Research Paper

Cornus officinalis Extracts Alleviate Amyloid Beta-Peptide 25-35-Induced Nerve Cell Injury in Alzheimer's Disease by Upregulating Long Non-Coding RNA Rpph1

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Zhao *et al.*: Effect of *Cornus officinalis* Extracts on Alzheimer's Disease Cell Injury Models

To explore the protective effect of *Cornus officinalis* extracts on Alzheimer's disease cell injury models and its underlying molecular mechanism. Pheochromocytoma cells were treated with 20 μM amyloid beta-peptide 25-35 to establish an Alzheimer's disease cell injury model *in vitro*, which was recorded as amyloid beta-peptide 25-35 group. Cells affected by amyloid beta-peptide 25-35 were treated with different-doses of *Cornus officinalis* extracts and recorded as low-dose *Cornus officinalis* extracts group, medium-dose *Cornus officinalis* extracts group and high-dose *Cornus officinalis* extracts group. Pheochromocytoma cells transfected with si-NC/si-long non-coding RNA Rpph1, treated with 20 μM amyloid beta-peptide 25-35 and 80 mg/ml of *Cornus officinalis* extracts were recorded as high-dose *Cornus officinalis* extracts+si-NC group, high-dose *Cornus officinalis* extracts+si-long non-coding RNA Rpph1 group. Cell viability and apoptosis were examined using cell counting kit-8 assay and flow cytometry. Protein expression was tested by Western blot. Malondialdehyde, superoxide dismutase and catalase levels were assessed by measuring cell oxidative stress. Amyloid beta-peptide, tumor necrosis factor-alpha, interleukin-6 and interferon gamma levels were examined using enzyme-linked immunosorbent assay. Long non-coding RNA Rpph1 expression was detected using quantitative reverse transcriptase polymerase chain reaction. Amyloid beta-peptide 25-35 treatment decreased pheochromocytoma cell viability, Cyclin D1, superoxide dismutase, catalase and long non-coding RNA Rpph1 levels, while increased apoptosis rate, cleaved-caspase-3, malondialdehyde, amyloid beta-peptide, tumor necrosis factor-alpha, interleukin-6 and interferon gamma levels. After treatment with different-doses of *Cornus officinalis* extracts, cell viability, Cyclin D1, superoxide dismutase, catalase and long non-coding RNA Rpph1 levels were enhanced, while apoptosis rate, cleaved-caspase-3, malondialdehyde, Aβ, tumor necrosis factor-alpha, interleukin-6 and interferon gamma levels were reduced in pheochromocytoma cells treated with amyloid beta-peptide 25-35. Downregulation of long non-coding RNA Rpph1 reversed the inhibitory effect of *Cornus officinalis* extracts on cell injury. *Cornus officinalis* extracts relieved amyloid beta-peptide 25-35-induced nerve cell injury by upregulating long non-coding RNA Rpph1, suggesting that *Cornus officinalis* extracts might be used in Alzheimer's disease treatment.

Key words: *Cornus officinalis* extracts, Alzheimer's disease, long non-coding RNA Rpph1, caspase-3, interferon gamma, tumor necrosis factor-alpha

Alzheimer's Disease (AD), a chronic degenerative neurological disease, is the most common type of dementia in the elderly[1,2]. Clinically, AD is often characterized by the deterioration of cognitive and memory functions, accompanied by mental abnormalities and social life dysfunction[3,4]. AD incidence has increased significantly with the progress of population aging, which brings a huge burden to society[5,6]. Therefore, developing effective therapeutic drugs for AD has become a global research focus.

Traditional Chinese Medicine (TCM) has the

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effect of preventing and treating AD[7,8]. *Cornus officinalis* (CO) is a rare TCM in our country, which has antioxidant, anti-inflammatory and neuroprotective effects[9,10]. Loganin, an iridoid glycoside extracted from CO, had been confirmed to improve the cognitive impairment of AD mice models[11]. Besides, iridoid glycosides of CO could inhibit Tau hyperphosphorylation and aggregation by activating protein phosphatase 2A, thus preventing neuron loss[12]. Importantly, cornuside and gallanthin extracted from CO could reduce the activities of ChE and Beta-Amyloid Cleaving Enzyme 1 (BACE1)[13], the important enzymes for AD progression[14,15]. The above studies suggest that CO extracts may have anti-AD effects, but the specific effects and mechanisms remain unclear.

Long non-coding RNA (lncRNA) Rpph1 attenuated Amyloid Beta-peptide (Aβ) 25-35-induced SH-SY5Y cell endoplasmic reticulum stress and apoptosis[16]. Besides, Rpph1 could improve Aβ-induced neuronal apoptosis[17]. Thus, lncRNA Rpph1 may be an important regulator of AD progression. In this, we found that CO could inhibit lncRNA Rpph1 expression. However, whether CO extracts exert its neuroprotective effect by inhibiting lncRNA Rpph1 expression remains unclear. Our study aimed to investigate the effect of CO extracts on Aβ25-35-induced nerve cell injury and whether it was related to lncRNA Rpph1 expression.

MATERIALS AND METHODS

Cell culture and treatment:

Pheochromocytoma (PC12) cells (Procell, Wuhan, China) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium plus 10 % Fetal Bovine Serum (FBS) and 1 % penicillin-streptomycin. Cells were treated with 20 μM Aβ25-35 (Sigma-Aldrich, St. Louis, MO, United States of America (USA)) for 24 h to record as Aβ25-35 group. Cells treated with Aβ25-35 and different-doses (20, 40 and 80 mg/ml) of CO extracts (KINGREEN, Xian, China) were recorded as low-dose CO extracts group, medium-dose CO extracts group and high-dose CO extracts group. Cells were transfected with si-NC/si-LncRNA Rpph1, treated with 20 μM Aβ25-35 and 80 mg/ml CO extracts, which were recorded as high-dose CO extracts+si-NC group, high-dose CO extracts+si-LncRNA Rpph1 group.

Cell Counting Kit-8 (CCK-8) assay:

PC12 cells in each group were re-seeded in 96-well plates. Following, cells were incubated with CCK-8 reagent (Dojindo, Kumamoto, Japan) and Absorbance (A) value was tested by microplate reader to assess cell viability.

Flow cytometry:

Collected PC12 cells were dyed by Annexin V/ Fluorescein Isothiocyanate (FITC) and Propidium Iodide (PI) (Abcam, Cambridge, MA, USA). Cell apoptosis rate was examined under flow cytometer.

Western blot:

Protein samples isolated from PC12 cells were separated and transferred to Polyvinylidene Difluoride (PVDF) membranes, followed by incubated with anti-Cyclin D1 (1:200, ab16663), anti-cleaved-caspase-3 (1:1000, ab2302), anti-β-actin (1:200, ab115777) and secondary antibody (1:2000, ab205718). Protein signals were examined by Enhanced Chemiluminescence (ECL) reagent (Beyotime, Shanghai, China), ChemiDoc XRS+imaging system and Quantity One software.

Measurement of oxidative stress:

Malondialdehyde (MDA), Superoxide Dismutase (SOD) and Catalase (CAT) levels in PC12 cells were tested by MDA assay kit (ab118970, Abcam), SOD activity assay kit (ab65354, Abcam) and CAT activity assay kit (ab83464, Abcam) according to kit instructions, respectively.

Enzyme-Linked Immunosorbent Assay (ELISA):

Aβ, Tumor Necrosis Factor-Alpha (TNF-α), Interleukin (IL)-6 and Interferon Gamma (IFN-γ) levels in PC12 cells were analyzed by Aβ ELISA kit (JL10958-48T, Jianglai, Shanghai, China), TNF-α ELISA kit (JL13202-48T, Jianglai), IL-6 ELISA kit (JL20896-48T, Jianglai) and IFN-γ ELISA kit (JL13241-48T, Jianglai), respectively.

Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR):

Extracted Ribonucleic Acid (RNAs) from PC12 cells was reverse-transcribed into complementary Deoxyribonucleic Acid (cDNA). SYBR Green was used for PCR with specific primers as below. LncRNA Rpph1,

forward: 5'-CGAGCTGAGTGCGTCCTGTC-3', Reserved: 5'-TCGCTGGCCGTGAGTCTGT-3'; Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), forward: 5'-ACAGCAACAGGGTGGTGGAC-3', Reserved; 5'-TTTGAGGGTGCAGCGAACTT-3'. Relative lncRNA Rpph1 level was analyzed with the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis:

Results were presented as mean±Standard Deviation (SD) by Statistical Package for the Social Sciences (SPSS) 20.0 software. Differences were evaluated by Student's t-test or Analysis of Variance (ANOVA). $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

After Aβ25-35 treatment, PC12 cell viability was restrained and apoptosis was enhanced. Under the treatment of CO extracts, PC cell viability was increased and apoptosis was inhibited (fig. 1 and Table 1). Aβ25-35 treatment decreased Cyclin D1 level and improved cleaved-caspase-3 level. However, CO extracts markedly enhanced Cyclin D1 level and reduced cleaved-caspase-3 level in Aβ25-35-induced PC12 cells (fig. 2 and Table 2).

Aβ25-35 treatment promoted MDA level, while decreased SOD and CAT levels in PC12 cells. However, MDA level was reduced, while SOD and CAT levels were enhanced with the increasing of CO extracts (Table 3). Aβ25-35 treatment enhanced Aβ, TNF-α, IL-6 and IFN-γ levels in PC12 cells, while CO extracts reduced their levels in Aβ25-35-induced PC12 cells (Table 4). Aβ25-35 treatment reduced lncRNA Rpph1 expression in PC12 cells, while CO extracts could promote lncRNA Rpph1 expression in a dose-dependent manner (Table 5).

In High-dose CO extracts+si-LncRNA Rpph1 group, lncRNA Rpph1 expression, Cyclin D1 level, cell viability, SOD and CAT levels were reduced, while cleaved-caspase-3 level, apoptosis rate, MDA, Aβ, TNF-α, IL-6 and IFN-γ levels were increased (fig. 3 and Table 6).

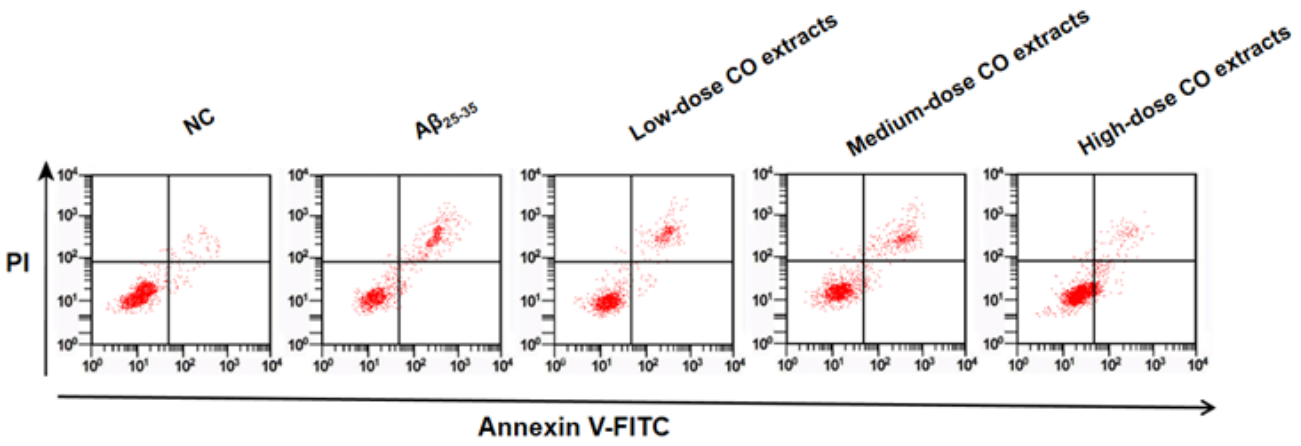

Fig. 1: Flow cytometry for detecting cell apoptosis

TABLE 1: CO EXTRACTS REGULATED Aβ25-35-INDUCED PC12 CELL VIABILITY AND APOPTOSIS

Group	A value	Apoptosis rate %
NC	0.902±0.07	7.12±0.34
Aβ25-35	0.448±0.03*	25.41±1.52*
Low-dose CO extracts	0.593±0.03#	21.35±1.25#
Medium-dose CO extracts	0.763±0.05#	14.81±1.03#
High-dose CO extracts	0.851±0.07#	9.43±0.52#
F	113.200	507.325
P	0.000	0.000

Notes: *P<0.05 to NC group and #P<0.05 to Aβ25-35 group

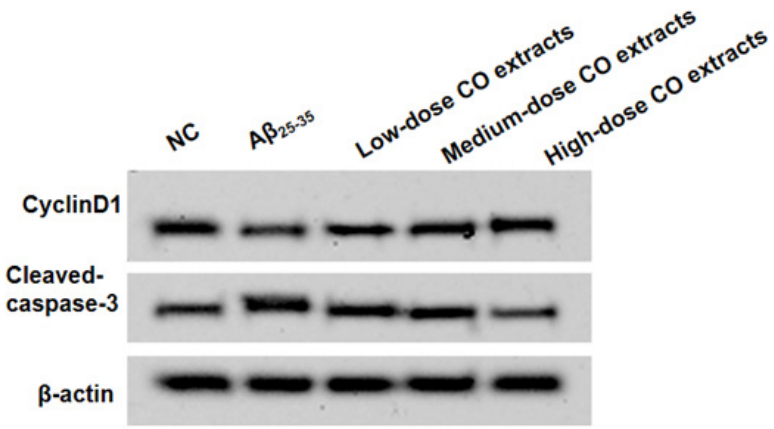

Fig. 2: Western blot for detecting Cyclin D1 and cleaved-caspase-3 levels

TABLE 2: DIFFERENT DOSES OF CO EXTRACTS REGULATED Cyclin D1 AND CLEAVED-CASPASE-3 LEVELS

Groups	Cyclin D1	Cleaved-caspase-3
NC	0.83±0.05	0.32±0.02
Aß25-35	0.42±0.02*	0.91±0.07*
Low-dose CO extracts	0.57±0.03#	0.70±0.05#
Medium-dose CO extracts	0.71±0.05#	0.52±0.03#
High-dose CO extracts	0.78±0.05#	0.37±0.02#
F	142.517	293.39
P	0.000	0.000

Notes: *P<0.05 to NC group and #P<0.05 to Aβ25-35 group

TABLE 3: CO EXTRACTS REGULATED MDA, SOD AND CAT LEVELS

Group	MDA (μmol/g)	SOD (U/mg)	CAT (U/mg)
NC	18.12±1.01	12.13±0.86	10.56±0.71
Aß25-35	34.25±2.17*	4.16±0.21*	4.89±0.27*
Low-dose CO extracts	30.13±1.23#	7.13±0.46#	6.07±0.41#
Medium-dose CO extracts	24.81±1.11#	10.05±0.73#	8.27±0.61#
High-dose CO extracts	21.06±1.24#	11.91±0.81#	9.48±0.53#
F	193.840	238.263	177.890
P	0.000	0.000	0.000

Notes: *P<0.05 to NC group and #P<0.05 to Aβ25-35 group

TABLE 4: EFFECTS OF CO EXTRACTS ON Aβ AND INFLAMMATION

Group	Aβ (ng/l)	TNF-α (ng/l)	IL-6 (ng/l)	IFN-γ (ng/l)
NC	283.16±15.13	75.46±4.12	35.69±2.16	15.67±1.02
Aß25-35	427.32±22.56*	179.26±11.26*	80.57±5.14*	45.97±3.14*
Low-dose CO extracts	383.72±18.49#	151.02±10.43#	61.27±4.86#	38.93±3.07#
Medium-dose CO extracts	327.17±21.43#	114.06±9.16#	48.62±3.19#	27.53±1.55#
High-dose CO extracts	298.52±18.83#	90.27±4.13#	39.02±1.76#	19.71±1.05#
F	86.581	234.117	222.217	307.410
P	0.000	0.000	0.000	0.000

Notes: *P<0.05 to NC group and #P<0.05 to Aβ25-35 group

TABLE 5: CO EXTRACTS ENHANCED LNCRNA RPPH1 EXPRESSION

Groups	LncRNA Rpph1
NC	1.00±0.10
Aß25-35	0.41±0.02*
Low-dose CO extracts	0.61±0.03#
Medium-dose CO extracts	0.79±0.05#
High-dose CO extracts	0.94±0.07#
F	141.618
P	0.000

Notes: *P<0.05 to NC group and #P<0.05 to Aβ25-35 group

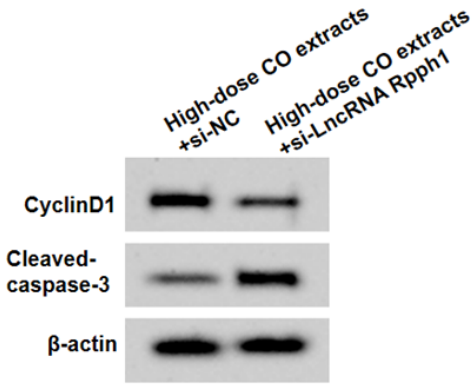

Fig. 3: Western blot for testing Cyclin D1 and cleaved-caspase-3 expression

TABLE 6: LNCRNA RPPH1 KNOCKDOWN REVERSED THE EFFECT OF CO EXTRACTS ON CELL INJURY

Group	LncRNA Rpph1	Cyclin D1	Cleaved-caspase-3	A value	Apoptosis rate (%)	MDA (μmol/g)	SOD (U/mg)	CAT (U/mg)	Aβ (ng/l)	TNF-α (ng/l)	IL-6 (ng/l)	IFN-γ (ng/l)
High-dose CO extracts+si-NC	1.00± 0.07	0.78± 0.05	0.38± 0.02	0.857± 0.05	9.31± 0.67	21.11± 1.32	11.88± 0.71	9.42± 0.69	291.52± 15.41	88.37± 5.12	39.46± 2.04	19.49± 1.15
High-dose CO extracts+si-LncRNA Rpph1	0.48± 0.02*	0.40± 0.02*	0.86± 0.07*	0.438± 0.03*	26.81± 1.54*	36.54± 2.47*	5.43± 0.35*	4.20± 0.31*	407.10± 28.13*	184.29± 10.44*	83.25± 6.73*	48.76± 2.91*
t	21.428	21.169	19.780	21.557	31.261	16.529	24.445	20.702	10.811	24.747	18.681	28.063
P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Notes: *P<0.05 to NC group and #P<0.05 to Aβ25-35 group

With the increasing number of AD patients, more and more attention has been paid to AD pathogenesis. AD development is not completely clear, mainly involving excessive deposition of Aβ, hyper phosphorylation of Tau protein, cholinergic dysfunction, inflammatory response, oxidative stress and apoptosis[18-20]. Studies have found that the AD *in vitro* cell model can be simulated by inducing PC12 cell injury with Aβ25-35[21,22]. MDA, SOD and CAT are oxidative stress-related factors[23] and TNF-α, IL-6 and IFN-γ are pro-inflammatory-related factors[24]. It had been reported that IL-6, TNF-α, IL-1β, IFN-γ and MDA levels were elevated, while SOD and CAT levels were reduced in AD models[25,26]. In this, PC12 cell viability was decreased, while cell apoptosis, oxidative stress, inflammation and Aβ level were increased under Aβ25-35 treatment. Therefore, AD cell injury model was successfully established in this study.

Ethanol extract of CO protected keratinocytes from oxidative stress caused by particulate matter[27]. Iridoid glycosides of CO had been confirmed

to repress the production of pro-inflammatory factors[28]. Cornuside, an iridoid glycoside from CO, could significantly reduce TNF-α, IL-6 and MDA levels in AD mice models[29]. These results indicate that CO extracts have obvious antioxidant and anti-inflammatory effects. To investigate whether CO extracts played a negative role in AD, PC12 cells were treated with Aβ25-35 and CO extracts. The results suggested that MDA, Aβ, TNF-α, IL-6 and IFN-γ levels were suppressed, while SOD and CAT levels were improved by CO extracts, indicating that CO extracts inhibited Aβ25-35-induced oxidative stress and inflammation in PC12 cells. Additionally, iridoid glycosides of CO had neuroprotective effects on traumatic brain injury by inhibiting apoptosis and promoting nerve repair[30]. Our data confirmed that CO extracts promoted PC12 cell viability and reduced apoptosis under Aβ25-35 treatment.

Aberrant expression of lncRNA Rpph1 has been implicated in human disease progression. For example, lncRNA Rpph1 was overexpressed in colorectal cancer, which could enhance cell proliferation and metastasis[31]. Also, high lncRNA Rpph1 was confirmed to promote hepatocellular carcinoma malignancy progression by increasing cell growth and metastasis[32]. LncRNA Rpph1 has been shown to restrain the apoptosis of nerve cells[16,17], indicating that it may participate in regulating AD progression. In this, we observed low lncRNA Rpph1 expression in PC12 cells after induced by Aβ25-35 and confirmed the increasing effect of CO extracts on lncRNA Rpph1 expression. Moreover, the reversal effect of lncRNA Rpph1 knockdown on CO extracts-mediated cell injury inhibition confirmed that CO extracts increased lncRNA Rpph1 expression to suppress Aβ25-35-induced PC12 cell injury.

In conclusion, our study reveals a novel molecular mechanism by which CO extracts inhibited AD progression. This study suggested that CO extracts might relieve Aβ25-35-induced nerve cell injury by up regulating lncRNA Rpph1. These results provide new evidence for the use of CO extracts in AD treatment and show that lncRNA Rpph1 may be a potential therapeutic target for AD.

Conflict of interests:

The authors declared no conflict of interests.

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