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Effects of miR-532-5p on human brain microvascular endothelial cells damage induced by ox-LDL via down-regulating CLIC4 expression

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Abstract: The effect of miR-532-5p on human brain microvascular endothelial cells damage induced by ox-LDL is studied. HBMEC-3 were cultured and treated with ox-LDL for 24 h to establish a model of cell oxidative damage. The expression of miR-532-5p was detected by qRT-PCR and that of CLIC4 was detected by Western blot. miR-NC, miR-532-5p mimics, si-NC, si-CLIC4, miR-532-5p mimics and pcDNA, miR-532-5p mimics and pcDNA-CLIC4 were transfected into HBMEC-3 cells, respectively, using ox -LDL processing for 24 h. Flow cytometry was used to detect the apoptotic rate. The dual luciferase reporting experiment verified the relationship between miR-532-5p and CLIC4. The ox-LDL treatment led to lower expression of miR-532-5p ($p<0.05$), higher expression of CLIC4 ($P <0.05$), enhanced content of MDA ($p<0.05$), decreased activities of SOD and CAT ($p<0.05$), increased apoptosis rate ($p<0.05$), higher protein level of Bax ($p<0.05$), and lower protein level of Bcl-2 ($p<0.05$). Compared with ox-LDL + miR-NC group and ox-LDL + si-NC group, ox-LDL + miR-532-5p group and ox-LDL + si-CLIC4 group had decreased content of MDA ($P <0.05$), increased activities of SOD and CAT ($p<0.05$), decreased apoptosis rate ($p<0.05$), lower level of Bax ($p<0.05$), and higher level of Bcl-2 ($p<0.05$). The miR-532-5p mitigates human brain microvascular endothelial cells damage induced by ox-LDL via down-regulating CLIC4 expression.

Keywords: CLIC4, miR-532-5p, ox-LDL, apoptosis.

INTRODUCTION

Brain microvascular endothelial cell is capable of maintaining brain function as well as normal microenvironment of the brain. Inflammation and oxidative stress in the brain tissue can cause endothelial cell injury and promote neurovascular disease (Fan *et al*., 2017). It has been reported that ox-LDL can promote injury of cerebral microvascular endothelial cells and give rise to cardiovascular and cerebrovascular diseases. Investigation into the molecular mechanism of endothelial cell injury can help prevent and treat vascular diseases. MicroRNA (miRNA) can inhibit the expression of target genes by binding to target genes. It has been reported that miRNA is associated with vascular endothelial cell inflammation and injury, angiogenesis. Bioinformatics analysis suggests that chloride intracellular channel 4 (CLIC4) may be a target gene of miR-532-5p. Studies have shown that increased expression of CLIC4 protein can promote apoptosis of cerebral cortex neurons and may also participate in the process of white matter injury (Ma *et al*., 2017). However, it is unknown whether miR-532-5p affects human microvascular endothelial cell injury by target regulation of CLIC4 expression. In this study, a model of cell oxidative damage is established to study the expressions of miR-532-5p and CLIC4 and their effects on apoptosis and oxidative stress (Pan *et al*., 2016).

MATERIALS AND METHODS

Human brain microvascular endothelial cell (HBMEC-3) was brought from Guangzhou Jennio Biotech Co., Ltd. ox-LDL was purchased from Beijing Solarbio Science & Technology Co., Ltd. DMEM and FBS were purchased from Gibco, USA. Lipofectamine 2000 was purchased from Invitrogen, USA. miR-532-5p oligonucleotide mimics (miR-532-5p mimics) and negative control mimic NC sequence (miR-NC), CLIC4 small interfering RNA (si-CLIC4), scrambled nonsense negative sequence (si-NC), anti-miR-532-5p and anti-miR-NC were purchased from Shanghai GenePharma Co., Ltd. pcDNA3.1 was purchased from Shanghai LMAIBio Engineering Co., Ltd . RIPA lysate was purchased from Beijing Baiao Laibo Technology Co., Ltd. Bicinchoninicacid (BCA) protein quantitative detection kit was purchased from Qiyi Biotechnology (Shanghai) Co., Ltd. Enhanced electrochemiluminescence reagent (ECL) was purchased from Beijing Notlas Biotechnology Co., Ltd. Rabbit anti-human CLIC4 antibody was purchased from Abcam, USA.

(1) Experimental grouping. HBMEC-3 cells were cultured in DMEM medium containing 10% FBS in a constant temperature. The cell was inoculated into a 24-well plate (100 μL/well), cultured in a medium containing 50 μg/mL of ox-LDL for 24 h to establish a cell oxidative damage model (Yang, *et al*., 2016), which was denoted as ox-LDL group. Con group: The same dose of PBS solvent was added for 24 h processing. With reference to Lipofectamine2000 instructions, transfect miR-NC, miR-532-5p mimics, si-NC, si-CLIC4, miR-532-5p mimics and pcDNA, miR-532-5p mimics and pcDNA-CLIC4 to HBMEC-3 cells, respectively, followed by 24h culture in a medium containing 50 μg/mL of

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ox-LDL, which were denoted as ox-LDL + miR-NC group, ox-LDL + miR-532-5p group, ox-LDL + si-NC group, ox-LDL + si-CLIC4 group, ox-LDL + miR-532-5p + pcDNA group, ox-LDL + miR-532-5p + pcDNA-CLIC4 group, respectively (Shown as table 1).

Total RNA in HBMEC-3 cells was extracted by Trizol method. With reference to the reverse transcription kit instructions, synthesize total RNA into cDNA. qRT-PCR reaction was performed using cDNA as a template. The reaction was carried out at 95℃ for 2 min, at 95℃ for 15 s, at 60℃ for 30 s, at 72℃ for 30 s, with 40 cycles in total. MiR-532-5p took U6 as internal reference, CLIC4 took GAPDH as internal reference, and the relative expression of miR-532-5p and CLIC4 mRNA was calculated using 2-ΔΔCt method (Yang *et al*., 2016).

HBMEC-3 cells in logarithmic phase were collected from each group, MDA content and SOD and CAT activities were detected in strict accordance with the kit instructions. (2) Detection of apoptotic rate by flow cytometry. HBMEC-3 cells in logarithmic phase were collected from each group, added with pre-cooled PBS, centrifuged for 6 min at 4°C. After supernatant was discarded, pre-cooled PBS was added for further centrifugation. The cells were resuspended by binding buffer. After addition of 5 μL PI, incubate it for 10 min, and detect the apoptosis rate of each group within 1 h using FACS Calibur flow cytometer and Cellauest software.

(3) Detection of target genes of miR-532-5p. StarBase predictions indicate that 3'UTR of CLIC4 contains complementary sequence of miR-532-5p. By inserting CLIC4-3'UTR sequence containing binding site and mutation site into the luciferase reporter gene vector, WT-CLIC4 and MUT-CLIC4 were constructed. WT-CLIC4, MUT-CLIC4, miR-NC, miR-532-5p mimics were co-transfected into HBMEC-3 cells. According to the instructions of Lipofectamine2000, miR-NC, miR-532-5p mimics, anti-miR-NC, anti-miR-532-5p were transfected into HBMEC-3 cells, and CLIC4 protein level in each group was detected by Western blot (Chen *et al*., 2017).

(4) Detection of CLIC4, Bax, Bcl-2 protein expression by Western blot. HBMEC-3 cells were collected, followed by addition of 400 μL of RIPA lysate to obtain total cell proteins. Protein samples were dissolved in SDS loading buffer and boiled in boiling water for 15 min. 50 μg of protein sample was taken for SDS-PAGE. After the completion of electrophoresis, the separated protein gel was transferred to a PVDF membrane, blocked at room temperature for 2 h, followed by addition of primary antibody diluent (1: 1000), incubated at 4℃ for 24 h, washed with TBST to incubate secondary antibody dilution (1: 2000) for 1 h, with addition of ECL drop wise for development. Gray value of each band was then analyzed using ImageJ software.

STATISTICAL ANALYSIS

Data were processed using SPSS 21.0. The measurement data was expressed as ($\bar{x}$ ±s), with t test conducted for comparison. The difference was considered statistically significant when $p < 0.05$.

RESULTS

Expression of miR-532-5p and CLIC4

Compared with Con group, ox-LDL group had a lower expression of miR-532-5p in human brain microvascular endothelial cells ($p<0.05$), but a higher expression of CLIC4 mRNA and protein ($p<0.05$), as shown in fig.1, table 2.

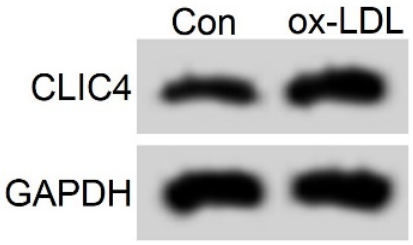

Fig. 1: CLIC4 protein expression

Effects of miR-532-5p overexpression on ox-LDL induced oxidative stress

Compared with Con group, the other three ox-LDL inducing groups had a significantly increased MDA content ($p<0.05$), but significantly lower SOD and CAT activities ($p<0.05$). Compared with ox-LDL+miR-NC, MDA content of human brain microvascular endothelial cells in ox-LDL + miR-532-5p group is significantly reduced ($p<0.05$), while SOD and CAT activities are increased ($p<0.05$), as shown in table 3.

Effects of miR-532-5p overexpression on ox-LDL-induced apoptosis

Compared with Con group, ox-LDL group had a higher apoptosis rate ($p<0.05$), a higher Bax protein level ($p<0.05$), but a lower Bcl-2 protein level ($p<0.05$). Compared with ox-LDL+miR-NC group, ox-LDL + miR-532-5p group had a lower apoptosis rate ($p<0.05$), a lower Bax protein level ($p<0.05$), but a higher Bcl-2 protein level ($p<0.05$), as shown in fig. 2 and table 4.

Effects of inhibited CLIC4 expression on ox-LDL-induced human brain microvascular endothelial cell injury

Compared with ox-LDL+si-NC group, ox-LDL+ si-CLIC4 group had a lower CLIC4 protein level ($p<0.05$), a lower MDA content ($p<0.05$), higher SOD and CAT activities ($p<0.05$), a lower apoptosis rate ($p<0.05$), a lower Bax protein level ($p<0.05$), and a higher Bcl-2 protein level ($p<0.05$), as shown in fig. 3, table 5.

Table 1: Experimental grouping parameters

Group	Treatment
Con Group	The same dose of PBS solvent was added for 24-h processing
ox-LDL Group	The medium containing 50μg /mL of ox-LDL was used for culturing for 24 h
ox-LDL+miR-NC Group	miR-NC was transfected into HBMEC-3 cells and incubated with medium containing 50g μg/mL of ox-LDL for 24 h
ox-LDL+miR-532-5p Group	miR-532-5p mimics was transfected into HBMEC-3 cells and incubated with medium containing 50g μg/mL of ox-LDL for 24 h
ox-LDL+si-NC Group	si-NC was transfected into HBMEC-3 cells and incubated with medium containing 50g μg/mL of ox-LDL for 24 h
ox-LDL+si-CLIC4 Group	si-CLIC4 was transfected into HBMEC-3 cells and incubated with medium containing 50g μg/mL of ox-LDL for 24 h
ox-LDL+miR-532-5p +pcDNA Group	miR-532-5p mimics and pcDNA were co-transfected into HBMEC-3 cells and incubated with medium containing 50g μg/mL of ox-LDL for 24 h
ox-LDL+miR-532-5p+pcDNA-CLIC4 Group	miR-532-5p mimics and pcDNA-CLIC4 were co-transfected into HBMEC-3 cells and incubated with medium containing 50g μg/mL of ox-LDL for 24 h

Table 2: Expression of miR-532-5p and CLIC4 (x ±s, n=9)

Group	miR-532-5p	CLIC4 mRNA	CLIC4 protein
Con	1.00±0.08	1.02±0.09	0.48±0.04
ox-LDL	0.39±0.04*	3.26±0.31*	0.92±0.08*
t	20.460	20.818	14.758
p	0.000	0.000	0.000

Table 3: Effects of miR-532-5p overexpression on ox-LDL-induced oxidative stress (x±s, n=9)

Group	miR-532-5p	MDA(nmol/mg prot)	SOD (U/mg prot)	CAT (U/mg prot)
Con	1.00±0.07	2.36±0.24	18.22±1.71	3.25±0.32
ox-LDL	0.43±0.04*	8.65±0.81*	7.36±0.74*	0.98±0.09*
ox-LDL+miR-NC	0.41±0.05	8.71±0.85	7.15±0.72	0.95±0.08
ox-LDL+miR-532-5p	0.79±0.07#	2.98±0.29#	15.36±1.51#	3.01±0.29#
F	213.993	286.634	181.886	281.581
p	0.000	0.000	0.000	0.000

Table 4: Effects of miR-532-5p overexpression on ox-LDL-induced apoptosis (x±s, n=9)

Group	Apoptosis rate (%)	Bcl-2 protein	Bax protein
Con	6.58±0.64	0.65±0.06	0.29±0.03
ox-LDL	23.69±2.37*	0.24±0.03*	0.74±0.07*
ox-LDL+miR-NC	24.85±2.44	0.23±0.02	0.75±0.06
ox-LDL+miR-532-5p	11.58±1.19#	0.56±0.05#	0.38±0.03#
F	218.488	228.649	200.621
p	0.000	0.000	0.000

Table 5: Effects of inhibition of CLIC4 expression on ox-LDL-induced human brain microvascular endothelial cell injury (x±s, n=9)

Group	CLIC4 protein	MDA (nmol/mg prot)	SOD (U/mg prot)	CAT (U/mg prot)	Apoptosis rate (%)	Bcl-2 protein	Bax protein
Con	0.41±0.04	2.24±0.23	18.14±1.77	3.31±0.33	7.25±0.73	0.67±0.06	0.28±0.03
ox-LDL	0.88±0.08*	8.59±0.85*	7.68±0.77*	0.91±0.09*	23.69±2.33*	0.23±0.03*	0.76±0.07*
ox-LDL+si-NC	0.89±0.07	8.55±0.82	7.65±0.78	0.84±0.08	25.14±2.41	0.22±0.03	0.77±0.06
ox-LDL+si-CLIC4	0.49±0.04#	3.46±0.34#	13.25±1.33#	2.87±0.28#	14.65±1.42#	0.51±0.05#	0.42±0.04#
F	159.290	256.851	150.292	297.650	18.562	221.886	198.627
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 6: Dual luciferase reporter experiment (x±s, n=9)

Group	WT-CLIC4	MUT-CLIC4
miR-NC	0.98±0.09	1.00±0.07
miR-532-5p	0.42±0.04*	0.97±0.08
t	17.058	0.847
p	0.000	0.410

Table 7: Regulation of CLIC4 protein expression by miR-532-5p (x±s, n=9)

Group	CLIC4 protein
miR-NC	0.43±0.04
miR-532-5p	0.22±0.02*
anti-miR-NC	0.41±0.04
anti-miR-532-5p	0.87±0.08#
F	272.490
p	0.000

Table 8: Up-regulation of CLIC4 expression reverses the effect of miR-532-5p overexpression on ox-LDL-induced human brain microvascular endothelial cell injury (x±s, n=9)

Group	CLIC4 protein	MDA (nmol/mg prot)	SOD (U/mg prot)	CAT (U/mg prot)	Apoptosis rate (%)	Bcl-2 protein	Bax protein
ox-LDL+miR-NC	0.87±0.07	8.81±0.88	7.24±0.72	0.88±0.03	24.69±2.41	0.22±0.03	0.76±0.07
ox-LDL+miR-532-5p	0.41±0.04*	2.84±0.26*	16.39±1.64*	3.11±0.31*	11.25±1.13*	0.58±0.05*	0.37±0.03*
ox-LDL+miR-532-5p +pcDNA	0.39±0.03	2.81±0.27	17.19±1.71	3.18±0.32	10.58±1.03	0.61±0.06	0.35±0.04
ox-LDL+miR-532-5p +pcDNA-CLIC4	0.75±0.07#	7.62±0.74#	9.36±0.94#	1.35±0.16#	20.36±2.04#	0.34±0.03#	0.64±0.06#
F	171.220	244.190	127.680	25.803	140.783	162.342	134.182
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000

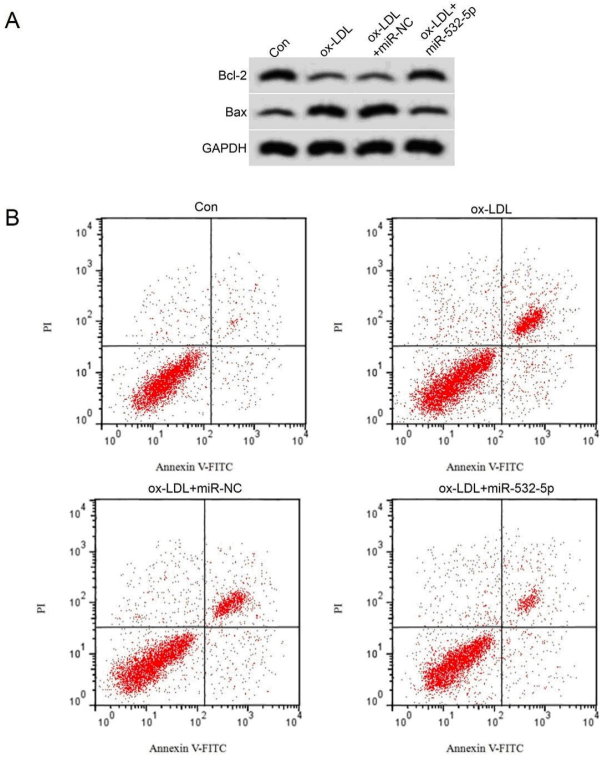

Fig. 2: Effects of miR-532-5p overexpression on ox-LDL-induced apoptosis (A: Apoptosis-associated protein expression; B: Flow cytometry of apoptosis)

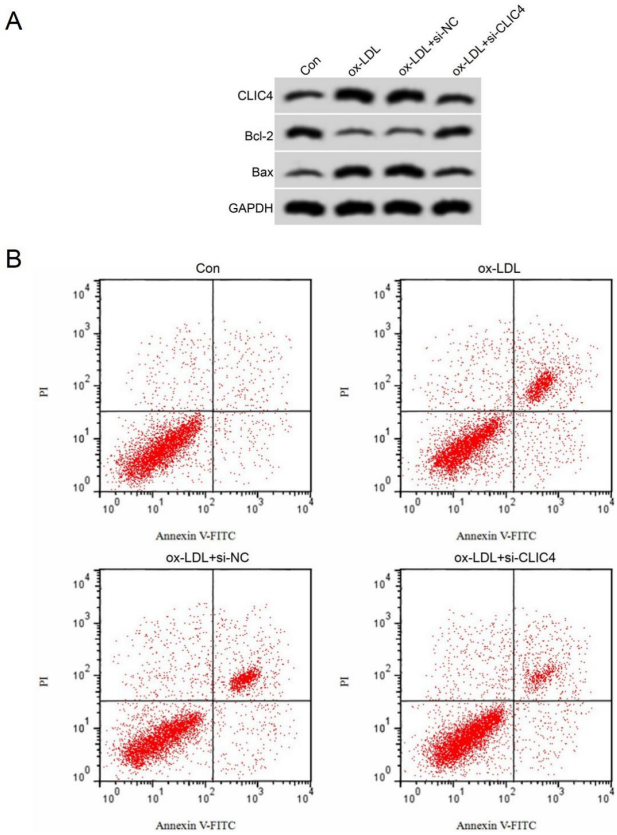

Fig. 3: Effects of inhibition of CLIC4 expression on ox-LDL-induced apoptosis (A: CLIC4 and apoptosis-related protein expression; B: Flow cytometry of apoptosis)

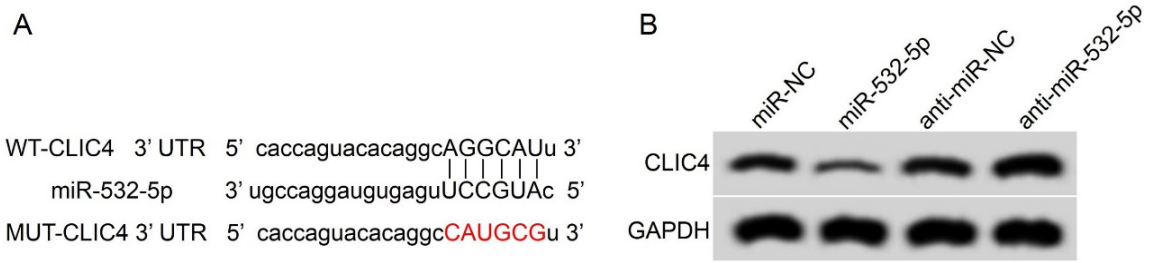

Fig. 4: Target regulation of CLIC4 expression by miR-532-5p

Target regulation of CLIC4 expression by miR-532-5p

StarBase prediction indicates that miR-532-5p has a binding site for CLIC4 (fig. 4A). According to dual luciferase reporter experiment, in the cell experiment transfected with WT-CLIC4, luciferase activity is lower in miR-532-5p group (p<0.05); in the cell experiment transfected with MUT-CLIC4, luciferase activity of miR-532-5p group has no difference from that of miR-NC group (p>0.05), as shown in table 6. Western blot results show that miR-532-5p group has a lower CLIC4 protein level than miR-NC group (p<0.05); anti-miR-532-5p group has a higher CLIC4 protein level than anti-miR-NC group (p<0.05), as shown in fig. 4B and table 7.

Up-regulation of CLIC4 expression reverses the effects of miR-532-5p overexpression on ox-LDL-induced human brain microvascular endothelial cell injury

Compared with ox-LDL+miR-532-5p+pcDNA group, ox-LDL+miR-532-5p+pcDNA-CLIC4 group has a higher CLIC4 protein level (p<0.05), a higher MDA content (p<0.05), lower SOD and CAT activities (p<0.05), a higher cell apoptosis rate (p<0.05), a higher Bax protein level (p<0.05), a lower Bcl-2 protein level (p<0.05), as shown in fig. 5, table 8.

DISCUSSION

It has been reported that miRNAs are abnormally expressed in brain microvascular endothelial cells after

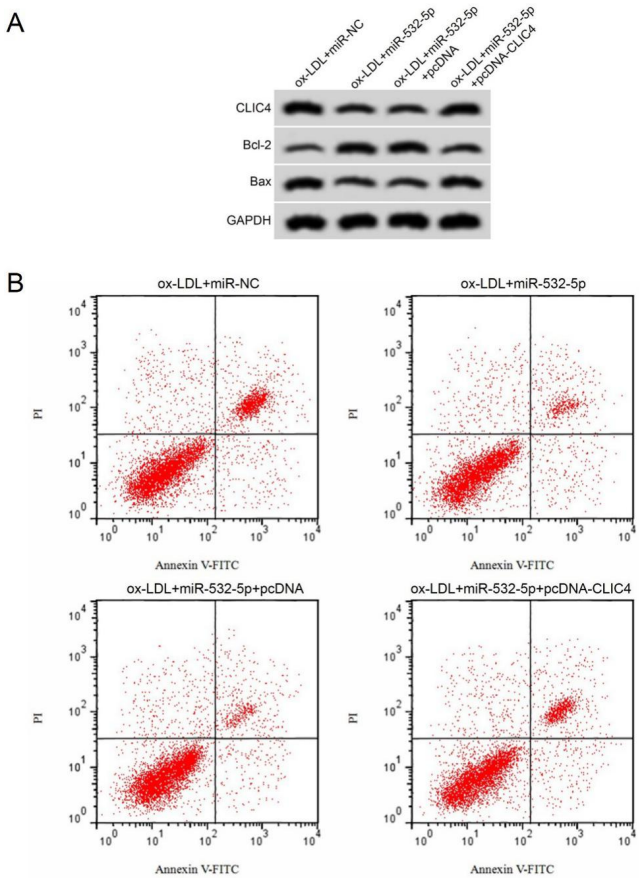

Fig. 5: Up-regulation of CLIC4 expression reverses effect of miR-532-5p over expression on apoptosis rate of ox-LDL-induced of human brain microvascular endothelial cell (A: CLIC4 and apoptosis-related protein expression; B: Flow cytometry of apoptosis)

hypoxia treatment or ox-LDL treatment, achieving regulation of processes like cell proliferation, angiogenesis and apoptosis (Pan, *et al*., 2016; Hu, *et al*., 2018). However, the molecular mechanism of some miRNAs in brain microvascular endothelial cell injury has not yet been elucidated (Yuan *et al*., 2015).

This study shows that reduced expression of miR-532-5p can promote brain microvascular endothelial cell injury. This study indicates that ox-LDL treatment leads to increased MDA content and reduced SOD and CAT activities in brain microvascular endothelial cells, which is consistent with the results reported in related literatures (Ma, *et al*., 2017). Also, it can be seen from this study that miR-532-5p overexpression could reduce MDA content and enhance SOD and CAT activities. Studies have shown that increased Bax protein expression can promote cell apoptosis by activating caspase cascade reaction, while Bcl-2 can inhibit cell apoptosis (Fan, *et al*., 2017). This study shows that apoptosis rate of cerebral microvascular endothelial cells is significantly increased, Bax expression is increased and Bcl-2 expression is reduced after ox-LDL treatment. However, MiR-532-5p overexpression treatment can significantly reduce the effects of ox-LDL on cerebral microvascular endothelial cell apoptosis and promote Bcl-2 expression while inhibiting Bax expression, suggesting that miR-532-5p overexpression may inhibit ox-LDL-induced apoptosis of human brain microvascular endothelial cells by regulating Bax and Bcl-2 expressions. CLIC4 expression is up-regulated during glial cell injury and can aggravate glial cell injury (Yuan, *et al*., 2015). Studies have shown that CLIC4 can activate the body's inflammation-related signaling pathways so as to facilitate the occurrence and development of inflammatory diseases. This study shows that CLIC4 expression is up-regulated in ox-LDL-induced human brain microvascular endothelial cells. This study found that inhibition of CLIC4 expression could significantly inhibit ox-LDL-induced apoptosis and oxidative stress response of cerebral microvascular endothelial cells, suggesting that inhibition of CLIC4 expression can relieve ox-LDL-induced brain microvascular endothelial cell injury. This study confirmed that miR-532-5p could achieve target binding with CLIC4 and negatively regulate CLIC4 expression. Further analysis shows that after up-regulation of CLIC4 expression, MDA content increases, SOD, SOD, CAT

activities decrease, cell apoptosis rate increases, Bax protein level increases, Bcl-2 protein level decreases.

CONCLUSION

In conclusion, miR-532-5p is down-regulated in ox-LDL-induced human brain microvascular endothelial cells while CLIC4 expression is up-regulated. MiR-532-5p overexpression can inhibit ox-LDL-induced apoptosis of cerebral microvascular endothelial cells by target interference with CLIC4 expression, thereby reducing oxidative stress response, enhancing cell antioxidant capacity, which lays a theoretical foundation for revealing the pathogenesis of cerebrovascular diseases.

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