Experimental and Molecular Pathology 106 (2019) 102–108

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Ligustrazine promoted hypoxia-treated cell growth by upregulation of miR-135b in human umbilical vein endothelial cells

Shujing Wei[a,b], Hui Wang[a,b,*]

[a] Operating Room, Jining No.1 People's Hospital, Jining 272011, China
[b] Affiliated Jining No.1 People's Hospital of Jining Medical University, Jining Medical University, Jining 272067, China

ARTICLE INFO

Keywords:
Pressure ulcers
Hypoxia
Ligustrazine
miR-135b
JNK/SAPK, PI3K/AKT/mTOR

ABSTRACT

Background: Pressure ulcers are a kind of troublesome disease which caused by long-term pressure and subsequently lead to tissue festering necrosis because of sustained ischemia, hypoxia and malnutrition. In our study, we used hypoxia to stimulate human umbilical vein endothelial cells (HUVECs) to mimic pressure ulcers and investigated the effects of Ligustrazine (Lig) with multi-activities on HUVECs.

Methods: HUVECs were treated by hypoxia to induce cell injury. HUVECs were administrated with Lig and/or transfected with miR-135b inhibitor or negative control. Cell viability and cell apoptosis were detected by Cell Counting kit-8 assay and flow cytometry, respectively. The protein expression of Cyclin D1 and p53, the apoptosis-related proteins (Bcl-2, Bax, pro-/Cleaved-Caspas-3), and the JNK/SAPK and PI3K/AKT/mTOR pathways related proteins was examined by western blot.

Results: Hypoxia-induced injury presented by decreasing cell viability and increasing cell apoptosis. Then Lig administration enhanced cell viability and inhibited cell apoptosis. Importantly, miR-135b was upregulated by the treatment of Lig. Further studies revealed that transfection with miR-135b inhibitor led to the opposite result with decreasing cell viability and increasing cell apoptosis. In addition, Lig increased the phosphorylation of JNK, SAPK, PI3K, AKT and mTOR.

Conclusion: Lig promoted hypoxia-treated HUVECs cell growth as evidenced by increasing cell viability and reducing cell apoptosis. This process might be modulated by upregulation of miR-135b and subsequent activation of JNK/SAPK and PI3K/AKT/mTOR pathways.

1. Introduction

Pressure ulcers, also known as pressure sores, are due to long-term pressure on local tissues, which cause tissue festering necrosis because of sustained ischemia, hypoxia, malnutrition (McInnes et al., 2015). Pressure ulcers are a kind of common issue for people with limited mobility, nerve damage, especially for seniors (Allman, 1997; Moore and Cowman, 2014). Recent years, pressure ulcers are frequently occurred with the increasing population of the elders. Therefore, different strategies and approaches have been used for preventing and decreasing the incident of pressure ulcers, or increasing cure rate (Niu et al., 2016). For disease prevention, pressure ulcer risk assessment tools are used for diagnosis and identify the possibility for developing pressure ulcers (Moore and Cowman, 2014). For disease treatment, surgical treatment and nonsurgical treatment are the two important methods (Niu et al., 2016). However, till now, the outcome for pressure ulcers patients are not optimistic. New therapies or medicine are urgently needed for the treatment of pressure ulcers.

Ligustrazine (Lig, Fig. 1) is an important ingredient extracted from traditional herb *Chuanxiong* (Chen et al., 2015). Currently, overwhelming evidence revealed protective effects in various different diseases. For instance, Lig could ease renal dysfunction in murine (Feng et al., 2004); Lig alleviated burned induced liver injury in rats (Zheng et al., 2006). Importantly, Lig has been reported to be closely associated with pressure ulcers. Study from Niu et al. elucidated that Lig administration added with holistic nursing could be applied as a novel combination therapy for the treatment of pressure ulcers (Niu et al., 2016). However, the underlying mechanisms are still not well studied. This crucial information motivated us to explore deeper relationship between Lig and pressure ulcers.

The aberrant expression of microRNAs (miRNAs) was closely related with incidence or management of diseases, such as acute

* Corresponding author at: Operating Room, Jining No.1 People's Hospital, No. 99, Shixian Road, High-Tech District, Jining 272011, Shandong, China; Affiliated Jining No.1 People's Hospital of Jining Medical University, Jining Medical University, No.133, Hehua Road, Jining 272067, Shandong, China.
E-mail address: huiw0015@sina.com (H. Wang).

https://doi.org/10.1016/j.yexmp.2018.12.005
Received 11 September 2018; Received in revised form 27 November 2018; Accepted 17 December 2018
Available online 18 December 2018
0014-4800/

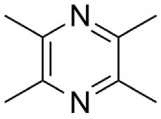

Fig. 1. The molecular formula of Ligustrazine.

myocardial infarction (Dong et al., 2009), liver injury (Meng et al., 2012), and pressure ulcers (Ji et al., 2017). Importantly, among these experimentally validated miRNAs, miR-135b was an interesting miRNA which revealed strongly response to hypoxia stimulation (Umezu et al., 2014; Zhang et al., 2013). Meanwhile, previous data suggested that tissue hypoxia was a main reason for the cause of pressure ulcers (Haleem et al., 2008). Therefore, we intend to elucidate whether miR-135b was involved in reducing hypoxia-induced response and the underlying mechanisms.

In our study, we used hypoxia to induce human umbilical vein endothelial cells (HUVECs) injury to establish an *in vitro* model of pressure ulcers. Our experiments were design to investigate the effects of Lig on hypoxia-induced injury and to identify the function of miR-135b in the biological process. We also detected the expression of cell cycle-related factors, such as CyclinD1. Because Cyclin D1 is a nuclear protein required for cell cycle progression in G1 (Baldin et al., 1993). Hope our study might provide a basement for the treatment of pressure ulcers in the future.

2. Material and methods

2.1. Cell model establish

HUVECs were purchased from Procell life science and technology Co., Ltd. (Cat. No.: CL-0122, Wuhan, China). The culture medium for HUVECs was Ham's F-12K medium (Thermo fisher scientific, Rockford, IL, USA), 100 μg/ml Heparin (Sigma #H3149), 50 μg/ml ECGs (BD #354006) plus 10% FBS (Gibco #10099141) according to the HUVECs cell culture instructions. Of note, HUVECs were enacted hypoxia treatment by cultured at 37 °C with 3% O_2 and 5% CO_2 while the normoxia culture was set at 21% O_2, meanwhile make sure that all the other conditions were same. The culture medium was renewed every 3 to 4 days.

2.2. Cell treatment

Lig was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). In our experiment, we dissolved Lig in dimethylsulfoxide (DMSO) and then pretreated cells were maintained in condition with or without Lig for 24 h.

2.3. Cell viability assay

Cell Counting Kit-8 (CCK-8, Yeasen, Shanghai, China) was used to examine cell viability. Firstly, HUVECs were seeded in 96-well plate at the density of 2×10^5 cells/well. Cells were maintained at 37 °C in humidified air with 5% CO_2. Secondly, added 10 μl CCK-8 solution and cells were incubated for 1 h. After incubation, absorption values for detecting cell viability were read at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

2.4. Apoptosis assay

Propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining (Yeasen, Shanghai, China) was used for determining cell apoptosis. In brief, the cells at the density of 100,000 cells/well were seeded in 6 well-plate. Treated cells were washed twice with precooling phosphate buffer saline (PBS) and centrifuged to resuspend in binding buffer. Then added 5 μl Annexin V-FITC, mixed gently and put in the dark and incubated for 15 min. In addition, added 5 μl PI to the sample. The apoptotic cells rate was measured with flow cytometer (Beckman Coulter, USA) according to the manufacture's instruction.

2.5. Cell transfection

miR-135b inhibitor and its negative control (NC, GenePharma Co., Shanghai, China) were transfected into HUVECs. Pre-treated cells at the density of 2×10^5 cells/well were seeded and incubated until the cells arrived at 70–80% confluence, they were transfected with miR-135b inhibitor and NC using Lipofectamine 2000 reagent (Invitrogen).

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

Total RNA was obtained from HUVECs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The Taqman MicroRNA Reverse Transcription Kit (Cat. No: 4366596, Thermo Fisher Scientific, Rockford, IL, USA) was used for converting miRNA to cDNA. The Taqman Universal Master Mix II (Cat. No: 4440040, Thermo Fisher Scientific) used for cDNA be generated in a reverse transcription reaction. These two cooperate with TaqMan MicroRNA Assay (Cat. No. 4427975, Assay ID: 002261, Thermo Fisher Scientific) were used for determining the miR-135b and U6 expression in HUVECs. U6 (Cat. NO. 4427975, Assay ID: 001973, Thermo Fisher Scientific) was the internal control for miR-135b.

2.7. Western blot

Western blot was used in our study to detect all the proteins expression. Protein was obtained from HUVECs using RIPA lysis buffer (Cat. No:R0010, Solarbio, Beijing, China) supplemented with protease inhibitors (Thermo Fisher Scientific). The BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used for determining proteins concentration. The western blot system was established using a Bio-Rad Bis-Tris Gel system following the manufacturer's instructions. Primary antibodies against the following: Cyclin D1 (ab134175), p53 (ab131442), β-actin (ab8227), Bcl-2 (ab32124), Bax (ab32503), pro-Caspase-3 (ab32499), cleaved-Caspase-3 (ab32042), all from Abcam (Cambridge, UK), stress-activated kinase/c-Jun N-terminal kinase (JNK/SAPK) (9252), phospho-SAPK/JNK (4668), phosphatidylinositol 3′-kinase (PI3K) (4249), Phospho-PI3K (13857), protein kinase B (AKT) (4685), phospho-AKT (4060), mammalian target of rapamycin (mTOR) (2983), phospho-mTOR (2971), all from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies were prepared in 5% blocking buffer and diluted according to the product instruction. These primary antibodies were incubated in membrane and maintained at 4 °C overnight at recommended concentration. Then for second antibody incubation, incubate with horseradish peroxidase (HRP) conjugated second antibody. Detection was performed by capturing the signals and analyzing the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

2.8. Statistical analysis

All data presented in our experiment showed as the mean ± standard deviation (SD) which based on at least three times experiments. Statistical analyses were performed using Graphpad 6.0 statistical software (GraphPad, San Diego, CA, USA). The *P*-values for Fig. 4A were calculated using student's *t*-test, while *P* values for all the rest figures were detected using a one-way analysis of variance (ANOVA). * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) all considered to be statistically difference.

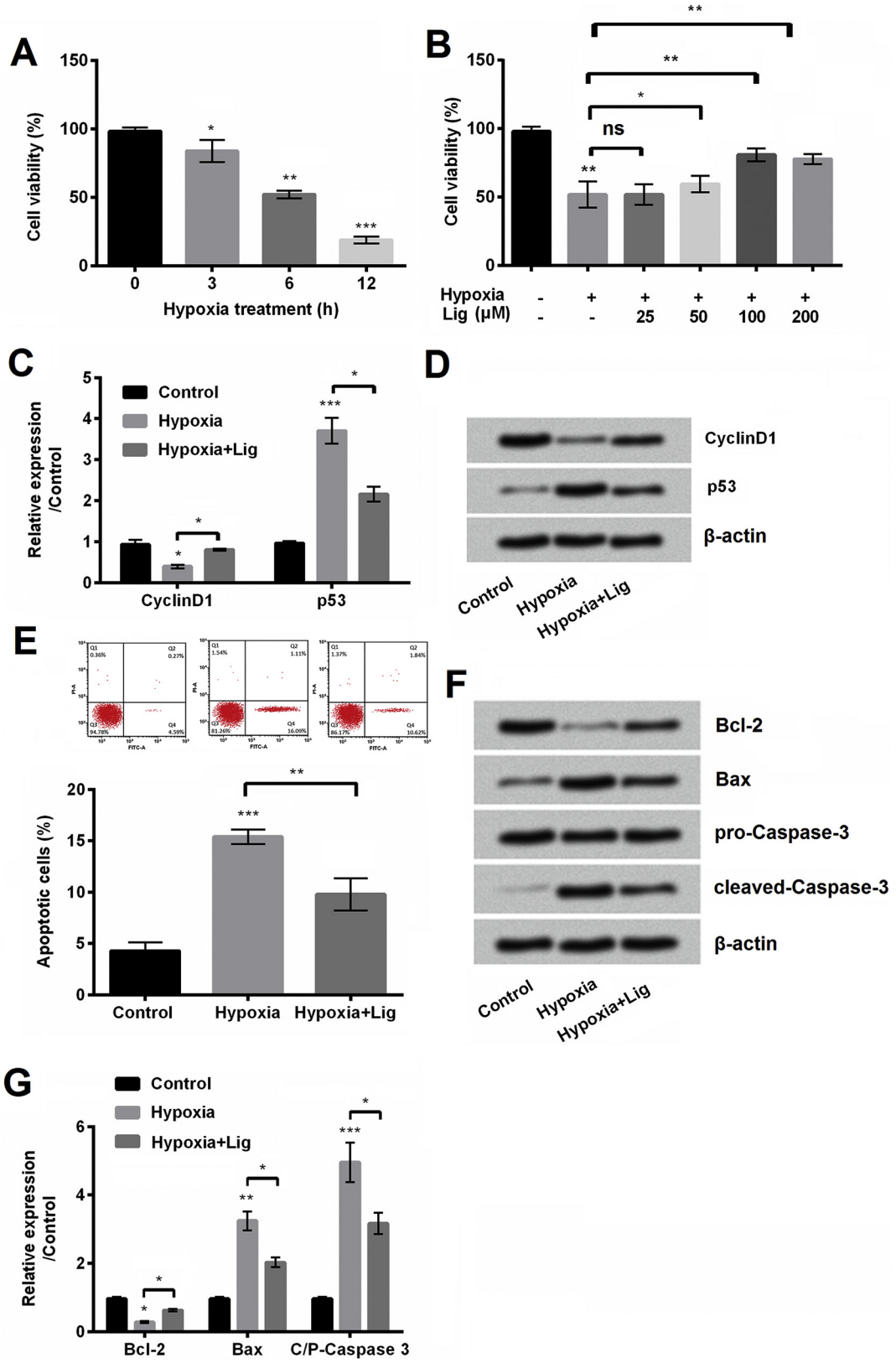

Fig. 2. Ligustrazine (Lig) alleviated hypoxia-induced human umbilical vein endothelial cells (HUVECs) injury. (A-B) Cell viability was detected by Cell Counting kit-8 with or without Lig administration in hypoxia treated HUVECs. (C-D) The protein expression of Cyclin D1 and p53 was analyses by western blot. (E) Cell apoptosis was examined by flow cytometry. (F-G) The expression of apoptosis-related proteins Bcl-2, Bax, pro-/Cleaved-Caspas-3 were detected by western blot. All data represented as mean ± standard deviation (SD). *P* value was detected by a one-way analysis of variance (ANOVA). Asterisk * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) were all considered as significant results.

3. Results

3.1. Lig alleviated hypoxia-induced HUVECs cell injury

HUVECs cells were exposed under hypoxia conditions in various time span (0, 3, 6 and 12 h), cell viability was detected. As shown in Fig. 2A, cell viability was significantly decreased with the increasing treatment time (3 h, $P < 0.05$; 6 h, $P < 0.01$; and 12 h, $P < 0.001$) as compared to the normoxia control. Hypoxia treatment time 6 h was chosen as the treatment time in the following experiments due to it was the half reducing time. Then we found that, for the hypoxia treated HUVECs cells, Lig supplement could statistically increase cell viability in a dose-dependent manner (50 μM, $P < 0.05$; 100 and 200 μM, $P < 0.01$). 100 μM was chosen in the subsequence experiments. Compared with control, hypoxia treatment significantly decreased the expression of Cyclin D1 ($P < 0.05$) and increased the expression of p53 ($P < 0.001$) while administration with Lig reversed the trend by upregulation of Cyclin D1 and downregulation of p53 (both $P < 0.05$, Fig. 2C–D). In the other hand, the percentage of apoptotic cells was dramatically increased by hypoxia treatment ($P < 0.001$) while decreased by after Lig administrated ($P < 0.01$, Fig. 2E). Simultaneously, the relative expression of apoptosis-related proteins was validated the results of apoptosis. As shown in Fig. 2F–G, anti-apoptotic protein Bcl-2 was downregulated ($P < 0.05$) while pro-apoptotic protein Bax ($P < 0.01$), and the ratio of cleaved-Caspase-3/pro-Caspase-3 were significantly increased ($P < .001$). On the other hand, the group with Lig led to the opposite results. Above all, Lig alleviated hypoxia-induced HUVECs cell injury.

3.2. Lig upregulated the expression of miR-135b

miR-135b was related with various cancers, such as myeloma (Umezu et al., 2014) and colorectal cancer (Xu et al., 2012). Importantly, miR-135b was reported to play vital roles in inflammation (Halappanavar et al., 2013), and miR-135b was also found to be involved in modulating hypoxia-treated HUVECs injury (Yang et al., 2018). In our study, we found that the expression of miR-135b was downregulated by hypoxia ($P < 0.05$) and upregulated by the administration with Lig ($P < 0.01$, Fig. 3). This result suggested that miR-135b might be involved in the protective effects of Lig on hypoxia-induced injury.

3.3. Lig alleviated hypoxia-induced HUVECs injury by upregulation of miR-135b

To validate the functions of miR-135b in HUVECs, miR-135b inhibitor and NC were transfected. As shown in Fig. 4A, downregulation of miR-135b by transfection with miR-135b inhibitor ($P < 0.01$) indicated high transfection efficiency. Further studies revealed that miR-135b silence significantly decreased cell viability ($P < 0.05$, Fig. 4B) and increased cell apoptosis ($P < 0.05$, Fig. 4E) as compared to the group transfection with NC. Meanwhile, we found that the expression of Cyclin D1 and p53 (Fig. 4C–D), and the apoptosis-related proteins Bcl-2, Bax and cleaved-Caspase-3 (Fig. 4F–G) were revealed the opposite results as compared with NC, which validated the results about the effects of miR-135b inhibitor on cell viability and cell apoptosis. Taken together, these results suggested that the protective effects of Lig on hypoxia-induced injury were through upregulation of miR-135b.

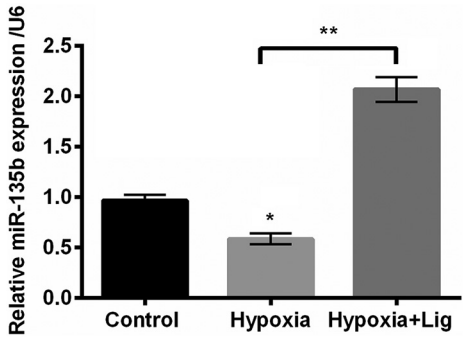

Fig. 3. Ligustrazine (Lig) upregulated the expression of miR-135b in hypoxia-treated human umbilical vein endothelial cells (HUVECs). The expression of miR-135b was detected by qRT-PCR. All data represented as mean ± standard deviation (SD). *P* value was detected by a one-way analysis of variance (ANOVA). Asterisk * ($P < 0.05$) and ** ($P < 0.01$) were considered as significant results.

3.4. Lig activated JNK/SAPK and PI3K/AKT/mTOR signal pathways through upregulation of miR-135b

JNK/SAPK and PI3K/AKT/mTOR signal pathways are closely related with pressure ulcer (Niu et al., 2016). In our study, we found that hypoxia treatment downregulated the phosphorylation of JNK, SAPK, PI3K, AKT, (all $P < 0.05$, Fig. 5A–B) and mTOR ($P < 0.01$, Fig. 5B) while supplementary of Lig led to the opposite results with significantly upregulated the phosphorylation of JNK, SAPK, PI3K, AKT and mTOR ($P < 0.01$ or $P < 0.001$, Fig. 5A–B). On the other hand, the upregulation trend induced by Lig was blocked by miR-135b silence (Fig. 5A–B). In a word, Lig activated JNK/SAPK and PI3K/AKT/mTOR signal pathways through upregulation of miR-135b.

4. Discussion

In our study, we investigated the effects of Lig on hypoxia-induced HUVECs injury. Results showed that Lig significantly increased cell viability and decreased cell apoptosis in hypoxia-stimulated HUVECs. Further studies demonstrated that Lig upregulated the expression of miR-135b which was dramatically downregulated by hypoxia. Subsequently, transfection with miR-135b inhibitor made the protective effects of Lig on hypoxia-induced injury disappeared in HUVECs, which indicated that effects of Lig on alleviating hypoxia-induced cell injury was through upregulation of miR-135b. Importantly, we also found that the protective effects of Lig might be through activation of JNK/SAPK and PI3K/AKT/mTOR signal pathways.

Pressure ulcers with low outcome and increasing ulcer-related death, bring sustainable burden both in physically and financially aspect to patients and their families (Bauer et al., 2016). Furthermore, for pressure ulcers, applied pressure also causes tissue distortion, resulting in shear stresses near the bony prominence (Dealey et al., 2015). An inflammatory response after pressure ulcers was also observed and it can further led to tissue damage and skin breakdown, and decreased functional outcomes (Krishnan et al., 2016). Therefore, the diagnosis, prevention and treatment for pressure ulcers are urgently needed. In our study, we used hypoxia to stimulate HUVECs to mimic hypoxia-induced pressure ulcers. Then the effects of Lig on hypoxia-induced injury were investigated. Lig is an important ingredient which possesses many diverse properties. Overwhelming evidence suggested that Lig revealed protective effects in cell injuries induced by various kinds of stimulants. For instance, Lig alleviated alcohol-induced liver injury *via* decreasing oxidative stress (Lu et al., 2015); Lig protected rat liver from acute econazole-induced injury (Liu et al., 2002); Lig revealed obviously protective effects in pulmonary tissues against scald-induced injury (Gao et al., 2012); Lig protected against tumor necrosis factor α (TNF-α)-induced endothelial dysfunction (Wu et al., 2012). Interestingly, in the current study, Lig administration increased cell viability and inhibited cell apoptosis in HUVECs, which indicated that Lig alleviated hypoxia-induced injury. The result of this study was similar to those previous studies, such as Lig alleviated hypoxia-induced vascular endothelial cell injury (Lin et al., 2004).

Cyclin D1 and p53 belong to cell cycle-related modulators (Conesa-

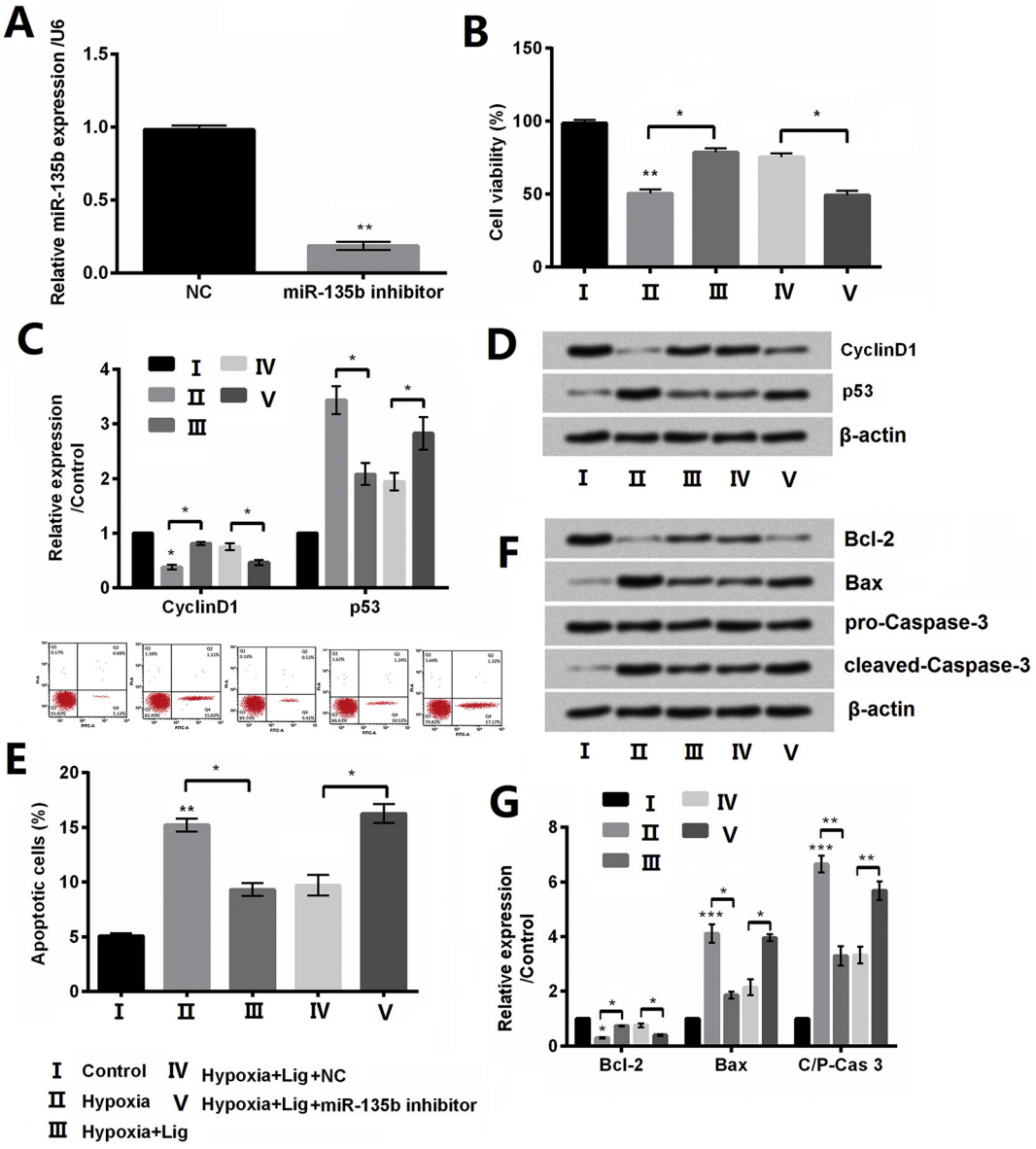

Fig. 4. Ligustrazine (Lig) alleviated hypoxia-induced human umbilical vein endothelial cells (HUVECs) injury *via* upregulation of miR-135b. (A) miR-135b inhibitor was transfected into HUVECs and miR-135b expression was detected by qRT-PCR. (B) Cell viability was detected by Cell Counting kit-8 with or without Lig administration in hypoxia treated HUVECs. (C-D) The protein expression of Cyclin D1 and p53 was analyses by western blot. (E) Cell apoptosis was examined by flow cytometry. (F-G) The expression of apoptosis-related proteins Bcl-2, Bax, pro-/Cleaved-Caspas-3 were detected by western blot. All data represented as mean ± standard deviation (SD). *P*-values for Fig. 4A were calculated using student's *t*-test, while *P* values for all the rest figures were detected using a one-way analysis of variance (ANOVA). Asterisk * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) were all considered as significant results.

Zamora et al., 2009). Cyclin D1 plays a vital role in developing of cell cycle progression in the stage of G1 (Sherr and Roberts, 1995) while p53 is responsible for cell cycle arrest (Bilancio et al., 2017). Importantly, p53 was also reported to be involved in cell apoptosis (Speidel, 2010), and apoptosis induced by p53 could be treated an effective approach for cancer suppression (Lebedeva et al., 2003). In our study, Cyclin D1 was significantly upregulated while p53 was downregulated by the treatment of Lig in hypoxia-stimulated cells. This result provided evidence in Lig increased cell viability and decreased cell apoptosis.

In addition, Bcl-2 is closely correlated with mitochondria, which exerts crucial functions in cell apoptosis (Zhang et al., 2015). Bcl-2 is an anti-apoptotic protein and Bax and cleaved-Caspase-3 are pro-apoptotic proteins (Il'in et al., 2016; Yang et al., 2011). The upregulation of Bcl-2, and downregulation of Bax and cleaved-Caspase-3 by the treatment of Lig validated the conclusion that Lig inhibited cell apoptosis. Previous study from Zhao et al. proved that Lig reduced OGD-induced PC12 cell apoptosis through decreasing the expression of Bax and cleaved-Caspase-3 and the ratio of Bax/Bcl-2 (Zhao et al., 2018), which revealed similar results compared to our study.

Further experiments were performed to explore the underlying mechanisms of Lig's protective effects on hypoxia-induced HUVECs injury. A wide range of previous literatures suggested that diversity of miRNAs were dysregulated by the stimulation of hypoxia (Du et al., 2012; Ghosh et al., 2010). Meanwhile, miR-135b was reported to be highly regulated (Jia et al., 2017) and acted as a tumor promoter (Zhang et al., 2013). On the other hand, miR-135b was found to be downregulated in response to hypoxia-induced vascular endothelial injury (Yang et al., 2018). In our study, we found that Lig dramatically increased the expression of miR-135b in hypoxia-treated HUVECs.

A serious of studies was conducted to elucidate the functions of miR-135b in hypoxia-induced injury. Results showed that transfection with

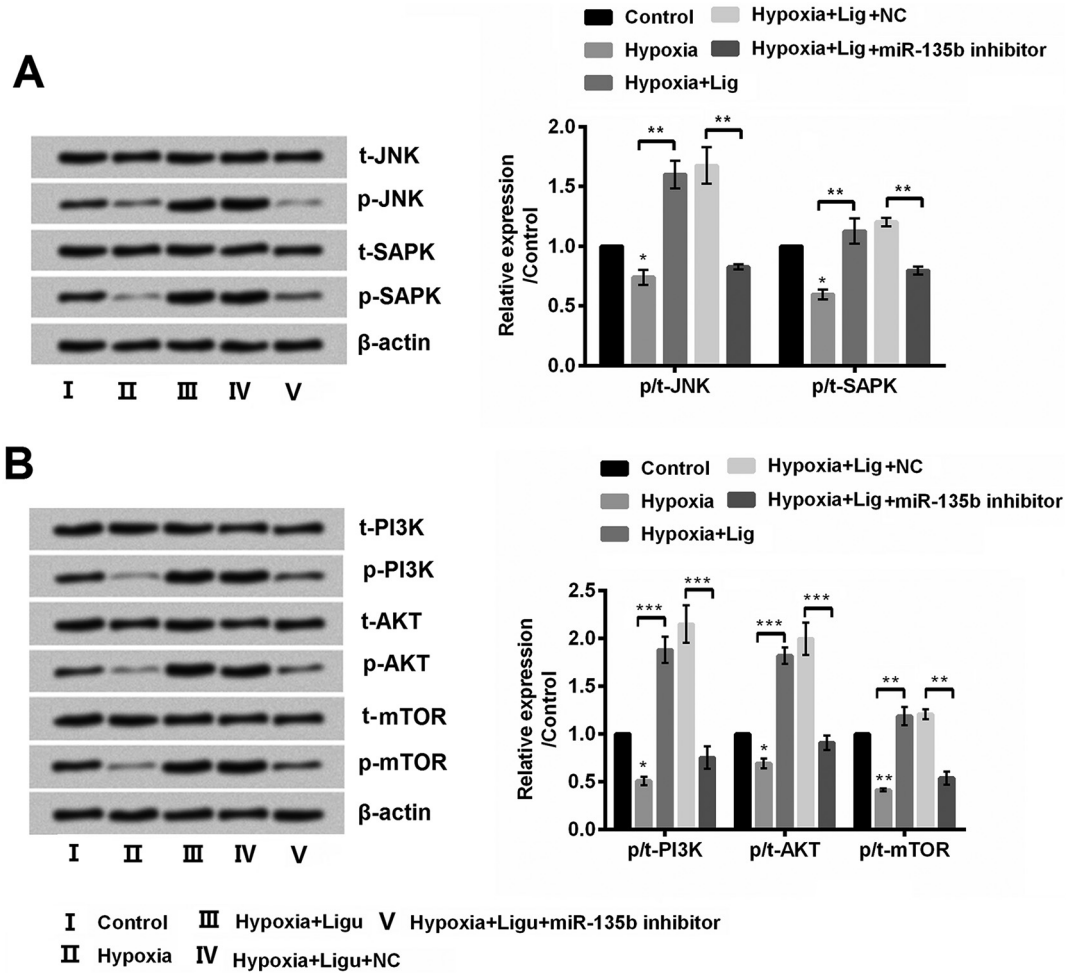

Fig. 5. Ligustrazine (Lig) activated c-Jun N-terminal kinase/stress-activated kinase (JNK/SAPK) and phosphatidylinositol 3′-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signal pathways through upregulation of miR-135b. (A-B) The phosphorylation of JNK, SAPK, PI3K, AKT and mTOR were detected by western blot. All data represented as mean ± standard deviation (SD). *P* value was detected by a one-way analysis of variance (ANOVA). Asterisk * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) were all considered as significant results.

miR-135b inhibitor (miR-135b silence) blocked the protective effects of Lig presented by decreasing cell viability and increasing cell apoptosis. These results were consistent with the previous studies that miR-135b silence enhanced hypoxia-induced vascular endothelial cell injury (Yang et al., 2018).

PI3K/AKT/mTOR are involved in pressure sores (Niu et al., 2016) and hypoxia stimulant was reported to activate JNK/SAPK signal pathway (Kunz and Ibrahim, 2003). JNK/SAPK and PI3K/AKT/mTOR play important roles in cell proliferation, growth and metastasis (Heras-Sandoval et al., 2014; Zhang et al., 2016). Also, previous evidence proved that various medicines revealed their protective effects through activation of JNK/SAPK and PI3K/AKT/mTOR signal pathway (Gong et al., 2004; Zhang et al., 2014). Similar results were showed in our results that Lig activated JNK/SAPK and PI3K/AKT/mTOR signal pathways through upregulation of miR-135b.

5. Conclusions

In conclusion, our study investigated the effects of Lig on hypoxia-induced cell injury, and we found that Lig could alleviate hypoxia-induced HUVECs injury through upregulation of miR-135b and activation of JNK/SAPK and PI3K/AKT/mTOR signal pathways. These findings presented here, of potential beneficial impact of Lig on pressure ulcers should be strengthened by additional preclinical and clinical studies *in vivo*. Our study might provide a fundamental research for the treatment of pressure ulcers in the future.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

None.

References

Allman, R.M., 1997. Pressure ulcer prevalence, incidence, risk factors, and impact. Clin. Geriatr. Med. 13, 421–436.
Baldin, V., et al., 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. 7, 812–821.
Bauer, K., et al., 2016. Pressure ulcers in the United States' inpatient population from 2008 to 2012: results of a retrospective nationwide study. Ostomy Wound Manage 62, 30–38.
Bilancio, A., et al., 2017. Bisphenol A induces cell cycle arrest in primary and prostate cancer cells through EGFR/ERK/p53 signaling pathway activation. Oncotarget 8,

115620–115631.
Chen, Y., et al., 2015. Novel oxazolidinone antibacterial analogues with a substituted Ligustrazine C-ring unit. Chem. Biol. Drug Des. 86, 682–690.
Conesa-Zamora, P., et al., 2009. Effect of human papillomavirus on cell cycle-related proteins p16, Ki-67, Cyclin D1, p53, and ProEx C in precursor lesions of cervical carcinoma: a tissue microarray study. Am. J. Clin. Pathol. 132, 378–390.
Dealey, C., et al., 2015. Challenges in pressure ulcer prevention. Int. Wound J. 12, 309–312.
Dong, S., et al., 2009. MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. J. Biol. Chem. 284, 29514–29525.
Du, R., et al., 2012. Hypoxia-induced down-regulation of microRNA-34a promotes EMT by targeting the Notch signaling pathway in tubular epithelial cells. PLoS One 7, e30771.
Feng, L., et al., 2004. Effect of Ligustrazine on ischemia-reperfusion injury in murine kidney. Transplant. Proc. 36, 1949–1951.
Gao, C., et al., 2012. Effects of Ligustrazine on pulmonary damage in rats following scald injury. Burns 38, 743–750.
Ghosh, G., et al., 2010. Hypoxia-induced microRNA-424 expression in human endothelial cells regulates HIF-alpha isoforms and promotes angiogenesis. J. Clin. Invest. 120, 4141–4154.
Gong, Y., et al., 2004. Erianin induces a JNK/SAPK-dependent metabolic inhibition in human umbilical vein endothelial cells. In Vivo 18, 223–228.
Halappanavar, S., et al., 2013. IL-1 receptor regulates microRNA-135b expression in a negative feedback mechanism during cigarette smoke-induced inflammation. J. Immunol. 190, 3679–3686.
Haleem, S., et al., 2008. Pressure sores and hip fractures. Injury 39, 219–223.
Heras-Sandoval, D., et al., 2014. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cell. Signal. 26, 2694–2701.
Il'in, D.A., et al., 2016. In vitro study of cytophysiological characteristics of multinuclear macrophages from intact and BCG-infected mice. Bull. Exp. Biol. Med. 160, 668–671.
Ji, X., et al., 2017. Rs739837 polymorphism in MiR-885-3p binding site within 3'-untranslated region of vitamin D receptor is associated with a decreased risk of pressure ulcers. Cell. Physiol. Biochem. 44, 2129–2137.
Jia, L., et al., 2017. miR-182 and miR-135b Mediate the Tumorigenesis and Invasiveness of Colorectal Cancer Cells via Targeting ST6GALNAC2 and PI3K/AKT Pathway. Dig. Dis. Sci. 62, 3447–3459.
Krishnan, S., et al., 2016. Early detection of pressure ulcer development following traumatic spinal cord injury using inflammatory mediators. Arch. Phys. Med. Rehabil. 97, 1656–1662.
Kunz, M., Ibrahim, S.M., 2003. Molecular responses to hypoxia in tumor cells. Mol. Cancer 2, 23.
Lebedeva, I.V., et al., 2003. Restoring apoptosis as a strategy for cancer gene therapy: focus on p53 and mda-7. Semin. Cancer Biol. 13, 169–178.
Lin, R., et al., 2004. Effect of Ligustrazine on injury of vascular endothelial cells ECV-304 induced by hypoxia and lack of glucose. Zhongguo Zhong Yao Za Zhi 29, 462–465.
Liu, C.F., et al., 2002. Hepatoprotective and therapeutic effects of tetramethylpyrazine on acute econazole-induced liver injury. Planta Med. 68, 510–514.
Lu, C., et al., 2015. Ligustrazine prevents alcohol-induced liver injury by attenuating hepatic steatosis and oxidative stress. Int. Immunopharmacol. 29, 613–621.
McInnes, E., et al., 2015. Support surfaces for pressure ulcer prevention. Cochrane Database Syst. Rev. 341–342 Cd001735.
Meng, F., et al., 2012. Epigenetic regulation of miR-34a expression in alcoholic liver injury. Am. J. Pathol. 181, 804–817.
Moore, Z.E., Cowman, S., 2014. Risk assessment tools for the prevention of pressure ulcers. Cochrane Database Syst. Rev. 153–154 Cd006471.
Niu, J., et al., 2016. Therapeutic effect of external application of Ligustrazine combined with holistic nursing on pressure sores. Med. Sci. Monit. 22, 2871–2877.
Sherr, C.J., Roberts, J.M., 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 9, 1149–1163.
Speidel, D., 2010. Transcription-independent p53 apoptosis: an alternative route to death. Trends Cell Biol. 20, 14–24.
Umezu, T., et al., 2014. Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1. Blood 124, 3748–3757.
Wu, H.J., et al., 2012. Protective effects of ligustrazine on TNF-alpha-induced endothelial dysfunction. Eur. J. Pharmacol. 674, 365–369.
Xu, X.M., et al., 2012. Expression of miR-21, miR-31, miR-96 and miR-135b is correlated with the clinical parameters of colorectal cancer. Oncol. Lett. 4, 339–345.
Yang, L., et al., 2011. ZL11n is a novel nitric oxide-releasing derivative of farnesylthiosalicylic acid that induces apoptosis in human hepatoma HepG2 cells via MAPK/mitochondrial pathways. Biochem. Biophys. Res. Commun. 409, 752–757.
Yang, S., et al., 2018. Inhibition of miR-135b by SP-1 promotes hypoxia-induced vascular endothelial cell injury via HIF-1alpha. Exp. Cell Res. 370, 31–38.
Zhang, L., et al., 2013. MicroRNA-135b acts as a tumor promoter by targeting the hypoxia-inducible factor pathway in genetically defined mouse model of head and neck squamous cell carcinoma. Cancer Lett. 331, 230–238.
Zhang, J., et al., 2014. Sevoflurane postconditioning protects rat hearts against ischemia-reperfusion injury via the activation of PI3K/AKT/mTOR signaling. Sci. Rep. 4, 7317.
Zhang, X., et al., 2015. Taurine induces the apoptosis of breast cancer cells by regulating apoptosis-related proteins of mitochondria. Int. J. Mol. Med. 35, 218–226.
Zhang, Y., et al., 2016. miR-202 contributes to sensitizing MM cells to drug significantly via activing JNK/SAPK signaling pathway. Zhonghua Xue Ye Xue Za Zhi 37, 987–992.
Zhao, T., et al., 2018. Ligustrazine suppresses neuron apoptosis via the Bax/Bcl-2 and caspase-3 pathway in PC12 cells and in rats with vascular dementia. IUBMB Life 70, 60–70.
Zheng, H., et al., 2006. Effect of Ligustrazine on liver injury after burn trauma. Burns 32, 328–334.