doi.org/10.36721/PJPS.2020.33.3.SP.1427-1433.1

Effects of velvet antler polypeptides on Alzheimer's disease cell model via miR-613 / HDAC6 pathway

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Abstract: To study the effect of velvet antler polypeptides (VAP) on Alzheimer's disease (AD) cell model, $A\beta_{25-35}$ was used to induce SK-N-SH cells to obtain AD cell model. The MDA, SOD, GSH-Px levels were determined using relevant kits. Flow cytometry was conducted to detect apoptosis, Western blot was employed to measure Bcl-2, Bax, HDAC6 protein expression, and qPCR was used to assay microRNA (miR)-613 and HDAC6 mRNA levels. TargetScan prediction combined with dual luciferase reporting experiments was conducted to analyze the targeting relationship between miR-613 and HDAC6. miR-613 was transfected in SK-N-SH cells; Alternatively, anti-miR-613 was transfected, followed by $A\beta_{25-35}$ and 80 mg/L of VAP. The AD model cells showed increased MDA content, apoptosis rate, Bax protein expression, HDAC6 mRNA and protein expression, but lower SOD, GSH-Px activities, Bcl-2 protein level, and miR-613 expression ($p<0.05$). VAP reduced MDA content, apoptosis rate, Bax protein expression, HDAC6 mRNA and protein expression, but enhanced SOD, GSH-Px activities, Bcl-2 protein level, and miR-613 expression ($p<0.05$). Over-expression of miR-613 increased SOD, GSH-Px activities, and Bcl-2 protein expression in AD model cells, but reduced HDAC6 protein levels, MDA content, apoptosis rate, and Bax protein levels ($p<0.05$). VAP may regulate $A\beta_{25-35}$-induced apoptosis so as to treat Alzheimer's disease.

Keywords: Velvet antler polypeptides, Alzheimer's disease, miR-613, HDAC6, apoptosis, oxidative stress

INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disease which may lead to dementia symptoms. Although there are many studies on the pathogenesis of AD, no appropriate treatment has been found (Elufioye, Chinaka, Oyedeji, 2019). So far, the treatment for AD can only slow disease progress and relieve symptoms, but cannot realize curative effect. Therefore, it is quite necessary to find other more effective treatment options. Velvet antler polypeptides (VAP) are extracts of velvet antler. In addition to immunomodulatory effects, it also brings some biological benefits, including regeneration of neurons, blood vessels, connective tissue, cartilage and bones (Xin, et al., 2017). Studies have shown that velvet antler polypeptides play a role in protecting hydrogen peroxide-induced vascular endothelial cell damage, and its mechanism is possibly to improve intracellular oxidative stress levels. Velvet antler polypeptides have a reverse effect on oxidative damage of osteoarthritis chondrocytes (Li, Zhao, Zhou, 2011). MicroRNA (miRNA/miR) -613 has down-regulated expression in myocardial cell injury induced by ischemia-reperfusion, thus promoting myocardial cell apoptosis induced by ischemia-reperfusion (Wu, et al., 2016). Histone deacetylase 6 (HDAC6) has increased expression in Alzheimer's disease mouse model, whose knockout can significantly improve cognitive function of mice. HDAC6 inhibitors have protective effects on hypoxia-reoxygenation injury in diabetic cardiomyocytes (Li et al., 2016; Wang et al., 2020). Nevertheless, the effect of velvet antler polypeptides and miR-613 on Alzheimer's disease cell model, the targeting relationship between miR-613 and HDAC6, and whether velvet antler polypeptides affect the cell model damage of Alzheimer's disease by regulating miR-613/HDAC6 expression are yet unknown. In this work, amyloid β_{25-35} ($A\beta_{25-35}$) was adopted to induce neuroblastoma SK-N-SH cell damage, and an AD cell model was constructed to analyze the effect of velvet antler polypeptides on oxidative stress and apoptosis of cells. Moreover, its potential mechanism of action is explored by combining miR-613 and HDAC6.

MATERIALS AND METHODS

Main reagents

SK-N-SH cells were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, and velvet antler was purchased from Zuojia Institute of Specialty Products, Jilin Province. DMEM medium and fetal calf serum were provided by Gibco, US. TRIzol was purchased from Thermo Fisher, US. MDA, SOD, GSH-Px kits were purchased from Nanjing Jiancheng Bioengineering Institute. Annexin δ-FITC/PI kit was purchased from Nanjing Kaiji Biological Company. Bcl-2, Bax, HDAC6 and GAPDH were purchased from CST, US.

Preparation of Velvet Antler Polypeptides

According to the method of Zhu Wenhe et al., fresh velvet antler was crushed and added with homogenate (pH 3.5

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acetic acid) to make slurry. Add 65% ethanol, stir and soak for 2h, followed by centrifugation to collect the supernatant. Velvet antler polypeptides were obtained after vacuum concentration and freeze-drying.

Cell Culture and Processing

SK-N-SH cells were cultured in DMEM medium under a humid environment with 5% CO2 at 37°C. SK-N-SH cells in logarithmic growth phase were inoculated into a 96-well plate. When SK-N-SH cells were attached to the culture plate, the culture medium was removed and the cells were treated with DMEM medium containing 20, 40, 80 mg/L velvet antler polypeptides for 24 h of culture at 37°C. Subsequently, AD cell model was constructed by incubating SK-N-SH cells in DMEM medium containing $A\beta_{25-35}$ (5 moL/L) at 37°C for 24 h (Xu, et al., 2018). There were five groups, including normal control (Con) group with no treatment; model (AD) group treated with only $A\beta_{25-35}$; AD + VAP-L group (low-dosage velvet antler polypeptides treatment group) treated with $A\beta_{25-35}$ + 20 mg/L velvet antler polypeptides; AD+VAP-M group (middle-dosage velvet antler polypeptides treatment group) treated with $A\beta_{25-35}$ + 40 mg/L velvet antler polypeptides; AD + VAP-H group (high-dosage velvet antler polypeptides treatment group) treated with $A\beta_{25-35}$ + 80 mg/L velvet antler polypeptides.

Cell Transfection

When the cells density grew to 70%, miR-613, miR-NC, anti-miR-613, anti-miR-NC were used to transfect SK-N-SH cells in strict accordance with the steps specified in Lipofectamine 2000 reagent instructions. Cells transfected with miR-613 and miR-NC were treated with $A\beta_{25-35}$. Cells transfected with anti-miR-613 and anti-miR-NCwere applied with Aβ25-35combined with velvet antler polypeptides 80 mg/L.

Detection of MDA, SOD, GSH-Px levels by kit

SK-N-SH cells were washed with PBS, followed by centrifugation to collect the supernatant. MDA content and SOD, GSH-Px activity were detected using MDA, SOD, GSH-Px detection kit.

Detection of apoptosis by flow cytometry

SK-N-SH cells were washed with PBS. 1×10^5 cells were treated with Binding Buffer to make a cell suspension, followed by addition of Annexin V-FITC (5 μL) and PI (5 μL)for incubation for 15 min in the dark. Cell apoptosis was detected by flow cytometry.

Western blot detection of Bcl-2, Bax, HDAC6 protein expression

SK-N-SH cell proteins were extracted with RIPA lysis buffer. The protein content was determined by BCA kit. After incubation in sealing solution (10% skim milk) at room temperature for 2 h, PVDF membrane was incubated with anti-Bcl-2, Bax, HDAC6 and GAPDH antibodies at 4°C. The membrane was then washed three times with Tris-HCl-Tween buffered saline (TBST) and incubated with a horseradish peroxidase-labeled secondary antibody. The protein-antibody complexes were testedby ECL detection system.

qPCR detection of miR-613 and HDAC6 mRNA levels

Total RNA of cells was extracted using TRIzol reagent. Subsequently, reverse transcription was conducted to synthesize cDNA. QPCR amplification was performed according to SYBR Green Mix instructions. The miR-613 primer sequence is 5'-CTTCGTCGGCTCTTCCATAC ATACT-3'(forward),5'-TTCACTTAGATACAGCTACG T-3'(reverse). The HDAC6 primer sequence is 5'-AGGT AAAGGGGAAGAAACAAA-3'(forward), 5'-TGCGGA TGAGTTGTTTCTGGC-3'(reverse). The internal reference U6 primer sequence is 5'-AGAGCCTTCCG TATTAGGTGCC-3'(forward), 5'-GATCTACTGGCCA CTGGATGC-3'(reverse). Ct values of miR-613, HDAC6 and the control gene U6 were calculated. The expression of miR-613 and HDAC6 mRNA was calculated using $2^{-\Delta\Delta Ct}$ method.

Experimental verification of miR-613's targeted regulation of HDAC6 by dual luciferase report

The TargetScan website (http://www.targetscan.org/) predicts that the 3'UTR of HDAC6 contains a nucleotide sequence complementary to miR-613. WT-HDAC6 andMUT-HDAC6 luciferase reporter plasmids containing miR-613 binding targets were established and co-transfected with miR-NC and miR-613 using Lipofectamine 2000 reagent, and dual luciferase activity was measured 48 h later.

STATISTICAL ANALYSIS

The data were processed using SPSS 22.0 software. The results were expressed in the form of mean ± standard deviation ($\bar{x}\pm s$), with independent sample T test for comparison between two groups, one-way analysis of variance for comparison among multiple groups, and SNK-q test for multiple comparisons between groups. The difference was considered statistically significant when $p<0.05$.

RESULTS

Effect of velvet antler polypeptides on oxidative stress in AD cell models

The test results of cell oxidative stress indicate that compared with Con group, AD group cells had significantly increased MDA content and significantly reduced SOD and GSH-Px activities; compared with AD group, AD model cells treated with 20, 40 and 80 mg/L velvet antler polypeptides exhibited significantly reduced MDA content, but significantly enhanced SOD and GSH-Px activities ($p<0.05$, table 1).

Table 1: Effect of velvet antler polypeptides on oxidative stress in AD cell model ($\bar{x}$ ±s, n=9)

Group	MDA(μmol/L)	SOD(U/mg)	GSH-Px(U/mg)
Con	6.58±0.66	45.36±4.21	71.56±7.12
AD	29.36±2.47a	10.69±1.18a	25.14±2.53a
AD+VAP-L	22.41±2.35b	17.96±1.78b	38.46±3.88b
AD+VAP-M	15.66±1.72bc	28.22±2.86bc	51.39±5.18bc
AD+VAP-H	8.46±0.85bcd	39.65±3.58bcd	62.58±6.47bcd
F	260.210	217.527	109.914
p	0.000	0.000	0.000

Note: Compared with Con group, $^{a}p<0.05$; compared with AD group, $^{b}p<0.05$; compared with AD + VAP-L group, $^{c}p<0.05$; compared with AD + VAP-M group, $^{d}p<0.05$. Con; control; AD: alzheimer disease; VAP: velvet antler polypeptides; VAP-L: velvet antler polypeptides-lowdosage; VAP-M: velvet antler polypeptides-middle dosage; VAP-H: velvet antler polypeptides-high dosage; MDA: malondialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidise.

Table 2: Effect of Velvet Antler Polypeptides on Apoptosis in AD Cell Model ($\bar{x}$ ±s, n=9)

Group	Apoptosis rate (%)	Bcl-2protein	Baxprotein
Con	7.65±0.74	0.74±0.07	0.24±0.03
AD	26.36±2.61a	0.30±0.03a	0.65±0.06a
AD+VAP-L	20.58±2.45b	0.43±0.04b	0.52±0.05b
AD+VAP-M	15.33±1.87bc	0.55±0.05bc	0.40±0.04bc
AD+VAP-H	10.32±1.05bcd	0.67±0.06bcd	0.29±0.03bcd
F	144.513	105.566	133.342
p	0.000	0.000	0.000

Table 3: Effect of velvet antler polypeptides on the expression of miR-613 and HDAC6 in AD cell model ($\bar{x}$ ±s, n=9)

Group	miR-613	HDAC6 mRNA	HDAC6protein
Con	1.00±0.08	1.00±0.09	0.43±0.03
AD	0.41±0.04a	3.25±0.32a	0.88±0.08a
AD+VAP-L	0.53±0.05b	2.63±0.25b	0.75±0.07b
AD+VAP-M	0.66±0.06bc	2.06±0.21bc	0.64±0.04bc
AD+VAP-H	0.79±0.07bcd	1.53±0.15bcd	0.51±0.05bcd
F	124.507	147.420	90.193
p	0.000	0.000	0.000

Note: Compared with Con group, $^{a}p<0.05$; compared with AD group, $^{b}p<0.05$; compared with AD + VAP-L group, $^{c}p<0.05$; compared with AD + VAP-M group, $^{d}p<0.05$. Con: control; AD: alzheimer disease; VAP: velvet antler polypeptides; VAP-L: velvet antler polypeptides-lowdosage; VAP-M: velvet antler polypeptides-middle dosage; VAP-H: velvet antler polypeptides-high dosage

Table 4: Dual luciferase reporter experiment ($\bar{x}$ ±s, n=9)

Group	WT-HDAC6	MUT-HDAC6
miR-NC	1.00±0.09	0.97±0.08
miR-613	0.44±0.04a	0.99±0.07
t	17.058	0.564
p	0.000	0.580

Note: Compared with miR-NC group, $^{a}p<0.05$. WT: wild type; MUT: mutant

Table 5: Regulation of HDAC6 protein expression by miR-613($\bar{x}$ ±s, n=9)

Group	HDAC6 protein
miR-NC	0.42±0.04
miR-613	0.21±0.02a
anti-miR-NC	0.40±0.04
anti-miR-613	0.86±0.08b
F	272.490
p	0.000

Note: Compared with miR-NC group, $^{a}p<0.05$; compared with anti-miR-NC group, $^{b}p<0.05$

Table 6: Effect of miR-613 over expression on AD cell model damage ($\bar{x}$ ±s, n=9)

Group	miR-613	HDAC6 protein	MDA (μmol/L)	SOD (U/mg)	GSH-Px (U/mg)	Apoptosis rate (%)	Bcl-2protein	Baxprotein
AD+miR-NC	1.00±0.08	0.84±0.08	28.46±2.71	9.87±0.99	22.14±2.23	25.33±2.14	0.28±0.03	0.69±0.06
AD+miR-613	2.37±0.24a	0.38±0.04a	11.69±1.17a	32.14±3.22a	57.13±5.22a	12.47±1.36a	0.61±0.06a	0.33±0.03a
F	16.246	15.429	17.044	19.832	18.492	15.215	14.758	16.100
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: Compared with AD + miR-NC group, [a]p<0.05. AD: alzheimer disease; MDA: malondialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase

Table 7: Inhibition of miR-613 expression reverses the effect of velvet antler polypeptides on AD cell model damage ($\bar{x}$ ±s, n=9)

Group	miR-613	HDAC6 protein	MDA (μmol/L)	SOD (U/mg)	GSH-Px (U/mg)	Apoptosis rate (%)	Bcl-2 protein	Baxprotein
AD	1.00±0.09	0.86±0.08	30.69±3.11	9.96±0.98	23.65±2.33	26.33±2.61	0.27±0.03	0.71±0.06
AD+VAP	2.68±0.26a	0.48±0.04a	9.31±0.93a	38.42±3.54a	61.58±6.18a	11.47±1.15a	0.65±0.06a	0.30±0.03a
AD+VAP+anti-miR-NC	2.73±0.27	0.46±0.04	9.25±0.92	40.22±4.13	63.59±6.33	10.39±1.07	0.66±0.06	0.28±0.03
AD+VAP+anti-miR-613	1.42±0.14b	0.74±0.06b	22.69±2.31b	16.32±1.58b	31.47±3.12b	20.33±2.14b	0.38±0.03b	0.59±0.05b
F	165.837	105.727	240.517	256.825	161.758	149.225	153.333	208.101
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: Compared with AD group, [a]p<0.05 ; compared with AD + VAP + anti-miR-NC group, [a]p<0.05 AD: alzheimer disease; VAP: velvet antler polypeptides;

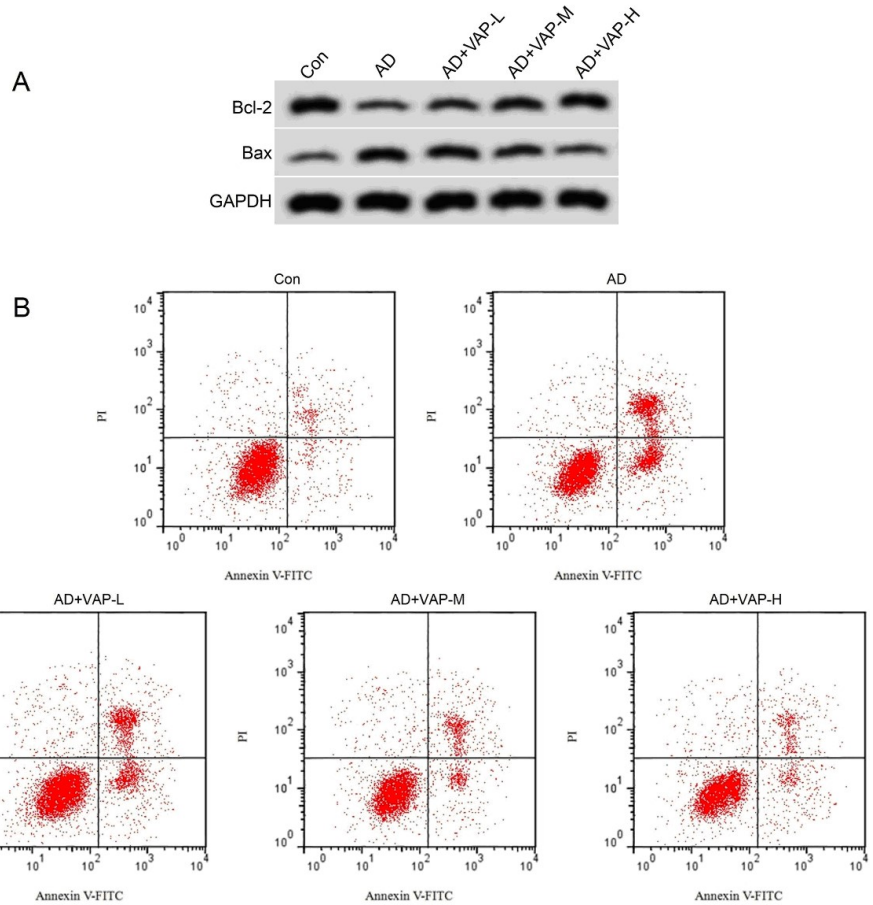

Fig. 1: Effect of velvet antler polypeptides on apoptosis in AD cell model (A: Apoptosis-related protein expression; B: Flow cytometry of apoptosis)

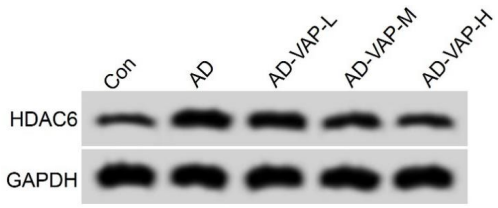

Fig. 2: HDAC6 protein expression

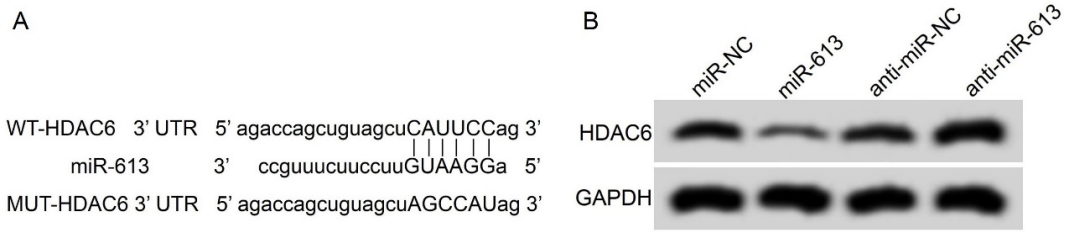

Fig. 3: Target expression of HDAC6 by miR-613 (A: 3'UTR of HDAC6 containing a nucleotide sequence complementary to miR-613; B: HDAC6 protein expression)

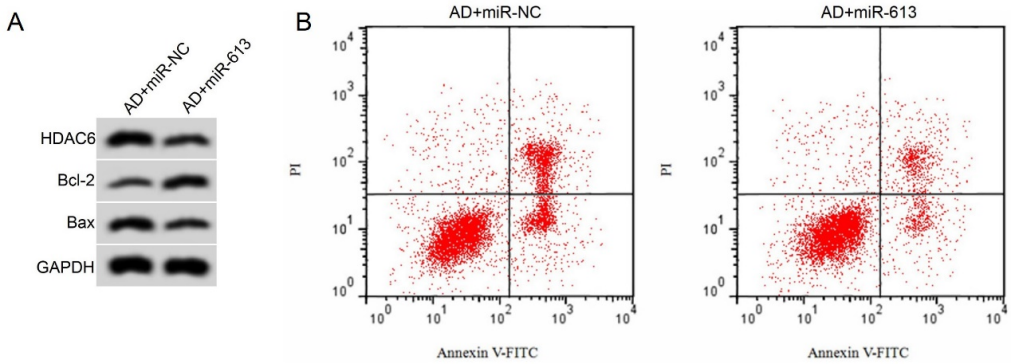

Fig. 4: Effect of miR-613 over expression on apoptosis in AD cell model (A: Expression of HDAC6 and apoptosis-related proteins; B: Flow cytometry of apoptosis)

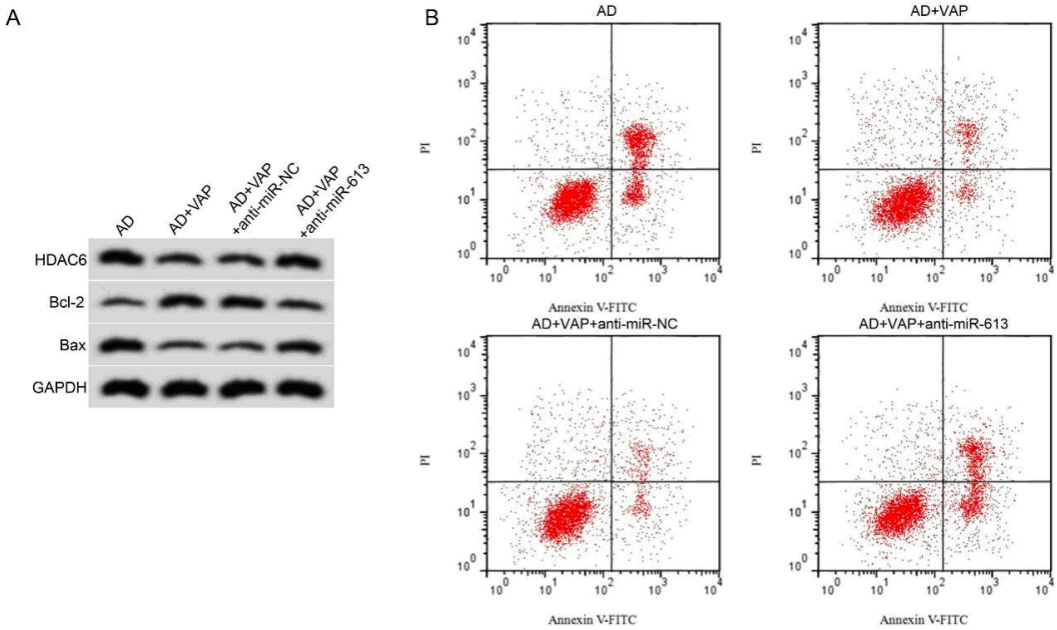

Fig. 5: Inhibited expression of miR-613 reverses the effect of velvet antler polypeptides on AD cell model apoptosis (A: Expression of HDAC6 and apoptosis-related proteins; B: Flow cytometry of apoptosis)

Effect of velvet antler polypeptides on apoptosis of AD cell model

The test results of cell apoptosis and related protein expression indicate that compared with Con group, AD group cells have significantly increased apoptosis rate and Bax protein expression, while Bcl-2 protein levels are significantly reduced; compared with AD group, AD model cells treated with 20, 40 and 80 mg/L velvet antler polypeptides have significantly reduced apoptosis rate and Bax protein expression, while Bcl-2 protein level is significantly increased ($p<0.05$, table 2, fig. 1).

Effect of velvet antler polypeptides on the expression of miR-613 and HDAC6 in AD cell model

The detection data of miR-613 and HDAC6 expression reveals that compared with Con group, AD group has significantly reduced miR-613 expression and significantly increased HDAC6 mRNA and HDAC6 protein expression; compared with AD group, AD model cells treated with 20, 40, 80 mg/L velvetantler polypeptides have significantly increased miR-613 expression, while expression of HDAC6 mRNA and HDAC6 protein is significantly reduced ($p<0.05$, table 3, fig. 2).

Targeted Regulation of HDAC6 Expression by MiR-613

TargetScan tool prediction results suggest that miR-613 and some bases in the 3'UTR of HDAC6 can form complementary pairs (fig. 3A). The experimental results of dual luciferase report shows that compared with co-transfection of miR-NC and WT-HDAC6, the relative luciferase activity of the cells is significantly reduced after co-transfection of miR-613 and WT-HDAC6 ($p<0.05$), while co-transfection of miR-NC or miR-613 with MUT-HDAC6 does not significantly change the relative luciferase activity of the cells (table 4). Transfection of miR-613 significantly reduces HDAC6 protein expression compared to transfection of miR-NC, while transfection of anti-miR-613 significantly increases HDAC6 protein level compared to transfection of anti-miR-NC ($p<0.05$, table 5, fig. 3B).

Effect of miR-613 over expression on AD cell model damage

The test results of AD cell model damage indicate that compared with AD + miR-NC group, over expression of miR-613 obviously enhanced the expression of miR-613, SOD, GSH-Px activity and Bcl-2 protein in AD model cells, whileHDAC6 protein level, MDA content, apoptosis rat and Bax protein level are significantly reduced ($p<0.05$, table 6, fig. 4).

Inhibition of miR-613 expression reverses the effect of velvet antler polypeptides (80 mg / L) on AD cell model damage

Compared with AD group, 80 mg/L velvet antler polypeptides significantly increase miR-613 expression, SOD, GSH-Px activity and Bcl-2 protein expression in AD model cells, while significantly reducing HDAC6 protein expression, MDA content, apoptosis rate and Bax protein expression ($p<0.05$, table 7, fig. 5). Compared with AD + VAP + anti-miR-NC group, AD + VAP + anti-miR-613 group has significantly reduced miR-613expression, SOD, GSH-Px activity and Bcl-2 protein expression in the cells, but improved HDAC6 protein level, MDA content, apoptosis rate and Bax protein level ($p<0.05$ table 7, fig. 5).

DISCUSSION

AD is one of the most common major neurocognitive disorders, which has significant social and economic implications (Xu, et al. 2018; Yan, et al. 2018) . Over the past few years, amyloid hypothesis has been serving a major driving force for drug development. However, phase III clinical trials of AD treatment by anti-Aβ drugs have not proven its efficacy(Long, Holtzman, 2019; Jia et al., 2019). For the important medical and social issue, effective AD treatments are urgently needed. Currently, comprehensive treatments targeting at other pathophysiological pathways of AD (such as oxidative stress) may be the key to improving the results of AD drug therapy research. In this study, AD cell damage model was simulated using Aβ25-35 to evaluate the effect of velvet antler polypeptides on oxidative stress and apoptosis in AD cell model, which provides a new clue for the development of AD clinical drugs.

Velvet antler polypeptides, as a promising biologically active resource, demonstrate extensive biological activities in anti-inflammation, learning and memory disorder relief, antioxidant and immunity enhancement. However, its effect on AD and its mechanism remain unclear. More and more reports demonstrate that neuronal apoptosis participates in the pathogenesis of AD (Chiroma, et al., 2019). Under normal circumstances, antioxidant enzymes such as SOD, GSH-Px can regulate active oxygen levels as free radical scavengers. SOD withholds oxidative stress by converting superoxide anions into more stable compound H2O2, which can be further converted into H2O by GSH-Px. MDA is an indicator of oxidative damage-induced toxic lipid peroxidation, which reflects the generation of reactive oxygen species. In this experiment, MDA content, apoptosis rate and Bax protein expression were significantly increased in the AD model, while SOD, GSH-Px activity and Bcl-2 protein levels were significantly reduced, which was consistent with previous studies (Yan, et al., 2018; Shao et al., 2017). Velvet antler polypeptides with different concentrations significantly reduced MDA content, apoptosis rate and Bax protein expression in AD model cells, while SOD, GSH-Px activity and Bcl-2 protein level were significantly. This suggests that velvet antler polypeptides can promote the survival of AD model cells, inhibit cell apoptosis and reduce oxidative stress injury, which is consistent with scholars' report (Li, et al., 2016) that velvet antler polypeptides protect cells from oxidative damage.

miRNA is a small non-coding RNA. Usually having 22-23 nucleotides, it controls gene expression by binding to 3'UTR region of mRNA. In this way, they inhibit translation or induce degradation of the target mRNA. Data show that miRNAs are significantly imbalanced in AD patients, and miRNAs can be used as candidate biomarkers for AD, such as miR-155, miR-34a, miR-9. miR-613participates in various cellular processes, such as proliferation, cycle, migration, drug resistance, etc. In myocardial cell injury induced by hypoxia/reoxygenation, miR-613 is significantly down-regulated, and over expression of miR-613 reduces hypoxia/reoxygenation-induced lactate dehydrogenase (LDH), MDA, Bax levels and myocardial apoptosis rate, showing activity of protecting cell from damage. The biological function of miR-613 in AD remains unknown. It was observed in this experiment that the expression of miR-613 was remarkably decreased in AD model cells, however velvet antler polypeptides could increase its expression level. Over expression of miR-613 significantly increased SOD, GSH-Px and Bcl-2 protein levels in AD model cells, while significantly reducing cell apoptotic rate, HDAC6 protein level, MDA content and Bax protein expression. This suggests that the over expression of miR-613 can alleviate oxidative stress and inhibit apoptosis in AD model cells, thus able to protect AD cell model from damage.

As a key enzyme in AD neurodegenerative disease, HDAC6 can be regarded as a new target for AD. Evidence has proved that HDAC6 expression is up-regulated in AD, and this study has consistent results. Inhibition of HDAC6 levels can help improve cognitive impairment in AD model and relieve AD phenotype. This study further discovered that in targeted regulation of HDAC6 expression by miR-613, up- or down-regulation of miR-613 can significantly inhibit or promote HDAC6 expression. At the same time, inhibition of miR-613 expression reverses the role of velvet antler polypeptides in inhibiting MDA content, apoptosis rate, Bax, HDAC6 protein expression in AD cell models, and reverses its role in promoting SOD, GSH-Px activity and Bcl-2 protein expression. These results suggest that the role of velvet antler polypeptides in protecting AD cell damage may be achieved by up-regulating miR-613 and down-regulating HDAC6 expression.

CONCLUSION

To conclude, velvet antler polypeptides can promote the survival of AD model cells, inhibit its apoptosis, reduce cell oxidative stress damage and relieve AD model cell damage. The mechanism of action is possibly to regulate miR-613/HDAC6 expression. It can be concluded that velvet antler polypeptides as an AD therapeutic agent have a great application potential.

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