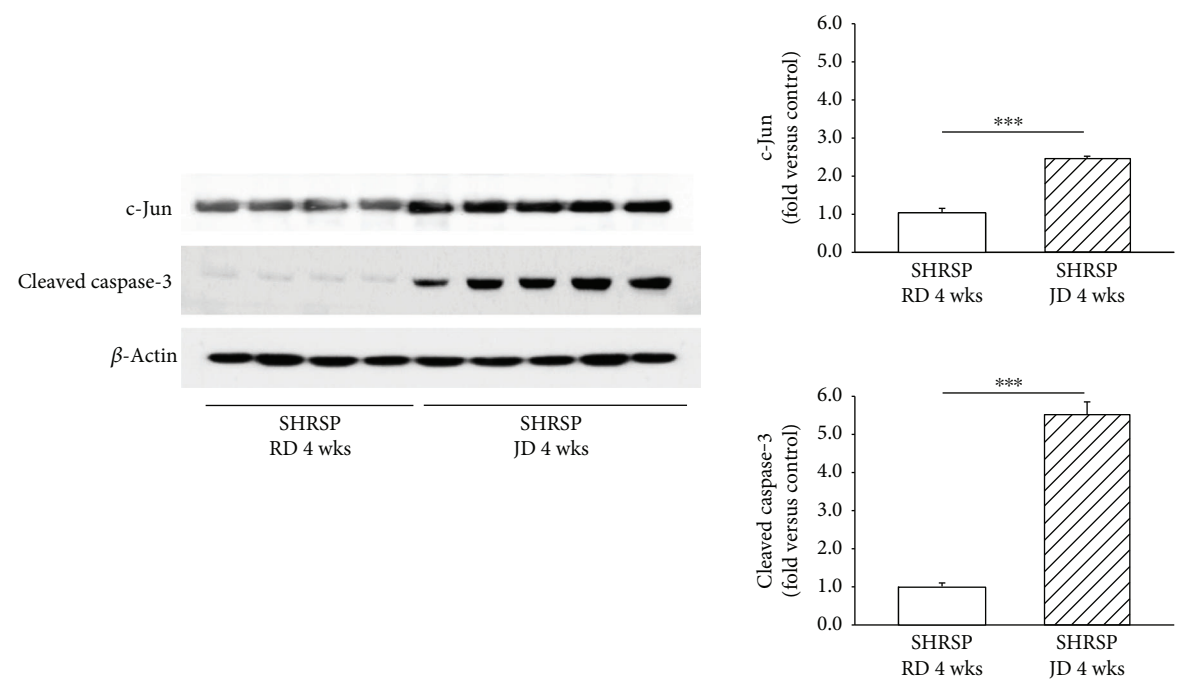
Treatment with either high-NaCl medium (Figures 7(a) and 7(b)) or medium supplemented with 200 μM H_2O_2 (Figures 7(c) and 7(d)) significantly reduced the viability of MECs, with a concomitant increase of cell apoptosis and necrosis. The transfection with mimic miR-122 of MECs exposed to either NaCl or H_2O_2 rescued the cell viability and reduced apoptosis and necrosis in a significant manner.

4. Discussion

In the current study, we pursued the hypothesis that an epigenetic modulation that appears to play a relevant contributory role in other experimental contexts [4] may be involved in the increased stroke susceptibility of the SHRSP animal model and may provide an early marker of disease. Interestingly, we detected a differential modulation of brain miR-122 expression level after 4 weeks of high-salt dietary regimen, before stroke occurrence, in the SHRSP as compared to the SHRSR strain. In particular, the brain miR-122 expression level was significantly increased in the SHRSR, whereas it was significantly decreased in the SHRSP. In the latter strain, the miR-122 decrease was associated with the early signs of cerebrovascular damage. In fact, substantial changes in markers of oxidative stress, inflammation, endothelial dysfunction, apoptosis, and necrosis could be detected along with the miR-122 decrease in the brains of SHRSP. In vitro, the overexpression of miR-122 revealed a significant



(a)



(b)

Figure 3: Characterization of brain expression level of cleaved caspase-3 and c-Jun in JD- versus RD-fed SHRSR and SHRSP strains. (a) Representative Western blots with corresponding densitometric analysis are shown for c-Jun and cleaved caspase-3 in the brains of RD- and JD-fed SHRSR. (b) Representative Western blots with corresponding densitometric analysis are shown for c-Jun and cleaved caspase-3 in the brains of RD- and JD-fed SHRSP. $^{***}p < 0.0001$ JD- versus RD-fed SHRSP. JD = high-salt Japanese style diet; RD = regular diet. $N = 8$ for each experimental group.

protective effect on survival in cerebral endothelial cells exposed either to excess NaCl or to hydrogen peroxide.

The SHRSP represents a suitable animal model to investigate the mechanisms involved in the increased stroke susceptibility associated with hypertension. Previous findings obtained in this model have been successfully translated to the human disease [20]. We have shown that SHRSP develops 100% stroke occurrence by the 7th week of a high-salt dietary regimen [12, 13] and that, by the end of 4 weeks of this dietary treatment, mechanisms of cellular

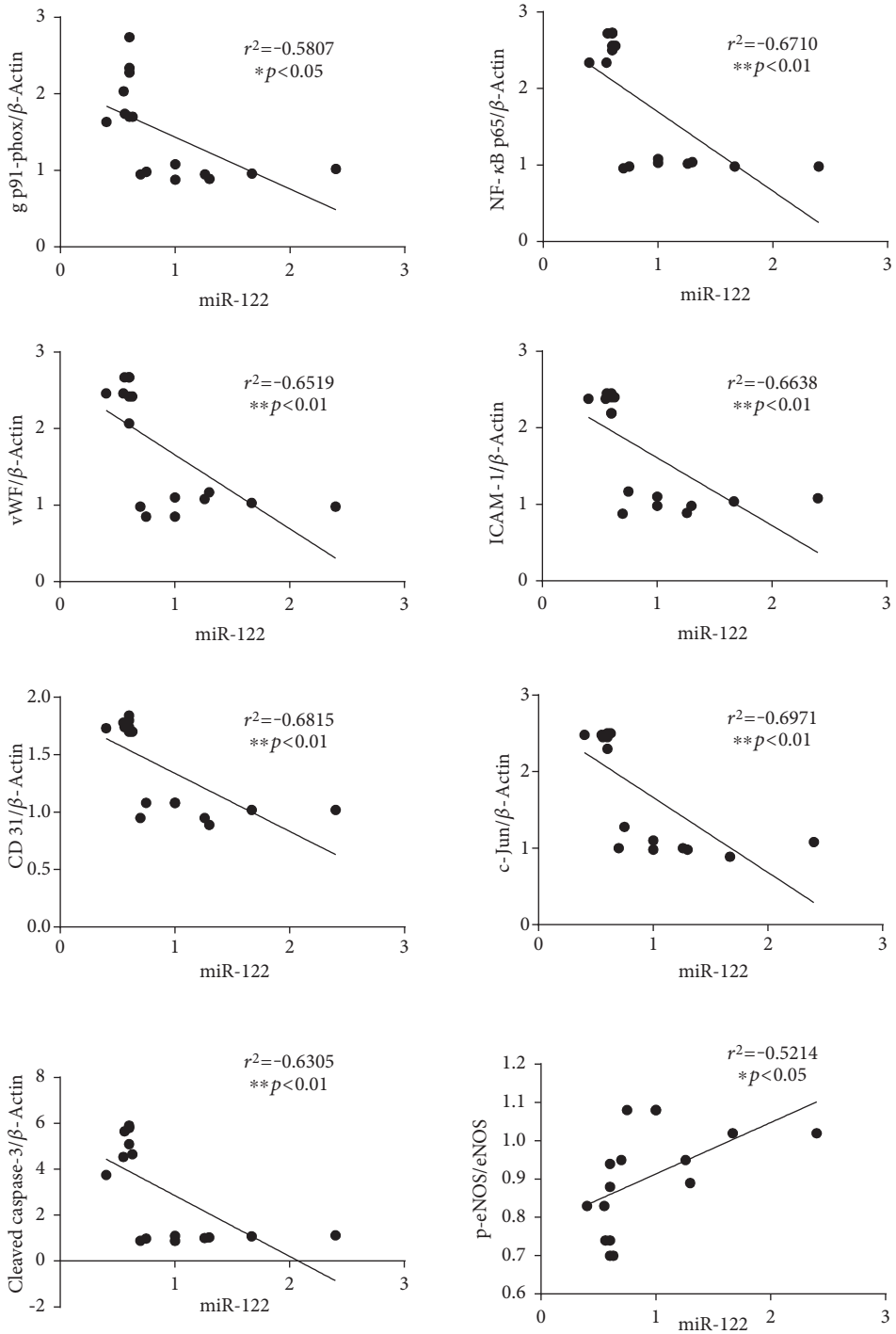


Figure 4: Correlation analysis between miR-122 and protein expression levels in the SHRSP strain. Scatter plots showing the results of the correlation analysis performed between miR-122 expression level and each examined protein level in the brain of SHRSP.

and vascular damage have been already activated with evidence of increased inflammation, increased oxidative stress, and endothelial dysfunction [14–16].

We had already reported an increase of NF-kB, a marker of inflammation, and of oxidized total proteins at the end of 4 weeks of JD in SHRSP [16]. In the current study, the expression level of gp91-phox confirmed the previous evidence of a substantial increase of oxidative stress at this experimental stage [16]. The parallel rise of ICAM-1, observed in the present study, goes along with previous findings obtained in the SHRSP, where a decrease of this inflammatory and endothelial integrity marker represented a substantial achievement of strategies aimed to combat stroke [21–23]. ICAM-1 is a cell surface glycoprotein that is typically expressed in injured endothelial cells, and it plays a well-known effect in facilitating endothelial leukocyte

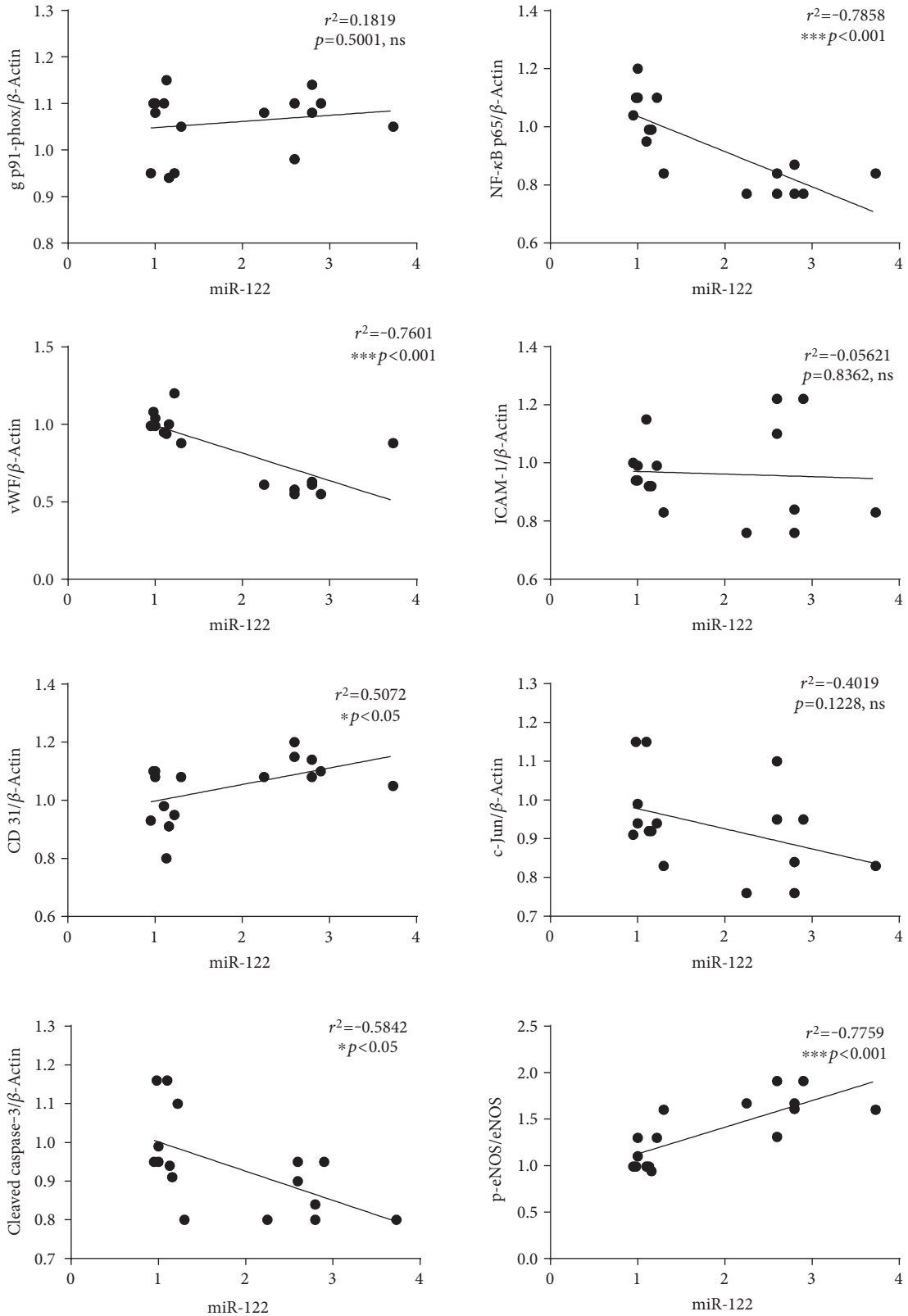


Figure 5: Correlation analysis between miR-122 and protein expression levels in the SHRSR strain. Scatter plots showing the results of the correlation analysis between miR-122 expression level and each examined protein level in the brain of SHRSR.

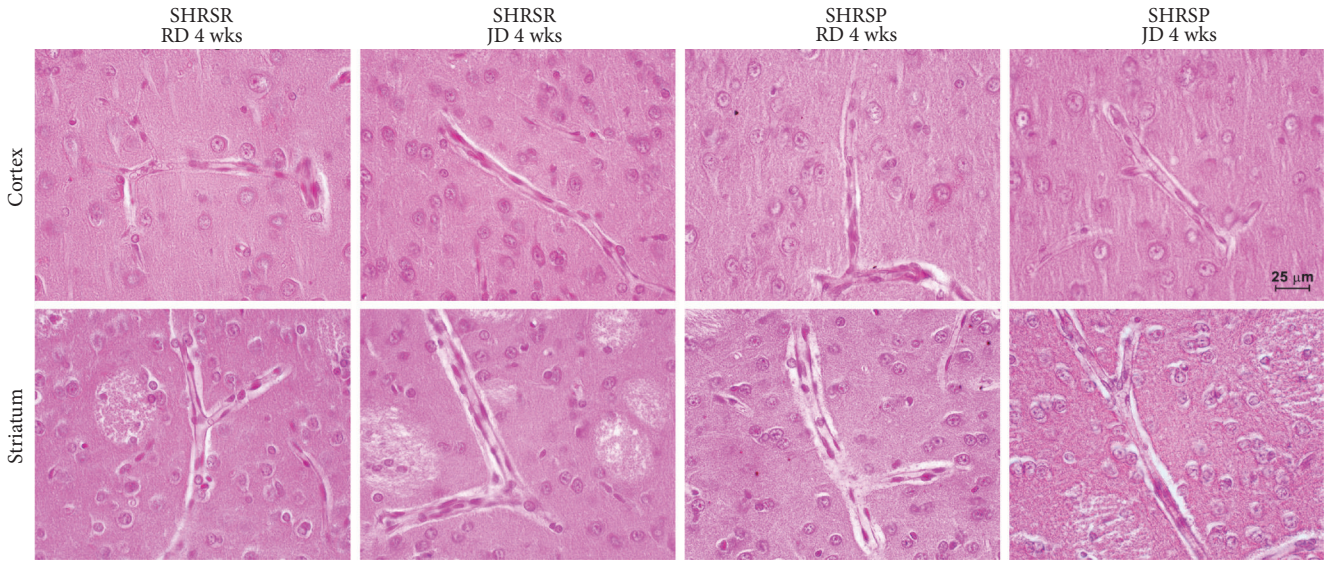


Figure 6: Brain histological analysis of SHRSP and SHRSR at the end of 4 weeks of either JD or RD treatment. Representative images of cortical (cortex) and striatal (striatum) blood vessels in the two strains at the end of 4 weeks of either RD or JD treatment. JD = high-salt Japanese style diet; RD = regular diet.

transmigration [24]. It has been previously associated with miR-122 levels in other experimental models of stroke [9]. In addition, herein, we report the first evidence that an increase of vWF and of CD31 expression is a distinct trait of the early phase of disease in SHRSP. The vWF is a blood glycoprotein, expressed also in endothelial cells, involved in hemostasis. Its increase has been associated with endothelial dysfunction and inflammation [25], and with increased risk of stroke [26]. CD31 is a cell adhesion molecule involved in inflammation and vascular biology including angiogenesis, leukocyte transmigration, leukocyte motility, thrombosis, vascular permeability, and numerous immune functions [27]. Its increased expression has been related, as a marker of angiogenesis, to the improvement of neurological outcomes in ischemic stroke rats treated with autologous peripheral blood-derived endothelial progenitor cells [28].

eNOS is the well-known nitric oxide producing enzyme in the endothelium [29] with potential therapeutic implications in stroke [30]. Its brain expression has been previously compared between SHRSP and Wistar Kyoto (WKY) rat strains with evidence of a significant downregulation in the stroke-prone rat [31]. Moreover, we reported a trend of eNOS to decrease with aging in the brains of SHRSP but not of SHRSR [32]. In the current study, we obtained the first demonstration that phospho-eNOS, as a hallmark of endothelial dysfunction, was significantly upregulated in the brains of SHRSR whereas it tended to decrease in the brains of SHRSP at the end of 4 weeks of JD feeding. Finally, the significant observed increase of markers of apoptosis and necrosis in the brains of SHRSP may represent the expected result of all above identified mechanisms and the most suitable pathological background for the subsequent cerebrovascular event. In fact, at this experimental stage, it is hard to observe evidence of histological brain damage [13].

The SHRSR strain is known for its stroke resistance, even when fed with a high-salt Japanese-style diet, along with the lack of brain inflammation and of oxidative stress after 4 weeks of JD feeding [13–16], as well as with the aging process [33]. The current data, showing a different expression of genes involved in inflammation, oxidative stress, endothelial integrity, apoptosis, and necrosis, as compared to SHRSP, are consistent with the previous findings, and they are certainly the consequence of a specific genetic background causing stroke resistance as opposed to stroke proneness [13]. Interestingly, based on our findings, miR-122 may be a contributor to stroke resistance in the SHRSR strain.

Importantly, to further support our hypothesis, we presently provide a clear cut in vitro demonstration of the significant protective effect of exogenous administration of miR-122 on cell survival upon exposure to stress stimuli. In particular, we showed that the overexpression of miR-122 was able to rescue the cell death induced by both excess NaCl and hydrogen peroxide in a line of brain endothelial cells. Of note, the exposure to excess NaCl mimicked the in vivo administration of HS diet to SHRSP.

The overall findings of our investigation suggest that miR-122 plays a contributory role in the pathogenesis of cerebrovascular disease, therefore supporting previous evidence [6, 9]. In fact, miR-122 was shown to be downregulated both in animal models of ischemic stroke, including the MCAO rat model [7, 8], and in peripheral blood cells of patients suffering from ischemic stroke [7, 10, 11]. In addition, the exogenous administration of miR-122 to MCAO rats reduced the brain infarct size and the neurological deficits with improvement of stroke outcomes [9]. Consistently with our current evidence, the miRNA-122 has been involved in the regulation of inflammation and of cell proliferation through the interference with the NF-kB and toll-like

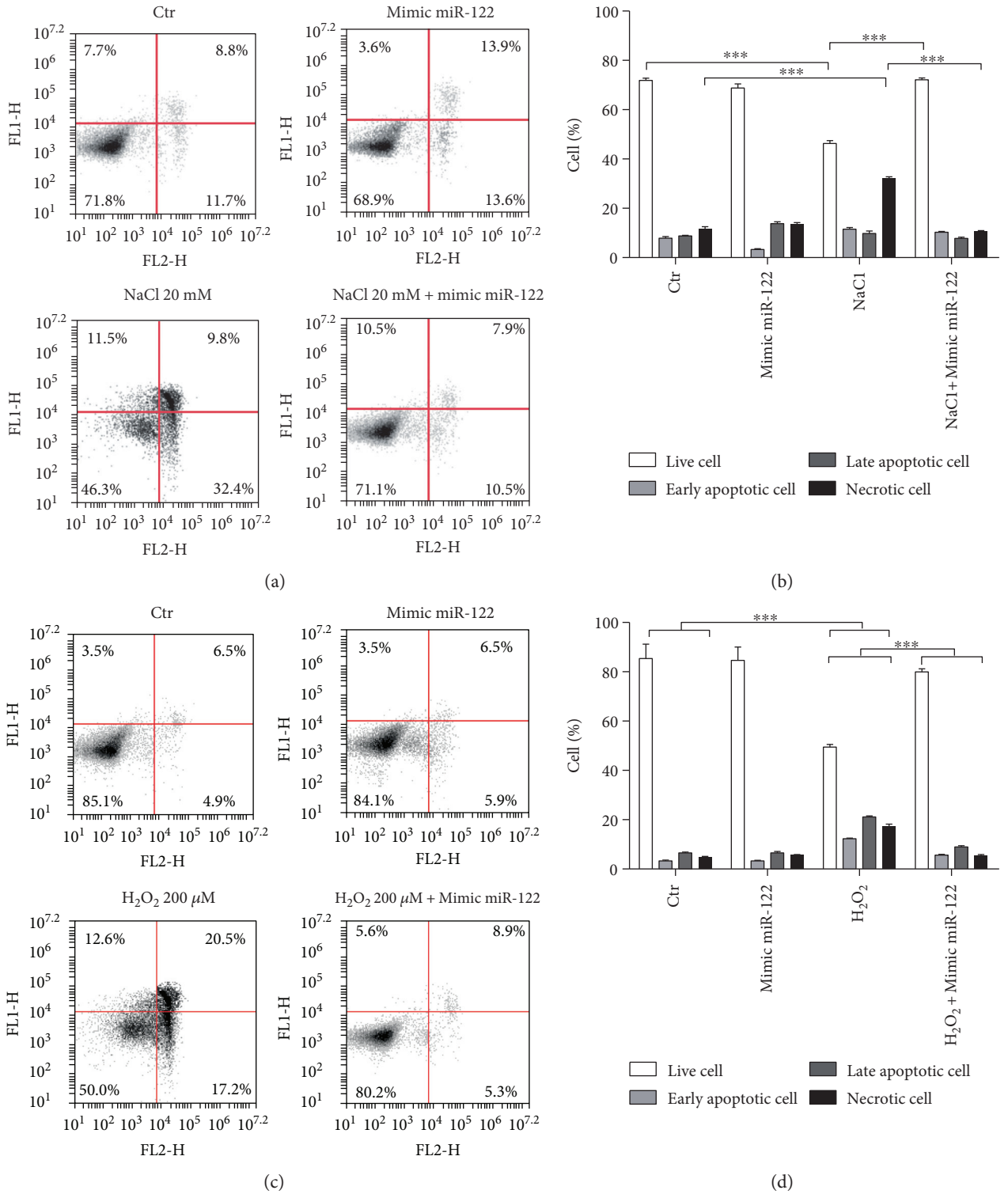


FIGURE 7: Impact of mimic miRNA-122 overexpression on cell viability, apoptosis, and necrosis in MECs upon exposure to stress stimuli. FACS analysis (a) and corresponding percent values (b) in MECs exposed for 72 hrs to high-NaCl medium (20 mM NaCl) either in the absence or in the presence of mimic miRNA-122 overexpression. FACS analysis (c) and corresponding percent values (d) in MECs exposed for 24 hrs to medium supplemented with 200 μM H_2O_2 either in the absence or in the presence of mimic miRNA-122 overexpression. Ctr: cells transfected with lipofectamine and miRNA negative control; mimic miR-122: cells transfected with mimic miR-122; NaCl: cells exposed to high-NaCl medium for 72 hrs; H_2O_2: cells exposed to medium supplemented with 200 μM H_2O_2 for 24 hrs; NaCl + mimic miR-122: cells transfected with mimic miR-122 and exposed to high-NaCl medium for 72 hrs; H_2O_2 + mimic miR-122: cells transfected with mimic miR-122 and exposed to medium supplemented with 200 μM H_2O_2 for 24 hrs. $^{***}P < 0.0001$ for each comparison.

receptor signaling pathways [10]. As a limitation of our current study, we did not test the impact of exogenous miR-122 administration on the stroke phenotype occurrence of JD-fed SHRSP.

In summary, the different modulation of miR-122 level, associated with the early signs of cerebrovascular damage, was detected in the brains of SHRSP after four weeks of the stroke permissive diet, before the occurrence of cerebrovascular events. Therefore, a decrease of brain miR-122, along with increased markers of oxidative stress, inflammation, endothelial dysfunction, apoptosis, and necrosis, can be considered an early marker of stroke associated with hypertension in our animal model. On the other hand, higher levels of miR-122, as those found in the brains of SHRSR, appear to be protective and, in fact, they were able to preserve cell survival from stress stimuli in vitro.

Our findings support the use of microRNAs, such as miR-122, as useful biomarkers for stroke prevention and diagnosis. Moreover, we support the concept that, by identifying genes and pathways regulated by microRNAs, we can better understand the mechanisms involved in the promotion of cerebrovascular damage for a better treatment of stroke.

Disclosure

The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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