



Mechanism of Action of Velvet Antler Polypeptide in Improving Mild Cognitive Impairment in Rats

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ABSTRACT

This study aimed to elucidate the mechanism of velvet antler polypeptide (VAP) in improvement of learning and memory function in mild cognitive impairment (MCI) rats model, specifically by investigating the PI3K, AKT and mTOR pathway. The type and content of amino acids (AA) in VAP were determined using pre-column derivatization-high performance liquid chromatography (PD-HPLC). Wistar rats were randomly divided into 5 groups (n=10 per group): control, model, piracetam (PIR), VAPH (300 mg/kg VAP) and VAPL (200 mg/kg VAP) groups. After intragastric administration, the rats' behavioral indexes were observed through the open field test (OFT), and the levels of superoxide dismutase (SOD), malondialdehyde (MDA), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) in serum were detected using ELISA. The protein expression levels of phosphoinositide 3-kinase (PI3K), protein kinase B (Akt) and mammalian target of rapamycin (mTOR) in the hippocampus were detected through western blotting. In the results, VAP contained 17 kinds of AA, including 7 essential AA, with a content of 394.08 g/kg, the total AA content was 831.55 g/kg. In the OFT, the PIR, VAPH and VAPL groups showed significant decreases in silent period, significant increases in exercise time and behavior path, and center residence time in the PIR and VAPH groups were also increased significantly. The MDA content in serum of PIR, VAPH and VAPL groups significantly decreased, while the SOD, BDNF and NGF content in serum of PIR and VAPH groups significantly increased. The expression levels of PI3K and mTOR in the hippocampus of PIR, VAPH and VAPL groups were significantly increased, and the expression level of Akt in the hippocampus of PIR and VAPH groups was significantly increased. In summary, VAP could improve the learning and memory abilities of MCI model rats, and its mechanism may be related to the regulation of PI3K, Akt and mTOR expression levels in the hippocampus of rats. This study provides a basis for further development and utilization of VAP and a better understanding of its mechanism of improving MCI.

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Key words

Velvet antler polypeptide (VAP), PI3K/Akt/mTOR, Signaling pathway, Mild cognitive impairment (MCI), Neurotrophic factors, Rat model, Scopolamine

INTRODUCTION

Mild cognitive impairment (MCI) is a transitional stage between brain aging and dementia that belongs to the syndrome of cognitive impairment, with the decline

of cognitive function as the main symptom (Anderson, 2020; Weil *et al.*, 2018). Cognitive deficit is related to alterations of neurotrophic factors level such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and so on (Budni *et al.*, 2015). BDNF and NGF can activate the downstream intracellular signaling pathways, such as the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway, thereby promoting neuroprotection (Numakawa *et al.*, 2010; Feng and Pei, 2022; Numakawa and Odaka, 2022). PI3K/Akt/mTOR pathway plays a vital role in cell growth, proliferation, differentiation, metabolism, motility transcription, and protein synthesis (Thorpe *et al.*, 2015). At present, there are many studies on PI3K/Akt/mTOR pathway in cancer (Zughaibi *et al.*,

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2021), but few researches on improving MCI. Shang *et al.* (2020) found that aluminum exposure can cause cognitive impairment through PI3K/Akt/mTOR pathway, with mTOR activity being a critical player involved in this mechanism. Akt and mTOR, as important regulatory molecules of oligodendrocytes, play important roles in the formation and development of the myelin sheath (Roher *et al.*, 2002). Zheng *et al.* (2021) found that Shenzhiling oral liquid could highly activate the PI3K/Akt/mTOR signaling pathway, thus heightening myelin protein expression and protecting the myelin sheath against Alzheimer's disease (AD).

Cornu cervi pantotrichum (deer antler) is a yang-invigorating agent used in traditional Chinese medicine that can nourish the blood, tonify qi, invigorate the spleen, and invigorate bones and tendons (Zhang *et al.*, 2016; Li, 2020). Velvet antler polypeptide (VAP) is one of its main active components, which has many pharmacological effects, such as anti-inflammatory, antioxidant and the repairment of damaged nervous system. VAP is a compound formed by dehydration and condensation of several amino acid (AA) molecules, and its specific AA constitution have been basically determined (Guo *et al.*, 2019). AA can inhibit the production of free radicals in brain, eliminate active oxygen and protect neurons (Hall *et al.*, 2019). Yang *et al.* (2020) studied the effect of VAP on the *in vitro* model of H₂O₂-induced SH-SY5Y cell injury and *in vivo* mouse model of cognitive impairment and found that VAP can reduce neuronal damage and apoptosis of neurons *in vitro* and *in vivo*. Wu *et al.* (2018) found that velvet antler polypeptides ameliorated hypoxic ischemic encephalopathy in rats by activating neural factors and SDF1/CXCR4 axis. Our previous studies have also shown that VAP can improve the BDNF and NGF content in the serum and hippocampus of the model rats, and has a protective effect on MCI model rats (Liu *et al.*, 2019). However, whether VAP can further alter PI3K/Akt/mTOR signaling pathway to improve cognitive impairment in MCI rats is unknown.

In this study, the contents of 17 kinds of AA in VAP were determined by Pre-column Derivatization High Performance Liquid Chromatography, and the effects of VAP on PI3K/Akt/mTOR pathway was observed to study the mechanism of VAP. The related indexes were detected by enzyme-linked immunosorbent assay (ELISA) and Western blotting.

MATERIALS AND METHODS

Materials and reagents

The VAP was prepared by the laboratory of the School of Pharmaceutical Sciences, Changchun University

of Chinese Medicine. Each 28.7 g of velvet antler contains about 1.0 g of VAP. AA mixed standard stock solutions were purchased from Tmrm Quality Inspection Technology Co., Ltd, Jiangsu, China. Scopolamine was obtained from Aladdin Biochemical Technology Co., Ltd, Shanghai, China and Piracetam was supplied by Northeast Pharmaceutical Group Co., Ltd, Shenyang, China. BDNF Elisa kit, NGF Elisa kit, SOD Elisa kit and MDA Elisa kit were purchased from Sanying Biotechnology Co., Ltd, Wuhan, China. PI3K, AKT, mTOR, GAPDH primary and secondary antibodies, total protein extraction kit, BCA protein concentration assay kit and ECL western blotting substrate were purchased from Solarbio Science and Technology Co., Ltd, Beijing, China.

Determination of VAP content

The VAP content was determined by pre-column derivatization high performance liquid chromatography (HPLC). The test conditions are as follows: Inertsil ODS-3 column (250 mm × 4.6 mm, 5 μm); mobile phase A: 0.1 mol/L sodium acetate buffer (pH = 6.5)/acetonitrile (93:7, v/v); mobile phase B: acetonitrile/water (8:2, v/v); column temperature: 40°C; flow rate: 1 mL/min; the detection wavelength: 252 nm; injection volume: 10 μL. The elution sequence is shown in Table I.

Animals and experimental groups

Fifty Wistar rats (half male and half female, 220 ± 20 g) were purchased from Yisi Experimental Animal Technology Co., Ltd. (Changchun, China), and were subjected to an adaptation period of 7 days, during which they were housed under environmental conditions of 24 ± 1°C, 12/12 h light/dark cycle, and had free access to food and water.

Table I. Linear gradient elution.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	100	0
14	85	15
29	66	34
30	0	100
37	0	100
37.1	100	0
45	100	0

Following adaptation, rats were randomly distributed into 5 groups (n = 10 per group): Control (administered an equal volume of distilled water), Model (equal volume of distilled water), PIR (piracetam 500 mg/kg), VAPH (VAP 300 mg/kg) and VAPL (VAP 200 mg/kg) groups. All groups were administered their respective treatments by

gavage for 18 days. Two hours after the last administration, all groups except the Control were subjected to an intraperitoneal injection of scopolamine 2 mg/kg to induce the MCI rat model.

Open field test (OFT)

To investigate the exploratory behavior of rats, all experimental groups were subjected to a 2-min period of OFT as described by Li *et al.* (2018) with slight modifications. Two hours after administration on the 11th-17th days, each group of rats were placed in the center of an open field box (bottom: 100 cm × 100 cm, height: 40 cm) for adaptability experiment in a quiet environment. The silent period, exercise time, behavior path, center residence time, corner residence time, and side residence time of the rats in each group were observed within 2 min. On the 18th day, 2 h after administration, the formal experiment was conducted, and the same indicators were recorded.

ELISA test

After the open field experiment, the rats in each group were anesthetized with 3% pentobarbital sodium (35 mg/kg). The blood was then collected from the abdominal aorta and centrifuged at 4000 rpm for 5 min to collect the upper serum. The expression levels of oxidative stress factors including superoxide dismutase (SOD) and malondialdehyde (MDA), and neurotrophic factors including BDNF and NGF in rat serum were detected through the ELISA kit according to the instructions.

Western blotting

After collecting blood from the abdominal aorta of rats, they were sacrificed by cervical dislocation and the hippocampus was removed immediately and stored at -80°C. Total protein of the hippocampal tissues were extracted by the total protein extraction kit. The protein concentration were determined using the BCA protein concentration assay kit. Protein samples (2 g/L) were mixed with 2× sample buffer, placed at 100°C for 5 min, then cooled to room temperature. Proteins were separated by 10% SDS-PAGE and transferred to PVDF membrane for 60 min. The membrane was then blocked with skim milk powder for 2 h, following by addition of the primary antibodies of appropriate concentration (PI3K, AKT,

mTOR and GAPDH) for 24 h at 4°C. After washing the membrane, the secondary antibody (1:2000, diluted with 5% skim milk powder) was added, and soaked in blocking solution for 2 h. Protein bands were visualized using an automated chemiluminescent gel imaging system with ECL substrate. The results were analyzed using Image-ProPlus 6.0 software (Olympus, Hatayaga, Japan) was used to measure the gray value for each target band.

Data analysis

All data are presented as mean±standard deviation (SD). SPSS software (Version 21.0, IBM, Armonk, NY, USA) was used for data analysis. One-way analysis of variance (ANOVA) was used to analyze the differences between treatments, $p < 0.05$ or $p < 0.01$ was considered as significant difference.

RESULTS

Analysis of AA composition and content in VAP

The contents of 17 AA in VAP were detected by pre-column derivatization HPLC (Table II). The total AA content was about 831.55 g/kg, including 7 essential AAs, the content was about 394.08 g/kg. The contents of different AA components were stable, which could be used in this study.

Behavioral effects of VAP on MCI rat in OFT

Compared with control group, the silent period and corner residence time of Model group significantly increased within 2 min, while the exercise time, behavior path, center residence time, and side residence time significantly reduced ($p < 0.01$). Compared with Model group, the silent period of PIR, VAPH and VAPL groups reduced significantly, and the exercise time and behavior path increased significantly ($p < 0.01$) (Figs. 1, 2A-C). In addition, the center residence time of PIR and VAPH groups increased significantly ($p < 0.05$). The corner residence time of PIR group significantly decreased and the side residence time significantly increased ($p < 0.01$). In VAPH and VAPL groups, the corner residence time decreased and the side residence time increased, but without significant difference (Table III, Fig. 2D-F).

Table II. Contents of amino acid components in VAP.

Sample (n = 3)	AA content (g/kg)																		
	Aap	Glu	Cys	Ser	Gly	His	Arg	Thr*	Ala	Pro	Tyr	Val*	Met*	Ile*	Leu*	Phe*	Lys*	E	T
1	36.61	36.10	36.14	44.73	79.91	35.78	46.37	67.38	45.03	42.23	42.23	68.38	56.71	56.46	60.10	43.95	41.95	394.93	843.04
2	38.71	38.12	34.50	46.86	72.55	33.74	44.85	67.07	45.44	42.69	42.69	66.75	53.01	59.26	59.62	44.86	42.48	393.05	825.64
3	38.86	34.49	34.42	46.58	73.60	33.11	44.60	66.39	44.22	42.55	42.55	71.31	51.16	59.08	59.23	44.81	42.27	394.25	825.96
Average	38.06	36.24	35.02	46.06	75.35	34.21	45.27	66.95	44.90	42.49	42.49	68.81	53.63	58.27	59.65	44.54	42.23	394.08	831.55

*; essential AA; E, total essential AA content; T, total AA content.

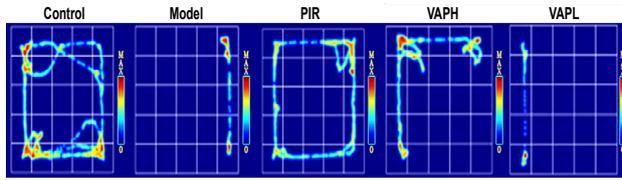


Fig. 1. Representative heat maps of the movement of rats within an open-field box over a two-minute period.
Notes: Control, control group; Model, model group; PIR, Piracetam group; VAPH, 300 mg/kg VAP group; VAPL, 200 mg/kg VAP group. Red indicated more time, and blue indicated less or no time. The time periods were given in seconds.

Table III. Residual time distribution of rats in each group in open field test.

Groups	Center (%)	Corner (%)	Side (%)
Control	10.4	41.7	48.0
Model	0.0	89.0	11.0
PIR	2.3	43.6	54.2
VAPH	10.5	57.2	32.3
VAPL	0.0	96.5	3.5

Notes: Control, control group; Model, model group; PIR, Piracetam group; VAPH, 300 mg/kg VAP group; VAPL, 200 mg/kg VAP group.

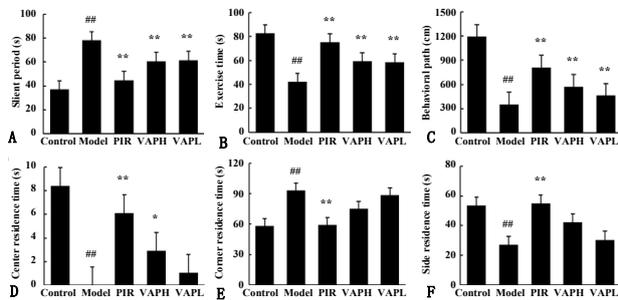


Fig. 2. Results of the open field test during a 2-min period for each group of rats.

(A) Silent period: total inactivity time; (B) Exercise time: total activity time; (C) Behavioral path: total travel distance; (D) Center residence time: time spent in the central area; (E) Corner residence time: time spent in the corners; (F) Side residence time: time spent on the side.

Notes: Data are presented as mean \pm SD ($n = 10$ per group). Compared with Control group, # $P < 0.01$; Compared with Model group, * $P < 0.05$, ** $P < 0.01$.

Effects of VAP on SOD, MDA, BDNF and NGF contents in rat serum

In order to evaluate the effect of VAP on oxidative stress and nerve factors, the levels of SOD, MDA, BDNF and NGF in serum of each group were determined by

ELISA. As shown in Figure 3A, B, compared with the control group, SOD level in the model group were obviously decreased ($p < 0.01$), while MDA content was increased significantly ($p < 0.01$). After VAP treatment, compared to the model group, the expression of SOD in VAPH group increased significantly ($p < 0.05$), while MDA levels in VAPH and VAPL groups decreased significantly ($p < 0.01$). Similarly, BDNF and NGF levels in the model group were significantly lower than in the control group ($p < 0.01$). After VAP treatment, their content in VAPH group was significantly higher than in model group ($p < 0.05$ or $p < 0.01$), whereas their content in VAPL group was higher than that in the model group, but not statistically significant ($p > 0.05$) (Fig. 3C, D). Taken together, these results indicated that VAP significantly increased the contents of SOD, BDNF and NGF and decreased MDA level in a dose-dependent manner.

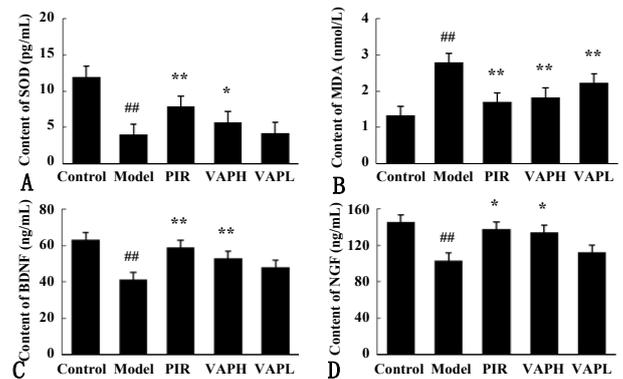


Fig. 3. Contents of SOD (A), MDA (B), BDNF (C) and NGF (D) in serum of rats in each group.

Notes: Data are presented as mean \pm SD ($n = 10$ per group). Compared with Control group, # $P < 0.01$; Compared with Model group, * $P < 0.05$, ** $P < 0.01$. SOD, superoxidase dismutase; MDA, malondialdehyde; BDNF, brain-derived neurotrophic factor; NGF, neural growth factor.

Effects of VAP on PI3K, AKT and mTOR expression levels in rat hippocampus tissues

To explore the underlying mechanism of action of VAP, the protein levels of PI3K, AKT and mTOR were detected by western blotting. Compared with the control group, the expression levels of PI3K, AKT and mTOR in the hippocampus of model group were significantly lower ($p < 0.01$). Compared with model group, the expression levels of PI3K and mTOR in hippocampus of PIR, VAPH and VAPL groups were significantly increased, and the expression levels of AKT in hippocampus of PIR and VAPH groups were significantly increased ($p < 0.05$ or $p < 0.01$) (Fig. 4A-D).

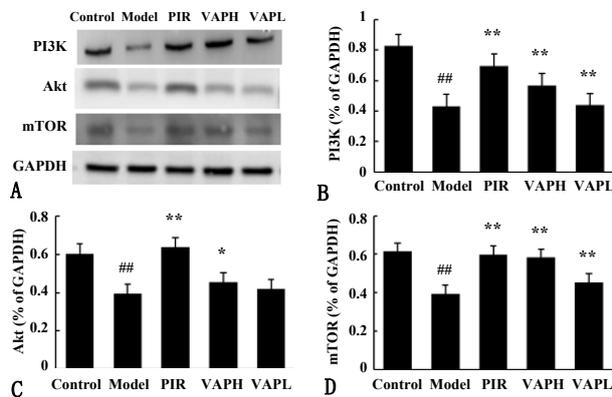


Fig. 4. Expression levels of PI3K, Akt and mTOR proteins in the hippocampus tissue of rats in each group.

Notes: (A) Western blot analysis; (B–D) Quantification of PI3K, Akt and mTOR in five groups. Data are presented as mean \pm SD (n = 10 per group). Compared with Control group, # $P < 0.01$; Compared with Model group, * $P < 0.05$, ** $P < 0.01$.

DISCUSSION

MCI is a transitional stage from normal aging to AD. Since there is no cure for AD, early intervention of MCI is of great significance for reducing the prevalence of cerebral dementia. VAP, which are rich in amino acids and bioactive peptides, is considered to be the main bioactive component of velvet antler (Li *et al.*, 2007; Sui *et al.*, 2014). This study measured the content of various AA components in VAP, and assessed whether it could improve MCI by modulating the PI3K/AKT/mTOR signal pathway. HPLC is a commonly used methods for the detection of AA. Because most AA can neither absorb ultraviolet light nor produce fluorescence reaction, it is necessary to derivatize the sample and convert it into components with ultraviolet absorption characteristics or capable of emitting fluorescence (Lee *et al.*, 2019). In this study, a total of 17 AAs were detected by pre-column derivatization HPLC, including 7 essential AAs. AAs are widely present in brain tissue, skeletal muscles and blood. They have the functions of increasing protein synthesis, reducing excessive inflammatory reaction, enhancing immune system function, anti-fatigue, improving body barrier, etc. (Tu *et al.*, 2018; Zhang *et al.*, 2017). When the body undergoes peroxide reaction due to ischemia, hypoxia or tissue damage, large amounts of free radicals are produced and cause damage to nerve cells. AAs in the cerebrospinal fluid can be absorbed by neurons, inhibit the production of free radicals, remove reactive oxygen species and protect neurons (Zhang *et al.*, 2017; Divakaruni *et al.*,

2017; Tramutola *et al.*, 2015). OFT was used to analyze the exploratory behavior of rats. The results showed that the exercise time, behavior path, center residence time and side residence time of each administration group were significantly higher than those of the model group, while the silent period and corner residence time were shorter than those of the model group. This indicates that VAP can improve the ability of learning and memory in MCI rats.

SOD, as an integral part of the antioxidant system, can catalyze superoxide anion radical disproportionation to generate oxygen and hydrogen peroxide, thus maintaining the balance of oxidation and oxidation resistance (Zhu *et al.*, 2019). MDA is a product of lipid peroxidation, which can lead to cell metabolic and functional abnormalities (Gęgotek and Skrzydlewska, 2019). Thus, the content of MDA is an important parameter reflecting the potential antioxidant capacity of the body. It can reflect the speed and intensity of lipid peroxidation and indirectly reflect the degree of tissue peroxidation damage. When the free radicals of the body act on lipid peroxidation, a large amount of MDA will be produced, leading to the cross-linking polymerization of proteins, nucleic acids and other macromolecules, resulting in severe cytotoxicity (Yi *et al.*, 2020). BDNF and NGF can promote the growth, development, differentiation, maturation and maintain the normal function of the nervous system, accelerate the repair of the nervous system after injury (Di Carlo *et al.*, 2019; Kowiński *et al.*, 2018; Ciafrè *et al.*, 2020; Jablochkova *et al.*, 2019). They could activate the downstream PI3K/Akt/mTOR pathway to produce brain protection (Li *et al.*, 2016; Zheng and Chen, 2020). In this study, the contents of SOD, BDNF and NGF in serum of MCI rats in VAP groups were significantly increased, while MDA levels were decreased, indicating that VAP can prevent and improve the cognitive impairment of MCI rats.

PI3K is a 3-hydroxy-phosphorylated intracellular phosphatidylinositol kinase, and Akt is a serine threonine protein kinase (Tsai *et al.*, 2018). PI3K/Akt pathway can regulate nerve cell apoptosis in a wide range (Hevner, 2015; Garabadu and Verma, 2019), and is particularly important for nerve cell survival (Luo *et al.*, 2019; Liu *et al.*, 2017). Our results demonstrated that VAP significantly increased the expression levels of PI3K, Akt, and mTOR proteins in hippocampus tissue, suggesting a protective and restorative role of VAP in the nervous system of MCI rats.

CONCLUSION

In conclusion, this study identified the content composition of AA in VAP, and demonstrated its ability to improve and enhance learning and memory performance

in MCI rat model. Furthermore, VAP was found to increase the contents of SOD, BDNF and NGF *in vivo*, and inhibit the production of MDA. Moreover, it could regulate the expression level of related proteins in the PI3K/AKT/mTOR signaling pathway. Thus, this study provides a valuable reference for the clinical treatment of MCI patients.

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IRB approval

Not applicable, this work did not involve humans.

Ethical statement

Research experiments conducted in this paper was approved by the Animal Care and Welfare Committee of Changchun University of Chinese Medicine (approved number: 2020172), following all guidelines, regulations, legal, and ethical standards as required for humans or animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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