Lactobacillus Plantarum -derived extracellular vesicles protect against ischemic brain injury via the microRNA-101a-3p/c-Fos/TGF- β axis cross-species

zhang yang (✉ yangzhang625@126.com)

Affiliated Hospital of Guizhou Medical University <https://orcid.org/0000-0003-2797-7535>

Zidan Gao

The Affiliated Hospital of Guizhou Medical University

Yifan Zhang

The Affiliated Hospital of Guizhou Medical University

Hongqun Chen

The Affiliated Hospital of Guizhou Medical University

Xuexia Yang

The Affiliated Hospital of Guizhou Medical University

Xuming Fang

The Affiliated Hospital of Guizhou Medical University

Yingwu Zhu

The Affiliated Hospital of Guizhou Medical University

Jiayan Zhang

The Affiliated Hospital of Guizhou Medical University

Fu Ouyang

The Affiliated Hospital of Guizhou Medical University

Jun Li

The Affiliated Hospital of Guizhou Medical University

Gang Cai

The Affiliated Hospital of Guizhou Medical University

Xiang Lin

The Affiliated Hospital of Guizhou Medical University

Xiaofang Shi

The Affiliated Hospital of Guizhou Medical University

Lan Chu

The Affiliated Hospital of Guizhou Medical University <https://orcid.org/0000-0002-1004-0300>

Research Article

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Abstract

Objective

As nanoscale membrane vesicles, extracellular vesicles (EVs) are actively released by cells and have recently been observed to assume an important role in the treatment of ischemic stroke. However, the source of EVs currently reported for the treatment of ischemic stroke is tightly restricted to mammals. Moreover, these EVs are limited in clinical translation due to the high cost of cell culture. In species other than mammals, *Lactobacillus Plantarum* culture is low-cost and high-yield. Notwithstanding, it is poorly identified whether *Lactobacillus Plantarum*-derived EVs (LEVs) can be applied across species for the treatment of ischemic stroke.

Methods

LEVs were injected into mice via the tail vein immediately after transient middle cerebral artery occlusion (tMCAO) modeling. Infarct volumes, neurological scores, and neuron apoptosis were examined 3 days following the ischemic episode. The expression of microRNA (miRNA) and the target gene in neurons and peri-infarct regions of the post-ischemic brain were measured for mechanistic analyses.

Results

LEVs crossed the blood-brain barrier and were taken up by neurons both in vitro and in vivo. LEV treatment attenuated neuron apoptosis following oxygen-glucose deprivation (OGD). In vivo findings displayed that LEV treatment substantially reduced neurological deficits and brain infarct size in tMCAO mice. High-throughput sequencing exhibited that miR-101a-3p expression was prominently elevated by LEV treatment in OGD-induced neurons, and corroborating findings were detected in tMCAO mice treated with LEVs. Mechanistically, c-Fos was directly targeted by miR-101a-3p. Moreover, c-Fos manipulated ischemia-induced neuron apoptosis in vitro and in vivo through the TGF- β 1 pathway. miR-101a-3p inhibition aggravated ischemia-induced neuron apoptosis in vitro and in vivo, and miR-101a-3p overexpression triggered opposite results.

Conclusion

Our results elaborated for the first time that LEVs might inhibit neuron apoptosis and injury across species via the miR-101a-3p/c-Fos/TGF- β axis, which may provide novel ideas and targets for the treatment of ischemic stroke and is also of significance for the clinical translation of EVs.

Introduction

As a prevalently occurring disorder, stroke represents the second leading cause of death and the third leading cause of disability among adults across the world ^[1]. To date, there exist only two effective therapeutic options for ischemic stroke, including tissue-type plasminogen activator and endovascular thrombectomy ^[2]. However, the therapeutic window is narrow for these two options (intravenous thrombolysis < 6 h; mechanical thrombectomy < 24 h) ^[3]. Therefore, only a small minority of people in the window receive timely treatment. In addition, 40% of treated stroke patients still show a poor prognosis ^[4]. Therefore, there is an urgent need for additional treatments to improve the neurological prognosis following ischemic stroke.

Extracellular vesicles (EVs) have recently been discovered to have the advantages of low immunogenicity, low toxicity, high blood-brain barrier permeability, and the modulation of intercellular communication in the treatment of ischemic stroke. It is because of these characteristics that extensive attention has been paid to the therapeutic potential of EVs in ischemic stroke ^[5]. As nanoscale membrane vesicles actively released by cells, EVs refer to vesicular bodies with a bilayer membrane structure detached from cell membranes or secreted by cells, which exist from prokaryotes to eukaryotes ^[6]. In prokaryotes, bacteria-derived EVs carry molecules similar to those of mammals, including small RNAs, mRNAs, and proteins ^[7]. They can affect a variety of biological processes, such as RNA transferring, intercellular communication, immunomodulatory activity, antibiotics, and eukaryotic host defense factors ^[8].

As currently reported, various kinds of mammal-derived EVs, such as mesenchymal stem cell-derived EVs ^[9], neural stem cell-derived EVs ^[10], M2 microglia-derived EVs ^[11], and brain endothelial cell-derived EVs ^[12], have been used to treat ischemic stroke by altering the expression of miRNAs in the organism. However, due to the high cost of mammal-derived cell culture, it is difficult to obtain culture medium for EV extraction on a large scale in the short term, which has limited the translation of EVs research to the clinic ^[13]. Therefore, it has more obvious advantages in clinical application to obtain culture media for EV extraction quickly, at a low cost, and in large quantities. Of note, *Lactobacillus Plantarum* can be used to extract EVs by harvesting culture media quickly and at a low cost. However, there has hitherto been no research demonstrating whether *Lactobacillus Plantarum*-derived EVs (LEVs) can be applied for the treatment of ischemic stroke. *Lactobacillus Plantarum* is included in the list of microorganisms with safety qualifications released by the European Food Safety Authority in 2020 ^[14]. *Lactobacillus Plantarum* has been reported to protect the small intestine against oxidative stress and inflammatory response induced by ischemia-reperfusion in animal models ^[15] and decrease infarct size in a rat model of myocardial infarction ^[16]. In addition, *Lactobacillus Plantarum* has been shown to mediate the expression of microRNAs (miRNAs) in mammals across species ^[17–20]. miRNAs assume a crucial role in the treatment of ischemic stroke via a wide range of mechanisms, including anti-oxidative stress, anti-inflammation, anti-apoptosis, blood-brain barrier protection, pro-angiogenesis, promotion of neuronal axon regeneration, and other tissue remodeling mechanisms. Besides, miRNAs exhibit effects in the occurrence and development of ischemic stroke in terms of diagnosis, disease monitoring, treatment, and prognosis improvement ^[21]. In our study, the results of miRNA microarray analyses documented that miR-

101a-3p expression was significantly increased after treatment of ischemic neurons with LEVs. Moreover, c-Fos is one of the direct target genes of miR-101a-3p. c-Fos is a transcription factor that modulates genes in the nucleus that are activated or repressed by external stimuli and is often utilized as a marker of neuronal activity, which plays a critical role in post-ischemic neuronal apoptosis and inflammation [22, 23]. The inhibition of c-Fos expression reduces ischemic brain injury (IBI). Another research reported that c-Fos downregulation contributed to the decline in the expression of transforming growth factor beta 1 (TGF- β 1) expression, an essential isoform of the TGF- β superfamily that orchestrates cell growth, differentiation, proliferation, and apoptosis [24]. TGF- β 1 participates in the regulation of ischemic stroke, and TGF- β 1 expression is lower in the serum of stroke patients than in that of control subjects [25]. The downregulation of TGF- β 1 diminishes neuronal apoptosis and further protects against cerebral ischemia-reperfusion injury.

Therefore, the present study was implemented to ascertain whether EVs from the probiotic strain *Lactobacillus Plantarum* YW11 reduced neuronal apoptosis and improved neurological recovery after stroke via the miR-101a-3p/c-Fos/TGF- β axis in an in vitro hypoxia model and an in vivo cerebral ischemia model.

Results

LEVs were identified and taken up by neurons across the blood-brain barrier

EVs were isolated from the *Lactobacillus Plantarum* medium by ultracentrifugation and were observed to exhibit the typical cup-shaped membrane vesicle morphology using the TEM (Figure 1A). Next, the diameter and concentration of LEVs were analyzed using NTA, which revealed that the diameter of LEVs was 60 ± 5.83 nm (Figure 1B). To explore whether LEVs could be taken up by neurons across the blood-brain barrier, LEVs were injected into mice via the tail vein after DiR (dark red fluorescence) labeling, followed by 6 h, 12 h, 18 h, and 24 h of observation in the IVIS system. It was found that the LEVs crossed the blood-brain barrier and entered the brain tissue (Figure 1C). To ascertain whether LEVs could be taken up by neurons, LEVs were subjected to PHK26 (a red fluorescent dye for EVs) labeling and were then injected into mice with cerebral ischemia via the tail vein or co-cultured with primary cortical neurons for immunofluorescence staining. The results suggested that PHK26-LEVs were taken up by neurons in vivo and in vitro (Figure 1D, E). In conclusion, LEVs crossed the blood-brain barrier and were taken up by ischemic neurons.

LEVs facilitated the viability but repressed the apoptosis of OGD-induced neurons

To investigate whether LEVs were involved in neuron protection, LEVs were co-cultured with primary neurons for 24 h to ensure adequate EV uptake, followed by 45-min OGD treatment and 12-h oxygenation. As discovered in Figure 2A, LEVs treatment significantly elevated the viability of OGD-induced neurons. To

further quantify the protective impacts of LEVs on OGD-induced neurons, an LDH assay was performed to rapidly quantify cell viability. The LDH assay was performed to rapidly assess cell damage by measuring the amount of LDH released from cells ^[24]. LDH levels were normalized to the control group. OGD treatment resulted in a 5-fold elevation in cell damage, but treatment with LEVs reduced cell death to 2-fold that of control neurons (Figure 2B).

Next, LEVs were isolated and co-cultured with primary neurons under OGD conditions. Next apoptosis-related proteins (Bax, Bcl-2, and Cleaved caspase3) were measured using western blot analysis, the results of which demonstrated obvious lower expression of Bax and Cleaved caspase3 and higher Bcl-2 expression in the LEV group than in the PBS group (Figure 2C, D). It was further displayed that LEV treatment diminished OGD-induced neuron apoptosis. TUNEL⁺ cell percentage was determined using TUNEL staining. The results depicted that TUNEL⁺ cell percentage was lowered in the LEV group compared with the PBS group (Figure 2E, F). In conclusion, treatment with LEVs substantially promoted the viability and suppressed the apoptosis of OGD-induced neurons.

LEVs attenuated neurological deficits and neuron apoptosis in tMCAO mice

To investigate whether LEVs exert neuroprotective effects in the brain of mice with cerebral ischemia, LEVs were injected via tail vein into tMCAO mice (Figure 3A). It was observed by mNSS scores that LEV treatment conspicuously alleviated neurological deficits 3 days after stroke compared to PBS treatment (Figure 3B). LEV treatment reduced the infarct size after 3 days of tMCAO versus PBS treatment (Figure 3C, D). Pathological observation of the cortical ischemic penumbra in mice was performed using HE staining. The results exhibited that cortical glial cells were proliferated and more swollen than before with mild degeneration in the neuron focal areas and obvious loose and softening neuropil in the PBS group. In the LEV group, cortical glial cells were mildly proliferated and basically normal with loose and softening neuropil only in the focal areas (Figure 3E). In addition, western blot analysis results documented that in contrast to the PBS group, Bax and Cleaved caspase3 protein expression was decreased but Bcl-2 protein expression was augmented in the cortices of mice with cerebral ischemia in the LEV group (Figure 3F, G). TUNEL⁺ and TUNEL⁺ NeuN⁺ cell percentages were assessed by TUNEL staining, which manifested that TUNEL⁺ and TUNEL⁺ NeuN⁺ cell percentages lowered in the LEV group compared with the PBS group (Figure 3H-J). In summary, LEVs restrained neurological deficits and neuron apoptosis in tMCAO mice.

LEVs altered miR-101a-3p expression to orchestrate it downstream targets c-Fos in the ischemic models

EVs are rich in miRNAs and can change miRNA expression to further mediate physiological functions in the recipient cells. Specific miRNAs expressed in ischemic neurons treated with LEVs were identified by conducting RNA sequencing, which are detailed in volcano, scatter, and heat maps (Figures 4A-C). The top 10 upregulated miRNAs were selected for qRT-PCR to validate the results of miRNA microarray

analysis. In OGD-induced neurons, miR-101a-3p, miR-148b-3p, and miR-186-5p were differentially expressed in the LEV group compared to the PBS group (Figure 4D). In tMCAO mice, miR-93-5p, miR-101a-3p, miR-20a-5p, and miR-103-3p were differentially expressed in the LEV group versus the PBS group (Figure 4E). In both OGD-induced neurons and tMCAO mice, only miR-101a-3p expression differed and the differences were more pronounced. Therefore, we hypothesized that LEVs exert neuroprotective effects by altering miR-101a-3p expression in ischemic neurons in vivo and in vitro. Afterward, 93 target genes of miR-101a-3p were predicted by miRmap, miRanda, microT, PicTar, and PITA databases (Figure 4F). As shown in Figure 4G, TargetScan was utilized to obtain the binding sites between miR-101a-3p and c-Fos in humans, rats, and mice. Besides, c-Fos, as a target gene, has been reported in several researches to play a role in the modulation of neuron apoptosis [26-29]. We, therefore, considered whether miR-101a-3p manipulated the function of neurons via c-Fos. Firstly, a dual-luciferase reporter gene assay was implemented to confirm whether miR-101a-3p targets c-Fos, which showed that in contrast to the mimic-NC + c-Fos-3'UTR-WT group, the luciferase activity was evidently declined in the miR-101a-3p mimic + c-Fos-3'UTR-WT group. However, there was no remarkable difference in luciferase activity between the mimic-NC + c-Fos-3'UTR-MUT group and the miR-101a-3p mimic + c-Fos-3'UTR-MUT group (Figure 4H). Collectively, miR-101a-3p could specifically bind to c-Fos.

miR-101a-3p decreased c-Fos expression and blocked the TGF- β 1 pathway to foster viability and restrict the apoptosis of OGD-induced neurons

We next explored the mechanism of miR-101a-3p-mediated neuroprotective effects after neuron ischemia. In the aforementioned experiments, we validated that miR-101a-3p orchestrated c-Fos that was involved in the regulation of OGD-induced neuron apoptosis. c-Fos is reported to be upstream of the TGF- β 1 pathway [24], so miR-101a-3p may mediate neuron survival through c-Fos and the downstream TGF- β 1 pathway. To further prove our hypothesis, miR-101a-3p inhibitor, inhibitor-NC, miR-101a-3p mimic, or mimic-NC was transfected into OGD-induced neurons. Cell viability was measured using the LDH assay and it was manifested that miR-101a-3p inhibitor treatment considerably diminished the viability of OGD-induced neurons, which was contrary to miR-101a-3p mimic treatment (Figure 5A). In addition, qRT-PCR analysis also demonstrated that miR-101a-3p inhibitor treatment contributed to the prominent decline of miR-101a-3p expression in OGD-induced neurons compared to treatment with PBS or inhibitor-NC, which was conflicting to miR-101a-3p mimic treatment versus treatment with PBS or mimic-NC (Figure 5B). As reflected by western blot analysis results, the increase of c-Fos and TGF- β 1 expression was noted in OGD-induced neurons after miR-101a-3p inhibitor treatment. However, opposite trends were triggered by miR-101a-3p mimic treatment (Figure 5C, D). As displayed in Figure 5E-H, miR-101a-3p inhibitor transfection appreciably enhanced Bax and Cleaved Caspase3 protein expression and TUNEL⁺ cell percentage and strikingly diminished Bcl-2 protein expression in OGD-induced neurons, which was opposite after miR-101a-3p mimic treatment. Conclusively, miR-101a-3p encouraged the viability and curtailed the apoptosis of OGD-induced neurons by disrupting the c-Fos/TGF- β 1 axis.

miR-101a-3p depressed c-Fos and the downstream TGF- β 1 pathway to improve neurological deficits and neuron apoptosis in tMCAO mice

To dissect whether miR-101a-3p exerted neuroprotective effects in the brain of mice with cerebral ischemia through c-Fos and its downstream TGF- β 1 pathway, tMCAO mice were treated with PBS, antagomir-NC, miR-101a-3p antagomir, agomir-NC, and miR-101a-3p agomir. As verified by qRT-PCR results, miR-101a-3p antagomir treatment remarkably reduced miR-101a-3p in contrast to treatment with PBS or antagomir-NC, whereas miR-101a-3p agomir treatment markedly augmented miR-101a-3p expression in tMCAO mice compared with treatment with PBS or agomir-NC (Figure 6A). Furthermore, western blot analysis results also documented that c-Fos and TGF- β 1 expression in tMCAO mice was elevated after miR-101a-3p antagomir treatment but lowered subsequent to miR-101a-3p agomir treatment (Figure 6B, C). Next, immunofluorescence was performed to detect c-Fos expression in mouse cortical peri-ischemic brain tissues. As presented in Figure 6D, E, miR-101a-3p antagomir treatment noticeably elevated but miR-101a-3p agomir treatment observably decreased the proportion of c-Fos⁺ NeuN⁺ cells in the cortical peri-ischemic brain tissues of tMCAO mice. In short, miR-101a-3p inactivated the c-Fos/TGF- β 1 axis in mouse cortical peri-ischemic brain tissues.

To evaluate whether miR-101a-3p exerted neuroprotective effects via the c-Fos/TGF- β 1 axis in the brain of mice with cerebral ischemia, tMCAO mice were injected with PBS, antagomir-NC, miR-101a-3p antagomir, agomir-NC, and miR-101a-3p agomir. As manifested in Figure 7A, injection of miR-101a-3p antagomir led to the increase of the infarct volume in tMCAO mice, whilst treatment with miR-101a-3p agomir dramatically declined the infarct volume 3 days post-ischemia. The mNSS assessment was carried out to examine the influences of miR-101a-3p on neurological deficits of mice after stroke. Neurological deficits of mice 3 days following tMCAO were signally enhanced by miR-101a-3p antagomir treatment but significantly ameliorated by miR-101a-3p agomir treatment (Figure 7B, C). The in vivo neuroprotective impacts of miR-101a-3p were assessed by western blot analysis and TUNEL staining. The results in Figure 7D-H exhibited that miR-101a-3p antagomir treatment substantially elevated Bax and Cleaved caspase3 protein expression and TUNEL⁺ and TUNEL⁺ NeuN⁺ cell percentages were obviously elevated and observably lowered Bcl-2 protein expression in tMCAO mice, which was contrary to miR-101a-3p agomir treatment. These findings suggested that miR-101a-3p mitigated neurological deficits and neuron apoptosis in tMCAO mice via the inactivation of the c-Fos/TGF- β 1 axis.

Discussion

Mammal-derived EVs exhibit neuroprotective and neuroregenerative effects in stroke models in vivo and in vitro. However, there existed no reports on EVs derived from other species in the treatment of ischemic stroke. *Lactobacillus Plantarum*, a non-toxic and edible probiotic, was utilized in this study, and our

results elucidated that LEV treatment reduced infarct size and lightened neurological deficits in the tMCAO mouse model. Moreover, LEVs can be taken up by neurons and significantly reduce ischemic neuron apoptosis. Mechanistically, LEVs constrained ischemic neuron apoptosis by increasing miR-101a-3p expression and blocking the c-Fos/TGF- β axis in neurons. In this study, we report for the first time that LEVs were taken up by ischemic neurons across species and further facilitated their survival, a process mediated by upregulating miR-101a-3p and inactivating the c-Fos/TGF- β axis in ischemic neurons.

Lactobacillus Plantarum assumes a beneficial role in a variety of diseases such as inflammatory bowel disease, metabolic syndrome, dyslipidemia, hypercholesterolemia, obesity, and diabetes, as well as nervous system-related disorders diseases^[30]. *Lactobacillus Plantarum* has been reported to protect the small intestine from oxidative stress and inflammatory damage caused by ischemia-reperfusion in animal models^[15] and to diminish infarct size in a rat model of myocardial infarction^[16]. Besides, it has been demonstrated that *Lactobacillus Plantarum* possesses a strong lead absorption capacity and tolerance^[31] and exerts anti-oxidant impacts in an aging mouse model^[32]. It was observed in palmitate-treated hepatocellular carcinoma cells that *Lactobacillus Plantarum* subdued glucose consumption and pro-inflammatory cytokine secretion by downregulating miR-212-targeted dual-specificity phosphatase-9^[17]. Also, it was found that *Lactobacillus Plantarum* suppressed obesity and fatty liver by manipulating the expression of miRNAs in the liver in high-fat diet-induced obesity rats^[18]. Additionally, *Lactobacillus Plantarum* altered cecal miRNA expression in neonatal broilers, thereby inhibiting *Salmonella* infection^[19]. More importantly, *Lactobacillus Plantarum* can assume a protective role in ischemia models and can change miRNA expression in mammals. However, little is known about the functional role of LEVs. Only a report has uncovered that LEVs could induce anti-inflammatory M2 macrophage polarization in vitro and play an anti-inflammatory part in inflammatory skin models^[33]. In addition, LEVs could trigger the upregulation of BDNF in hippocampal neurons in a mouse model with depression-like behaviors to achieve antidepressant effects^[34]. The above studies indicated that *Lactobacillus Plantarum* and their EVs can exhibit cross-species regulatory impacts with unknown specific mechanisms in mammal and cell models. Intriguingly, our study unraveled that *Lactobacillus Plantarum* and LEVs can mediate miRNA expression in mammals across species. Thus, LEVs assume a critical role in the mediation of neurological recovery in ischemic stroke. Indeed, the data obtained in our research elaborated that LEVs crossed the blood-brain barrier and improved neurological function, pathological changes in ischemic neurons, and changes in brain infarct size in a mouse model of ischemia-reperfusion-induced brain injury. The current research further displayed that LEV treatment suppressed neuron apoptosis and death in both in vitro and in vivo ischemia models. These results illustrated that LEVs can treat IBI.

Several labeling strategies are available for in vivo exosome tracking, such as luciferase reporter genes^[35, 36], fluorescent proteins^[37, 38], and lipophilic fluorescent dyes^[37, 39–42]. Among them, lipophilic fluorescent dyes, including Dil, DiR, PKH26, and PKH67, are widely applied due to their pivotal chemical properties. Therefore, DiR and PKH26 staining were employed for EV labeling, respectively, in our study. The in vivo imaging observation exhibited that DiR-labeled LEVs injected into mice via the tail vein were mainly present in the spleen and liver, and we did not find any swelling or inflammation in the spleen and

liver after LEV delivery. EV treatment did not cause weight loss and death of animals, indicating that EVs are not toxic to organs. Notably, mounting evidence revealed the effects of EVs on a wide range of organs in mice, including the thymus, heart, lung, liver, spleen, kidney, adrenal gland, ovary, uterus, and brain. Other studies also elaborated that the repeated administration of EVs to mice did not contribute to conspicuous toxicity, as assessed by the monitoring of body weight changes, immune responses, and histopathological changes for up to 3 weeks^[43]. EVs are rich in miRNA and exert functions mainly by transferring EV-miRNAs to the recipient cells, thereby modulating downstream gene expression to further control the function of the recipient cells.

miRNAs are implicated in the treatment of ischemic stroke by a variety of mechanisms, including anti-oxidative stress, anti-inflammation, anti-apoptosis, blood-brain barrier protection, pro-angiogenesis, promotion of neuronal axon regeneration, and other tissue remodeling effects. Meanwhile, miRNAs control the development of ischemic stroke in terms of diagnosis, disease monitoring, treatment, and prognosis improvement^[21]. Our research found multiple differentially expressed miRNAs after OGD/R induction and high-throughput sequencing using LEVs-treated and untreated primary cortical neuronal cells as controls. We also validated miR-101a-3p, which showed the most substantial differential expression, in in vitro and in vivo ischemia models. As documented, the inhibition of miR-101a-3p restricted hypoxia/re-oxygenation (H/R) injury in H9C2 cells and decreased cell apoptosis by inhibiting Bax/Bcl-2 pathway during H/R injury^[44]. Besides, miR-101a-3p upregulation lowered amyloid precursor protein (APP) expression and delayed the onset of Alzheimer's disease^[45]. In our research, the data exhibited that neuroprotective impacts could be exerted by overexpression of miR-101a-3p in ischemia models in vivo and in vitro and that miR-101a-3p downregulation exacerbated ischemic injury. Neuron protection after cerebral ischemia-reperfusion injury is a crucial factor for the determination of the prognosis of ischemic stroke^[46]. Of note, the apoptotic pathway is the main target to interfere with neuronal regression following ischemia-reperfusion injury. Apoptotic body morphology, characterized by deep DNA breaks, is a key feature of apoptotic cell death^[47]. Apoptosis is orchestrated by Bax and Bcl-2 of the Bcl-2 family^[48, 49]. Bcl-2 represses apoptosis, whilst Bax facilitates apoptosis. Recent researches have noted that the modulation of apoptosis is dependent on the Bcl-2/Bax ratio, that is, the lower the Bcl-2/Bax ratio, the higher the apoptosis rate^[50, 51]. The proteins in the caspase family participate in the initiation and mediation of apoptosis, and caspase-3 is a critical caspase activated by various apoptotic stimuli^[52, 53]. Our data manifested the considerable decline of Bcl-2 expression and the apparent activation of Bax and caspase3, accompanied by elevated TUNEL-positive cells in the peri-ischemic cortex following stroke and an evident increase of apoptotic rates subsequent to OGD/R induction, which was concurrent with previous researches^[54, 55].

miR-101a-3p can directly target c-Fos^[56, 57], and the downregulation of c-Fos curtails the development of IBI^[58]. Another study displayed that decreased c-Fos expression blocked the TGF- β 1 pathway^[59]. Furthermore, TGF- β 1 repression diminished neuron apoptosis and further protected against cerebral ischemia-reperfusion injury^[60]. This research aimed to dissect LEVs as a target of neuroprotection in

ischemic stroke mediated by miR-101a-3p. To validate miR-101a-3p-mediated neuroprotection, in vivo and in vitro ischemia models were intervened by overexpressing miR-101a-3p, which reduced c-Fos expression in neurons and depressed downstream TGF- β 1, thereby increasing neuron apoptosis. In conclusion, the suppression of miR-101a-3p activated the c-Fos/ TGF- β 1 axis to subdue neuron apoptosis. Our study demonstrated that the c-Fos/TGF- β 1 axis manipulated neuron apoptosis, which was coincident with literature reports.

Conclusions

Taken together, the findings in this research provide a new option for EVs in the treatment of ischemic stroke. Our data provided evidence that LEVs might improve neurological impairment in tMCAO by lowering neuron apoptosis, which was achieved by a mechanism associated with miR-101a-3p and c-Fos/TGF- β 1 (Figure 8). We speculate that LEVs are potential therapeutic targets for the treatment of ischemic stroke, which is partially correlated with miR-101a-3p and c-Fos/TGF- β 1.

Materials And Methods

Experimental design

All animal procedures were conducted as per the guidelines of the Institutional Animal Care and Use Committee of the Affiliated Hospital of Guizhou Medical University (Guizhou, China) and reported according to the guidelines of Animals in Research: Reporting In Vivo Experiments. Male C57BL/6 mice (Janvier Labs, Le Genest-Saint-Isle, France) aged 10-12 weeks were housed under circadian rhythm with free access to food and water. In all phases of the study, the researchers were blinded to the experimental conditions. Mice were randomly assigned to different treatment groups.

Mice were randomly arranged into sham group (mice undergoing sham operation), middle cerebral artery occlusion (MCAO) group (MCAO mice without any treatment), phosphate buffer saline (PBS) group (MCAO mice injected with 100 μ L PBS through the tail vein for 3 consecutive days), LEV group (MCAO mice injected with LEVs through the tail vein for 3 consecutive days), miR-101a-3p agomir + MCAO group, miR-101a-3p antagomir + MCAO group, negative control (NC) agomir + MCAO group, and NC antagomir group. For miR-101a-3p agomir treatment, miR-101a-3p agomir (2.5 μ g/2.5 μ L) was diluted with 1.25 μ L Entranster TM-in vivo transfection reagent (18668-11-1, Engreen, Beijing, China). The solution was gently mixed with 1.25 μ L PBS and placed at room temperature for 5 min. Thereafter, intracerebroventricular injections were performed using a microinjector (ZS Dichuang Co., Ltd., Beijing, China) under the guidance of a stereotaxic instrument (ZS Dichuang Co., Ltd.) as per the previously described method ^[61]. The treatment of NC agomir, miR-101a-3p antagomir, and NC antagomir were operated as that of miR-101a-3p agomir. The neurobehaviors of all mice were observed for three days.

Transient MCAO (tMCAO)

Adult male C57BL/6 mice weighing 22-26 g were anesthetized with 1.5-2% isoflurane and 30% or 70% oxygen or nitrous oxide. tMCAO was conducted as described previously [62]. Briefly, the common carotid artery, internal carotid artery, and external carotid artery were separated. A silicone-coated nylon suture with a diameter of 0.23 ± 0.02 mm was inserted from the external carotid artery, followed by the internal carotid artery, and gently inserted into the middle cerebral artery. The suture was withdrawn for reperfusion 1 h after tMCAO. Immediately after removal of the suture, LEVs were injected into mice through the tail vein, which was repeated for 3 days at 100 μ g per day.

Neurobehavioral observation

To determine neurobehavioral performance, all mice underwent a modified neurological severity score (mNSS) test 1 day before and 3 days after tMCAO operation, in which motor, reflex, and balance functions were assessed. The evaluation scores ranged from 0 to 14, and the higher the score, the more severe the neurological deficit.

2, 3, 5-Triphenyltetrazolium chloride (TTC) staining

Cerebral infarction areas were assessed by TTC staining. Briefly, freshly excised brains were cut into 2 mm sections along the coronal axis and incubated at 37°C in 2% TTC. Normal areas were red and infarcted areas were white.

Hematoxylin-eosin (HE) staining

The brain tissues were fixed, paraffin-embedded, and cut into 4 μ m slices. After dewaxing with xylene, the slices were treated with gradient ethanol, hydrated, and stained with hematoxylin for 4 min. Next, the slices were rinsed with tap water and differentiated with Hydrochloric acid ethanol for 25 s, followed by 10-min soaking in warm water, 2-min staining with eosin solution, routine dehydration, clearing, and sealing.

Preparation of LEVs

Lactobacillus Plantarum YW11 utilized in this research was isolated from Kefir grains collected in Tibet. The strain was preserved as a frozen (-80°C) stock in Man-Rogosa-Sharpe (MRS) broth supplemented with 20% glycerol. The sterile semi-defined medium (SDM) broth contained 10 g bactocasitone (Difco, Detroit, MI, USA), 5 g yeast nitrogen base (Difco), 2 g ammonium citrate, 5 g sodium acetate, 0.1 g MgSO₄·7H₂O, 0.05 g MnSO₄, 2 g K₂HPO₄, 20 g glucose, and 1.0 mL Tween 80 (per 1 L), with pH adjusted to 6.6 using 1 M acetic acid [63]. The subculture was then conducted under the same conditions at a dilution ratio of 1: 100. After two passages of activation in MRS liquid medium, the strain was centrifuged at 5 000 r/min for 10 min, and the supernatant was obtained and filtered through a sterile 0.22 μ m filter membrane for further use. LEVs were respectively purified from 10-fold culture concentrate or 1 L culture supernatant using ultracentrifugation and density gradient ultracentrifugation according to previously reported methods for purification of EVs from Gram-positive bacteria [64, 65]. Protein concentrations were measured using the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Waltham, MA, USA) and the harvested LEVs were stored at -80°C until use.

Transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA)

LEVs were observed under TEM (JEOL-1230, JEOL, Tokyo, Japan). The samples were fixed with 1% glutaraldehyde, placed on a formvar-carbon-coated grid, and stained with 1% phosphotungstic acid. NTA was implemented using a ZetaView system (Particle Metrix, Meerbusch, Germany) to track the Brownian motion of the EVs suspended in PBS and generate size distribution data by applying the Stokes-Einstein equation.

Labeling of LEVs and in vivo imaging observation

Fluorescent labeling of LEVs was performed using a PKH26 kit (Sigma-Aldrich, St. Louis, MO, USA) and DiR fluorescent labeling kit (Sigma-Aldrich), respectively, by referring to the reported protocol. Subsequently, PKH26-labeled LEVs were incubated with cultured neurons for 24 h. Then, the cells or brain tissues were fixed and stained with 4', 6-diamidino-2-phenylindole (DAPI) and microtubule-associated protein 2, and the stained images were observed under confocal microscopy. Observation under fluorescence microscopy confirmed that LEVs were stained with PKH26 (red). Three C57BL/6 mice were selected and injected with DiR-LEVs through the tail vein (200 µg/mice). An in vivo imaging system (IVIS) was applied to observe the targeting of DiR-LEVs at 6 h, 12 h, 18 h, and 24 h after injection.

Isolation of primary cortical neurons

Primary cortical neurons were isolated from specific pathogen-free C57BL/6 fetal mice aged 16-18 days using a modified Yavin method ^[66]. The isolated cortices were then cut into small pieces, mixed with Dulbecco's Modified Eagle Medium (DMEM), transferred to sterile culture flasks, and ground 30 times with a pasteurized tube. The isolated cells were cultured at 37°C with 5% CO₂ on culture plates pre-coated with 50 µg/mL Poly-L-lysine at a density of 1×10^6 cells/well. Subsequent to 4-h incubation, the medium was renewed with a neuron-specific medium. Finally, the growth of cells was observed under a microscope.

Standard and oxygen-glucose deprivation (OGD) model

OGD was performed following the previous protocol ^[67]. Specifically, primary neurons were incubated with DMEM encompassing 10% fetal bovine serum in a hypoxic chamber (94% N₂, 5% CO₂, and 1% O₂) at 37°C. Then the cells were re-oxygenated for 24 h under standard cell culture conditions.

Lactate dehydrogenase (LDH) and cell viability analysis

Primary neuronal death was assessed by LDH release from the culture medium. Following OGD treatment and transfection, primary neurons were cultured in 6-well plates. Neurons were harvested and resuspended in 96-well plates, followed by 30-min culture in a CO₂ incubator at 37°C. The level of LDH release from the supernatant of cultured cells was determined using an LDH kit (Beyotime, Shanghai, China). LDH levels in the control group were expressed as 100%, and LDH levels in other groups were normalized to this value. Cell viability was assayed using Cell Counting Kit-8 (CCK-8) solution (Dojindo,

Kumamoto, Japan). CCK-8 solution was added to each well 2 h before the time points of the sample collection. The optical density (OD) value at 450 nm was evaluated using a microplate reader (BioTek, Winooski, VT, USA).

Western blot analysis

Western blot analysis was carried out as previously described ^[68]. Brain tissues around the core infarct area were attained from the ipsilateral cerebral hemisphere. For cell and brain tissues, lysates were prepared using Radio-Immunoprecipitation assay lysis buffer (Millipore, Billerica, MA, USA) containing protease inhibitor mixture, and proteins were quantified by the BCA method (Pierce, Rockford, IL, USA). The primary antibodies consisted of antibodies from Cell Signaling Technologies (Beverly, MA, USA) against Bcl-2-associated X (Bax, 14796), B cell leukemia/lymphoma 2 (Bcl-2, 3498), cleaved-caspase3 (9664), and caspase3 (9662) and antibodies (1: 1000) from Abcam (Cambridge, UK) against c-Fos (ab214672), TGF- β 1 (ab215715), and β -actin (ab8227). The membrane was re-probed with secondary antibodies for 2 h, and the positive bands were detected using chromogenic reagents.

miRNA mimic transfection and dual-luciferase reporter gene assay

Neurons were transfected with miR-101a-3p mimic. In short, miR-101a-3p mimic or its NC was incubated with Lipofectamine 2000 (11668027; Thermo Fisher Scientific). The mixture was added to the cultured cells. OGD/R was induced at least 24 h following transfection.

HEK 293T cells (2×10^6 cells) (Sigma-Aldrich) were co-transfected with miR-101a-3p mimic (RiboBio, Guangzhou, China) and luciferase reporter plasmids containing wild-type (WT) or mutant-type (MUT) binding sites between miR-101a-3p and the 3' untranslated region (UTR) of Notch1 according to the manuals of Lipofectamine™ 2000 transfection reagent. Relative luciferase activity was measured at 24 h post-transfection using the Dual-Luciferase Reporter Gene Assay Kit (Promega, Madison, WI, USA).

High-throughput sequencing

Neurons were pretreated with LEVs and PBS, followed by OGD induction. Then, sequencing was conducted in three biological replicates on the Illumina HiSeq™ 2500 platform. Differential miRNAs with expression fold changes > 1 and p values < 0.05 between the OGD + LEVs and OGD + PBS groups were utilized for analysis. Volcano and heatmaps of differential miRNA expression profiles were plotted using the R package TRAPR ^[26].

Quantitative real-time polymerase chain reaction (qRT-PCR)

miRNA levels in neurons were measured by qRT-PCR. Total RNA was extracted as per the protocols of Trizol (Thermo Fisher Scientific) and miRNAs in LEVs were extracted using a miRcute miRNA Isolation Kit (Tiangen Biotech, Beijing, China) in the light of the manufacturer's protocols. RNA samples were quantified at OD 260/280, and reverse transcription was conducted using a PrimeScript™ RT kit (RR037A, TAKARA, Tokyo, Japan). qRT-PCR was performed on an LightCycler 96 (Roche Group, Basel,

Switzerland) using a SYBR® Premix Ex Taq™ II kit (Tli RNaseH Plus) (RR420A, TAKARA). The fold changes in expression were calculated by the $2^{-\Delta\Delta C_t}$ method. The primers are listed in Appendix 1.

Immunofluorescence

The immunofluorescence method was implemented as previously reported [68]. In a word, the samples were fixed in 4% paraformaldehyde for 15 min, sealed in 5% serum at 37°C for 60 min, and then probed at 4°C overnight with antibodies (Abcam) against Neun (ab177487; 1: 200) and c-Fos (ab214672; 1: 500). After 3 PBS washes, the samples were re-probed with secondary antibody (1: 100; Cell Signaling Technology, Beverly, MA, USA) for 2 h at 37°C and stained with DAPI for 2 min at 37°C. Finally, the samples were washed once with PBS. Confocal microscopy (Olympus, Tokyo, Japan) was adopted to capture immunofluorescence images.

TdT-mediated dUTP Nick-End Labeling (TUNEL) staining

Cells or brain tissues were fixed with 4% paraformaldehyde. Subsequent to PBS washing of cells and brain tissues, TUNEL staining was performed using a One-Step TUNEL Apoptosis Assay Kit (C1086, Beyotime) following the manufacturer's manuals. After DAPI staining of cell nuclei, slides were washed and imaged with a fluorescent microscope. Cell apoptosis was analyzed using an image analyzer to calculate the positive cell rate.

Statistical analysis

SPSS version 21.0 (IBM Corp., Armonk, NY, USA) was utilized for data analysis. Econometric data were expressed as mean $\pm$ standard deviation. The unpaired *t*-test was employed to compare data between two groups. Data were compared among multiple groups using one-way analysis of variance (ANOVA) with Tukey's for post hoc tests. Data comparison between groups at different time points was performed by two-way ANOVA with Bonferroni for post hoc tests. $p < 0.05$ indicated statistically significant differences.

Declarations

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ETHICS APPROVAL

The animal study protocol was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University.

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AUTHOR CONTRIBUTIONS

Zhang Yang and Zidan Gao conceived and designed the study. Xiao fang Shi conducted experiments. Yifan Zhang and Hongqun Chen analyzed the data and wrote the manuscript. Lan Chu revised the manuscript, and all the authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated/analyzed in preparing this study are available upon request.

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Figures

Figure 1

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Lactobacillus plantarum

Figure 8

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