

Original Article

Jatrorrhizine can improve nerve cell injury induced by A β 25-35, acting through miR-223-3p/HDAC4 axis

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Abstract: Objective: The purpose of this research is to probe the mechanism of Jatrorrhizine (JAT) improving A β 25-35-induced nerve cell injury through the miR-223-3p/HDAC4 axis. Methods: SH-SY5Y cells were treated with A β 25-35 to simulate nerve injury in the pathogenesis of Alzheimer's disease (AD), and JAT-treated SH-SY5Y cells were assessed for HDAC4 and miR-223-3p. The HDAC4 and miR-223-3p levels were tested by qRT-PCR. Proliferation was determined through MTT. Apoptosis was assessed by flow cytometry, and the related indexes of oxidative stress (OS) were examined by an OS kit. Results: Compared with AD group, OD value increased, apoptosis rate decreased, and OS was inhibited in the AD+JAT group (all $P < 0.05$). In SH-SY5Y cells, miR-223-3p can specifically inhibit the HDAC4 expression. The miR-223-3p expression increased and HDAC4 decreased after JAT acted on SH-SY5Y cells stimulated by A β 25-35 (all $P < 0.05$). The addition of over-expression HDAC4 vector or miR-223-3p inhibitor could inhibit proliferation, and promote apoptosis and OS on the basis of JAT (all $P < 0.05$). In addition, over-expressing miR-223-3p can suppress over-expressed HDAC4's effects on proliferation, apoptosis, and OS of SH-SY5Y cells (all $P < 0.05$). Conclusion: JAT can improve the nerve injury induced by A β 25-35 by up-regulating miR-223-3p and inhibiting the HDAC4 expression, suppress apoptosis and OS, and induce proliferation. This research further clarified the mechanism of JAT in AD.

Keywords: Jatrorrhizine, A β 25-35, SH-SY5Y, miR-223-3p, nerve cell injury

Introduction

Alzheimer's disease (AD) is a familiar chronic neurodegenerative disease marked by memory loss and progressive cognitive dysfunction [1]. With the continuous growth of the elderly population, the morbidity of AD is also rising rapidly [2]. At the moment, there is no effective treatment. The current treatment can only appropriately slow down the disease progression, and the individual difference of patients receiving treatment is great. It is urgent to analyze the pathogenesis of AD and explore new molecular therapeutic targets for treatment.

The deposition of β -amyloid peptide (A β) and hyperphosphorylation of senile plaques (SP) and Tau proteins leading to neurofibrillary tangles are two main pathologic manifestations of AD [3]. The accumulation of A β in brain and cerebral vessels is effective in the occurrence and progression of AD. There is evidence that many factors including apoptosis, inflamma-

tion, and oxidative stress (OS) are relevant to AD's pathogenesis [4].

Jatrorrhizine (JAT) is a tetrahydroisoquinoline alkaloid with bactericidal effect extracted from *Coptis chinensis*, which is often used as a detoxifying and hypoglycemic agent [5]. Research has shown that JAT has many biologic functions such as anti-inflammatory and anti-oxidant [6]. Previous research has shown that JAT can be used to protect neurons from cell damage induced by hydrogen peroxide or A β oligomer [7]. JAT's mechanism in AD needs to be explored in depth. Therefore, this study aims to explore the regulatory mechanism of JAT in AD by combining traditional Chinese medicine treatment with molecular biology.

It has been reported that HDAC4, a member of HDAC family IIa, can regulate the survival of neurons. Under the action of some proteins in neurodegenerative diseases, HDAC4 can translocate from the cytoplasm to the nucleus and

inhibit the activity of MEF2 after binding to myocyte specific enhancer 2 (MEF2), a transcription factor related to neuronal survival in the nucleus, resulting in neuronal death [8]. The expression of HDAD4 in the frontal cortex of patients with cognitive impairment of AD is enhanced, which is related to early neuron dysfunction and cognitive impairment [9]. It is speculated that JAT may control AD progression by regulating HDAC4, and HDAC4 is chosen as an important research point to find the possible upstream regulation mechanism.

microRNAs (miRNAs), non-coding RNAs with 18-22 nucleotides, exert a variety of biological functions by targeting the 3' untranslated region (3'UTR) of their target mRNA [10, 11]. Recently, they have become new therapeutic targets for neurodegenerative diseases [12]. This research predicted that HDAC4 could specifically bind miR-223-3p by using TargetScan and StarBase online prediction websites. It has been reported that miR-223-3p can protect isolated cortical neurons from conditioned medium-mediated degeneration in multiple sclerosis [13]. Furthermore, miR-223 in serum exosomes can be used as a crucial diagnostic and prognostic marker of dementia [14]. Nevertheless, a functional study of miR-223-3p in AD has not been confirmed. It is speculated that miR-223-3p may regulate AD development and progression.

Thus, this research speculated that JAT might influence the neural cell behavior of AD by adjusting the miR-223-3p/HDAD4 axis. This provides a new research basis for further understanding the role of JAT in the occurrence and development of AD, and also creates a new target for treatment.

Materials and methods

Cell culture

Human neuroblastoma cell line SH-SH-SY5Y was provided by American Type Culture Collection (ATCC) and stored in DMEM basic medium. It was added with 10% FBS and 1% penicillin/streptomycin, and placed in a 5% CO₂ incubator at 37°C. SH-SY5Y cells were pre-treated by 20 μ M/L A β 25-35 (80-90% fusion) for 24 h to construct AD cell models [15].

For in vitro cell research, JAT (CAS: 3621-38-3, National Institute of Food and Drug Administration, China) was dissolved in dimethyl sulfoxide (DMSO) to form 10 mM concentration, which was stored in the dark at 4°C and further diluted to 10 μ M in fresh cell culture medium for later use [16]. Cells were cultured under 5% CO₂ and 95% humidity, and they were in logarithmic growth phase.

Cell transfection and grouping

miR-223-3p mimics, inhibitors, and their negative controls (miR-NC), HDAC4 overexpression vectors (pcDNA3.1-HDAC4), and their negative controls (pcDNA3.1-NC) were constructed at Yuanjing Biotechnology Co., Ltd., China. According to the instructions, miR-223-3p mimic/inhibitor and its negative control (miR-NC) and pcDNA3.1-HDAC4 (pcDNA3.1-NC) were transfected into SH-SY5Y cells through Lipofectamine 3000.

The cell groups were as follows: control group (blank control SH-SY5Y cells), AD group (SH-SY5Y cells induced by A β 25-35), AD+JAT group (those induced cells+JAT), AD+JAT+pcDNA3.1-HDAC4 group (those induced cells+JAT+HDAC4 over-expression vector), AD+JAT+pcDNA3.1-NC group (those induced cells+JAT+HDAC4 negative control), AD+JAT+miR-223-3p inhibitor group (those induced cells+miR-223-3p inhibitor), AD+JAT+miR-NC group (those induced cells+miR-223-3p negative control), AD+JAT+pcDNA3.1-HDAC4+miR-223-3p mimic group (those induced cells+JAT+HDAC4 over-expression vector+miR-223-3p mimics) and AD+JAT+pcDNA3.1-HDAC4+miR-NC group (those induced cells+JAT+HDAC4 over-expression vector+miR-223-3p negative control).

MTT

Cell proliferation was measured by the MTT cell proliferation test kit (C0009M, Shanghai Beyotime Biotechnology Co., Ltd., China). SH-SH-SY5Y cells grown in logarithmic phase were collected and inoculated on 96-well plates with 1 \times 10⁴ cells per well. A total of 20 μ L MTT (5.0 mg/mL) was added to each well at 24, 48 and 72 h and cultured at 37°C for 4 h. The MTT solution was removed and 100 μ L dimethyl sulfoxide (DMSO) was added. The

Table 1. qRT-PCR sequences

Name		Sequence (5'-3')
miR-223-3p	Forward	CGCUAUCUUUCUAUUAACUGACCAUAA
	Reverse	CGCUAUCUUUCUAUUAUGACUCCAUA
HDAC4	Forward	CGGTCCAGGCTAAAGCAGAA
	Reverse	TCTGTGACATCCAACGGACG
GAPDH	Forward	ACCACAGTC CATGCCATCAC
	Reverse	TCCACCACCCT GTT GCTGTA
U6	Forward	GCUUCGGCAGCACAUUACUAAA
	Reverse	CGCUUCACGAAUUUGCGUGUCAU

absorbance at 490 nm was tested by a strip reader after shaking for about 10 min.

Flow cytometry

Apoptosis was assessed by Annexin V-FITC/PI apoptosis detection kit (C1062S, Shanghai Beyotime Biotechnology Co., Ltd., China) based on the instructions. Transfected cells were dissolved with trypsin, cleaned with cold PBS, centrifuged, and resuspended in buffer. They were double-stained with Annexin-V-FITC (10 μ L) and propidium iodide (PI; 5 μ L) under dark conditions for 15 min. Then, apoptosis was tested by flow cytometry (RS001, Shanghai Ruishi Technology Co., Ltd., China).

Determination of OS levels

The OS levels were tested by micro method, using malondialdehyde (MDA) content detection (BC0025), lactate dehydrogenase (LDH) (BC0685) and superoxide dismutase (SOD) activity detection kits (BC0175) (Beijing Solarbio Technology Co., Ltd., China). The supernatant was collected by centrifugation at 3000 rpm at 4°C for 15 min. The MDA levels and the LDH and SOD activities were determined by using the corresponding test kits in light of the manufacturer's instructions.

qRT-PCR

Total RNA was obtained from SH-SY5Y cells by TRIzol[®] reagent, and the same amount was transcribed into cDNA by cDNA synthesis kit (BTN131005, Beijing Biolab Technology Co., Ltd., China). The SYBRPremixEx-Taq kit was applied for real-time PCR in Step One Plus[™] (RR420A, Beijing Zhijie Fangyuan Technology Co., Ltd., China). The reaction conditions were as follows: pre-denaturation at 95°C for 30 s,

pre-denaturation at 95°C for 35 s, pre-denaturation at 60°C for 60 s. The gene relative expression was calculated by 2^{- $\Delta\Delta$ Ct} method, and GAPDH and U6 were regarded as internal references. Primer sequences are shown in **Table 1**.

Dual luciferase report experiment

The targeted binding relationship between miR-223-3p and HDAC4 was examined by a dual luciferase gene detection kit (D0010, Beijing Solarbio Technology Co., Ltd., China). The 3'UTR fragments of wild-type and mutant HDAC4 containing miR-223-3p binding site were inserted into pGL3 vector and named HDAC4-WT and HDAC4-MUT, respectively. For luciferase report analysis, SH-SY5Y cells were co-transfected with HDAC4-WT or HDAC4-MUT and miR-223-3p mimics or simulated NC under Lipofectamine 3,000. The relative luciferase activity was normalized by Renilla luciferase activity.

Statistical analysis

SPSS 22.0 was applied in statistical analysis. The measurement data were under normality test through Jarque-Bera, and those in accordance with normal distribution were represented in the form of mean \pm standard deviation ($\bar{x} \pm sd$). The inter-group comparison was made by t-test, and that among multiple groups was assessed through one-way analysis of variance and Tukey back testing. If they did not conform to the normal distribution, the inter-group comparison was made by Mann-Whitney U test, and that among multiple groups was evaluated by Bonferroni method. P<0.05 denotes a significant difference.

Results

JAT can improve SH-SY5Y cell injury stimulated by A β 25-35

In order to probe into JAT's role in AD progression, after SH-SY5Y cells were stimulated by A β 25-35 to construct AD cell models in vitro, 10 μ M JAT was added to A β 25-35 induced SH-SY5Y cell culture medium to observe JAT's effect. Cell proliferation experiments manifested that the proliferation of SH-SY5Y cells was enhanced after JAT was added (all P<0.05; **Figure 1A**). Flow cytometry revealed that JAT

Jatrorrhizine improves nerve cell damage induced by A β 25-35

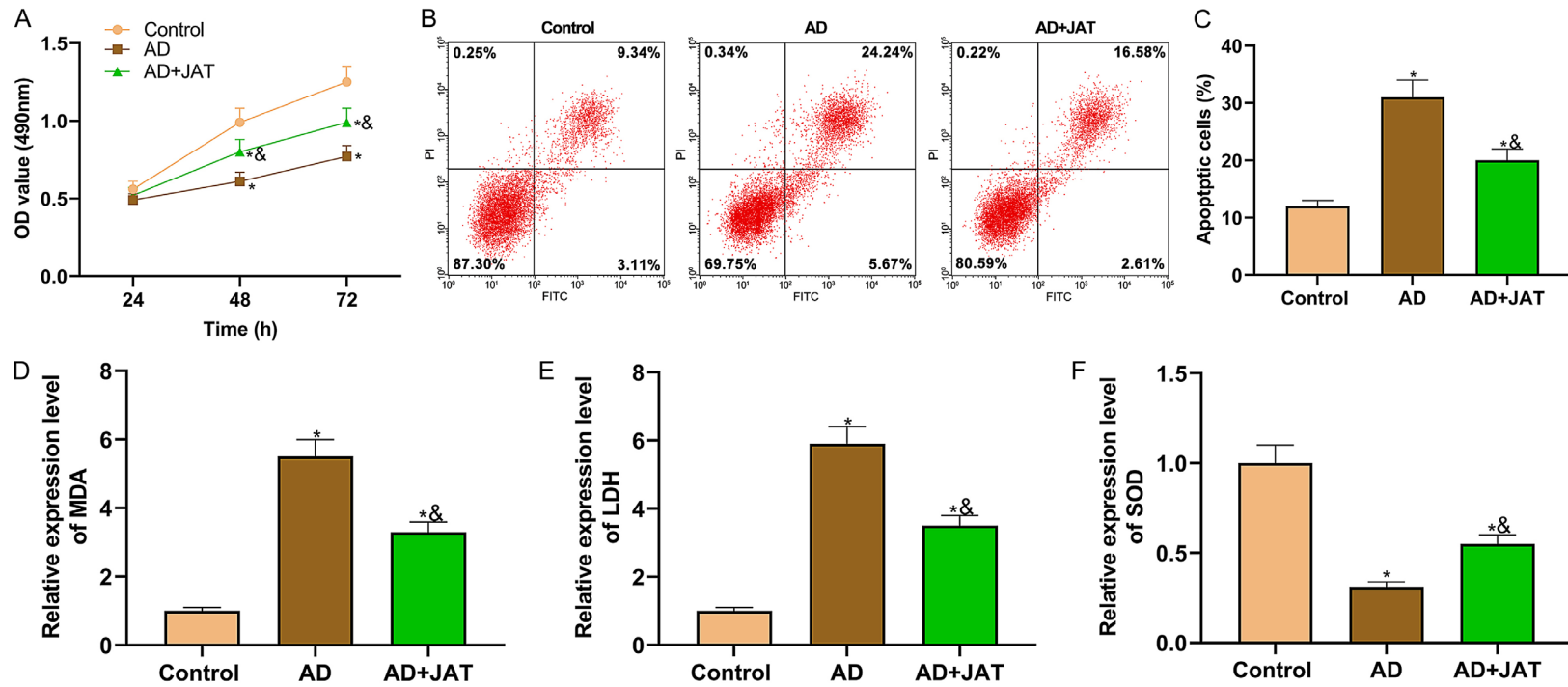


Figure 1. Effect of JAT on SH-SY5Y cells in AD. A: Cell proliferation is tested by MTT assay; B and C: Apoptosis is tested by flow cytometry; D-F: Detection results of OS parameters (MDA, LDH and SOD). Compared with control group, *P<0.05, while compared with AD group, &P<0.05. JAT: Jatrorrhizine; MDA: malondialdehyde; LDH: lactate dehydrogenase; SOD: superoxide dismutase.

reduced the apoptosis rate of SH-SY5Y cells stimulated by A β 25-35 ($P < 0.05$; **Figure 1B, 1C**). The OS parameters showed that the levels of oxidative products (MDA and LDH) decreased and those of antioxidant substances (SOD) increased after JAT treatment, suggesting that JAT enhanced the antioxidant capacity of SH-SY5Y cells and reduced cell damage (all $P < 0.05$; **Figure 1D-F**).

Ove-expressing HDAC4 can partially reverse JAT's protective effect on SH-SY5Y cells

qRT-PCR manifested that the HDAC4 expression decreased after JAT was supplemented to SH-SY5Y cell culture medium stimulated by A β 25-35 ($P < 0.05$; **Figure 2A**). To study JAT and HDAC4's mechanism in AD, HDAC4 was overexpressed and transfected into SH-SY5Y cells treated with JAT and A β 25-35 for functional experiments. The HDAC4 expression in each group was shown in **Figure 2B**. Compared with AD group, the HDAC4 expression in AD+JAT group was inhibited, while compared with AD+JAT+pcDNA3.1-NC group, the expression in AD+JAT+pcDNA3.1-HDAC4 group was enhanced (all $P < 0.05$). MTT assay manifested that JAT improved the proliferation of SH-SY5Y cells decreased by A β 25-35, while HDAC4 overexpression vector partially reversed it ($P < 0.05$; **Figure 2C**). The experimental data of apoptosis revealed that the apoptosis rate decreased by JAT increased by pcDNA3.1-HDAC4 (**Figure 2D, 2E**). Compared with AD+JAT+pcDNA3.1-NC group, MDA, LDH increased and SOD decreased in AD+JAT+pcDNA3.1-HDAC4 group, suggesting that HDAC4 overexpression vector aggravated the OS of SH-SY5Y cells stimulated by A β 25-35 ($P < 0.05$; **Figure 2F-H**).

HDAC4 targets miR-223-3p

HDAC4 was chosen as the end point and the possible upstream regulation mechanism was sought. With HDAC4 as the research target, Targetscan and Starbase were used to predict the upstream miRNA that might regulate HDAC4, and the top 50 with higher binding strength in Starbase and those with binding scores below -0.05 in Targetscan were selected and intersected. Three miRNAs (miR-29a-3p, miR-29b-3p and miR-223-3p) were obtained (**Figure 3A**). We focused on miR-223-3p (**Figure 3B**). Research has shown that miR-223 in serum exosomes can be used as an impor-

tant diagnostic and prognostic marker of dementia [14]. The expression of serum miR-223-3p can be used as a key diagnostic index of AD [17]. Hence, miR-223-3p was selected for follow-up study.

The interaction between HDAC4 and miR-223-3p was verified by dual luciferase report experiment. Compared with miR-NC and HDAC4-WT co-transfection group, the luciferase activity of miR-223-3p mimic and HDAC4-WT co-transfection group decreased ($P < 0.05$; **Figure 3C**). This manifested that after miR-223-3p was overexpressed in SH-SY5Y cells, the HDAC4 expression decreased, and miR-223-3p could negatively regulate the expression ($P < 0.05$; **Figure 3D**).

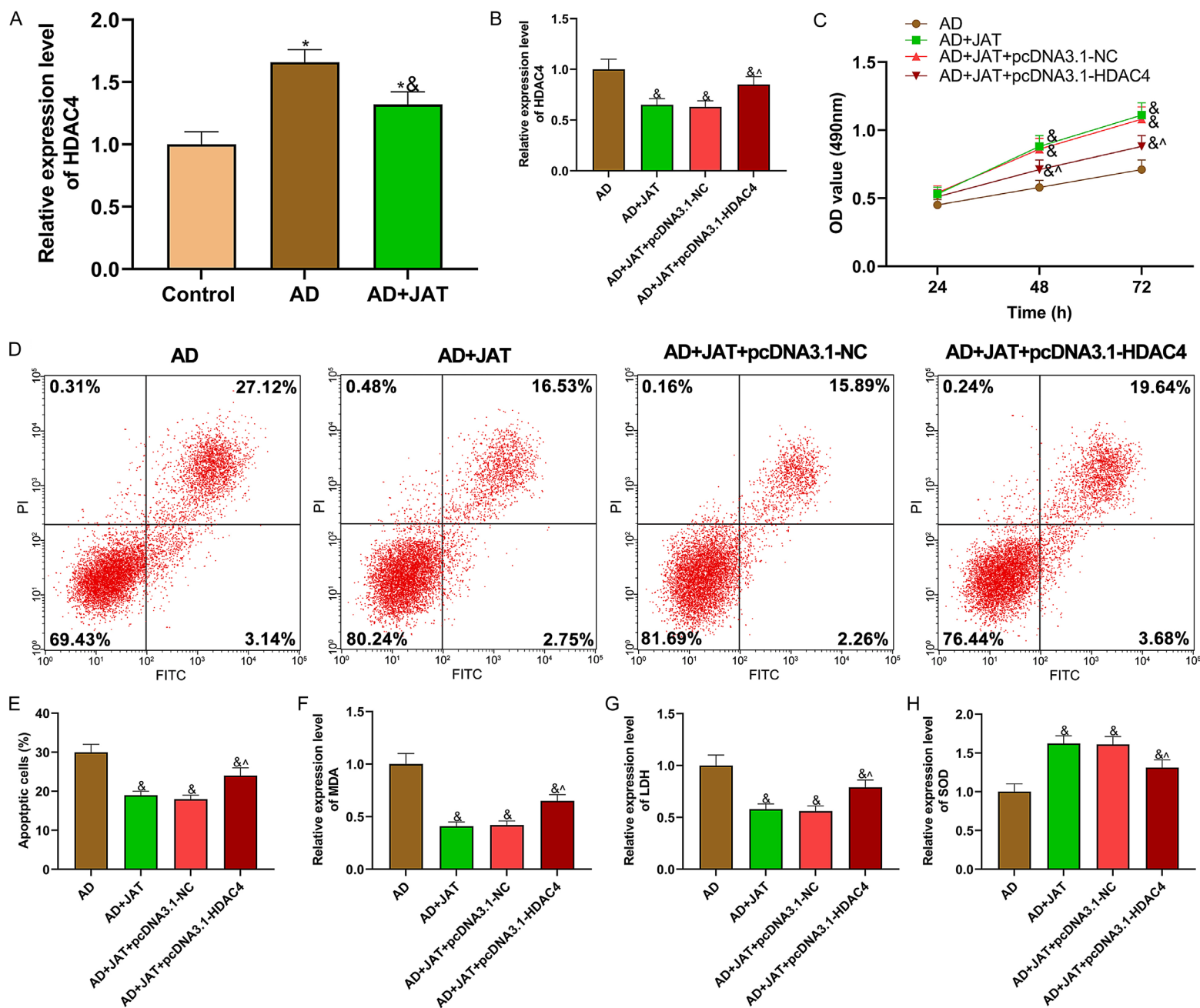
miR-223-3p inhibitor can partially reverse JAT's protective effect on SH-SY5Y cells

qRT-PCR detection revealed that the miR-223-3p expression in SH-SY5Y cells treated with A β 25-35 increased after JAT was added ($P < 0.05$; **Figure 4A**). To study JAT and miR-223-3p's mechanism in AD, the miR-223-3p inhibitor was transfected into SH-SY5Y cells for functional analysis. The data showed the miR-223-3p expression in each group of cells (**Figure 4B**). Compared with AD group, the cell proliferation of AD+JAT group increased ($P < 0.05$), and that of SH-SY5Y cells decreased after miR-223-3p was inhibited ($P < 0.05$; **Figure 4C**). Flow cytometry analysis manifested that the apoptosis rate in the AD+JAT group was lower than that in the AD group ($P < 0.05$), while transfecting miR-223-3p inhibitor increased the apoptosis rate that was decreased by JAT ($P < 0.05$; **Figure 4D, 4E**). The OS parameters manifested that miR-223-3p inhibitor partially reversed the decreased levels of oxidation products (MDA and LDH) and increased those of antioxidant substances (SOD) in JAT ($P < 0.05$; **Figure 4F-H**).

JAT regulates miR-223-3p/HDAC4 to reduce SH-SY5Y cell injury stimulated by A β 25-35

To study the mechanism of JAT combined with miR-223-3p/HDAC4 in AD, HDAC4 and miR-223-3p overexpression vectors were co-transfected into JAT-treated AD cell models. **Figure 5A, 5B** shows the HDAC4 and miR-223-3p expression in each group of cells. The functional experimental data signify that overexpressing HDAC4 can inhibit the proliferation of

Jatrorrhizine improves nerve cell damage induced by A β 25-35



Jatrorrhizine improves nerve cell damage induced by A β 25-35

Figure 2. Overexpressing HDAC4 can partially reverse JAT's protective effect on SH-SY5Y cells. A, B: Expression of HDAC4 in each group of cells; C: Cell proliferation is tested by MTT assay; D, E: Apoptosis is tested by flow cytometry; F-H: Detection results of OS parameters (MDA, LDH and SOD). Compared with control group, * $P < 0.05$, compared with AD group, $^{\#}P < 0.05$, while compared with AD+JAT+pcDNA3.1-NC group, $^{\wedge}P < 0.05$. JAT: Jatrorrhizine; MDA: malondialdehyde; LDH: lactate dehydrogenase; SOD: superoxide dismutase.

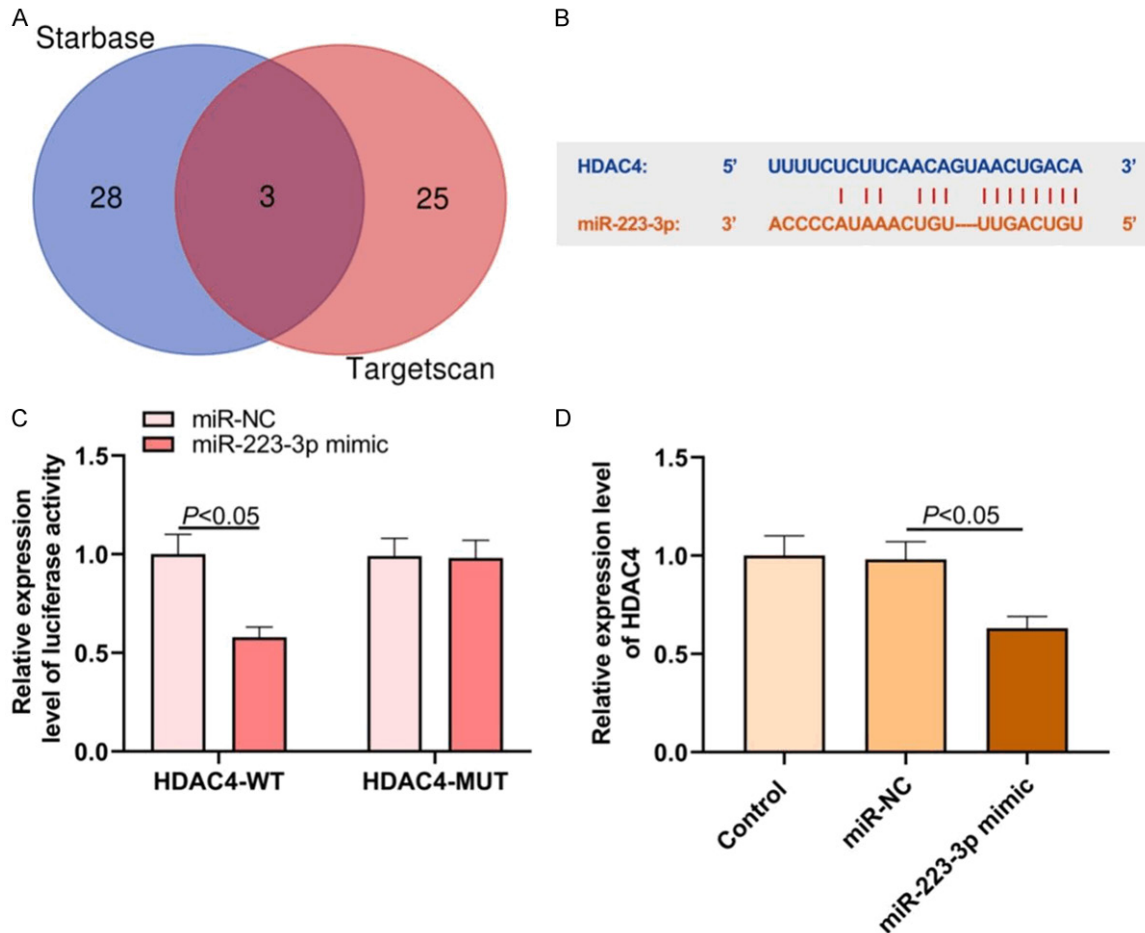


Figure 3. Bioinformatic tools predict possible molecular mechanisms in AD. A: Common targeted miRNAs predicted by HDAC4 in Targetscan and StarBase databases; B: Binding site of HDAC4 and miR-223-3p; C: Dual luciferase report experimental results; D: Effect of miR-223-3p on HDAC4 expression in SH-SY5Y cells.

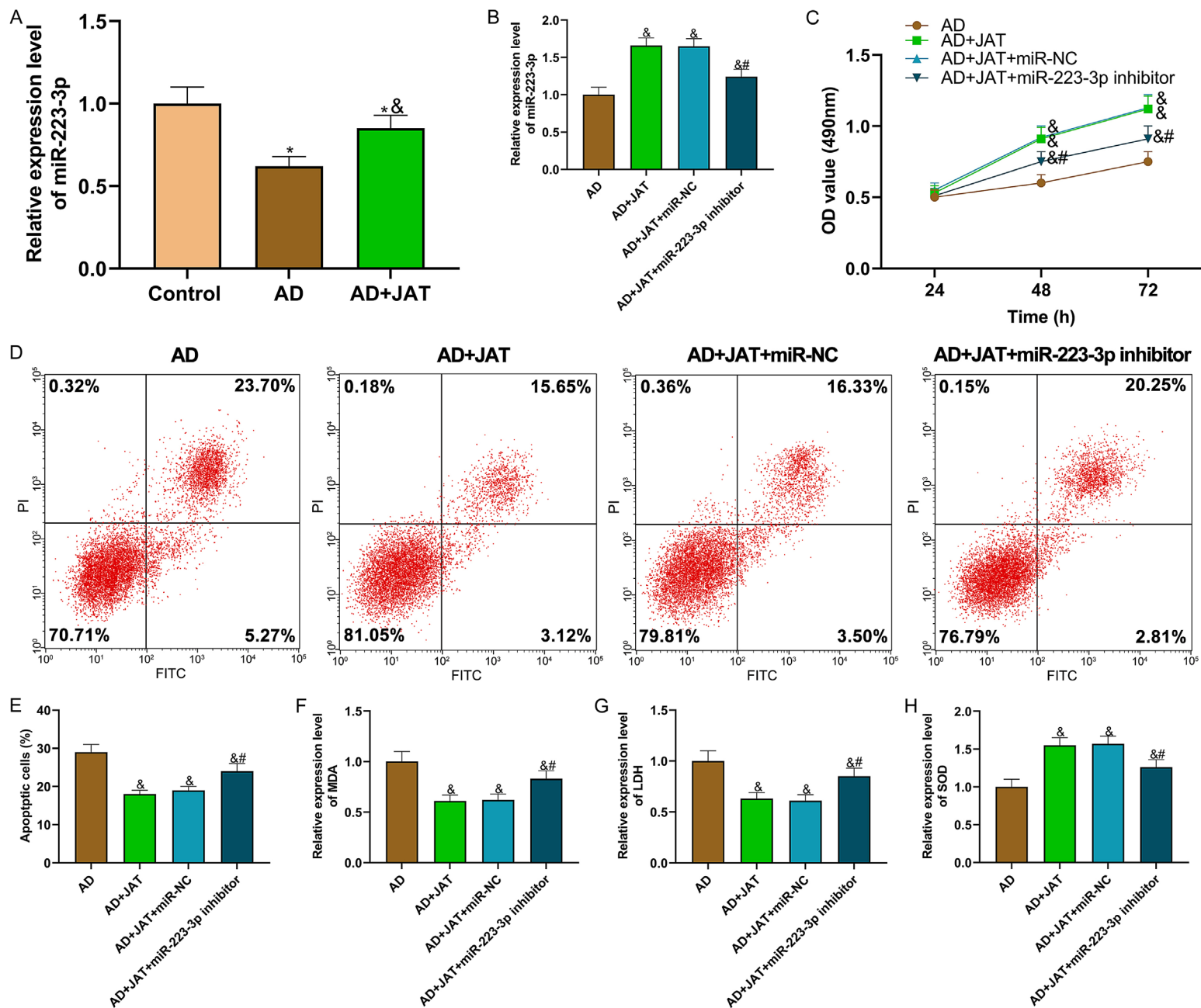
JAT, increase the apoptosis rate, and aggravate OS, while over-expressing miR-223-3p can partially reverse the effect mediated by pcDNA3.1-HDAC4, improve the proliferation and antioxidant capacity of SH-SY5Y cells in AD, and reduce the apoptosis rate ($P < 0.05$; **Figure 5C-H**). The above results suggest that JAT can protect SH-SY5Y cells induced by A β 25-35 by up-regulating miR-223-3p and inhibiting HDAC4.

The mechanism diagram of this research is shown (**Figure 6**).

Discussion

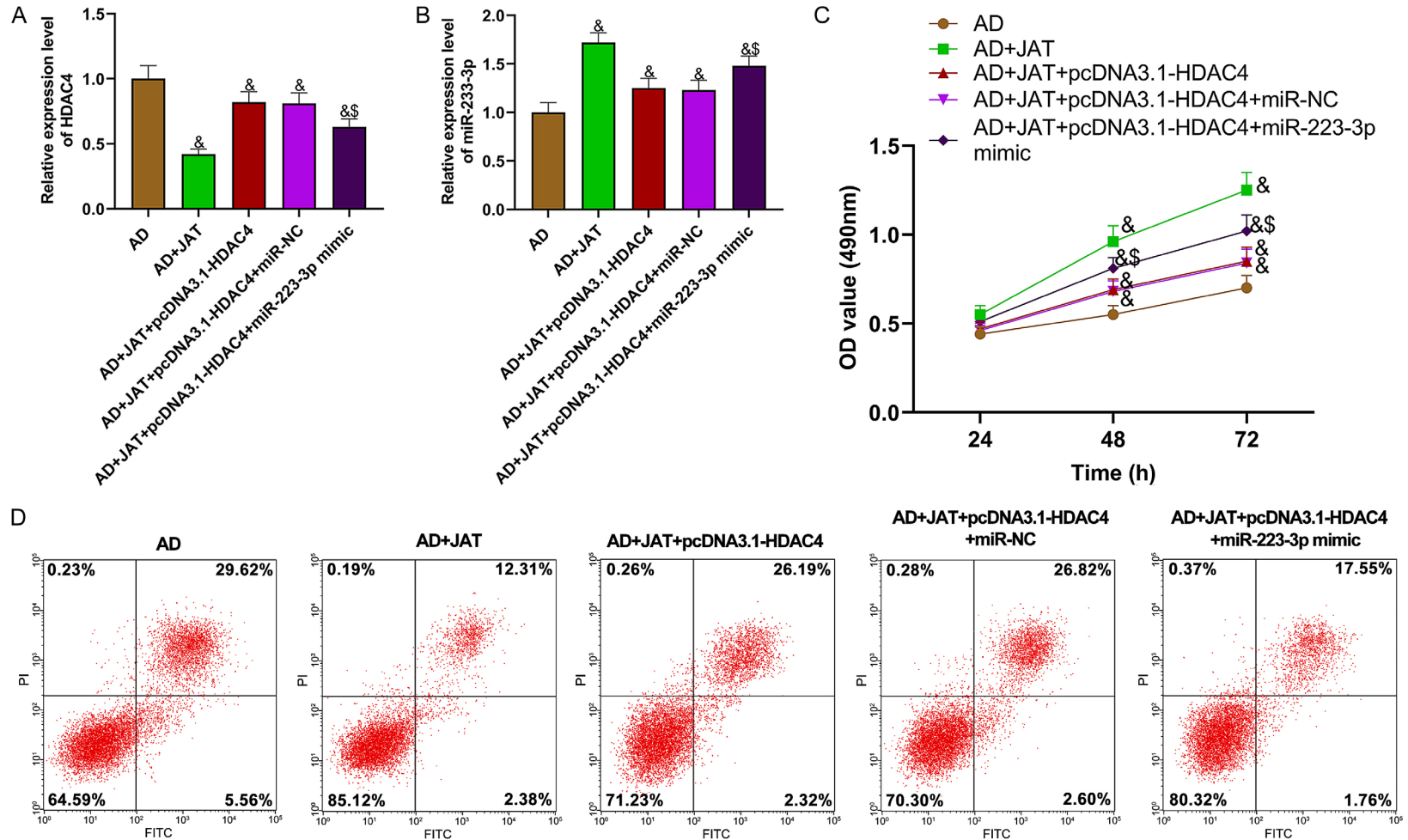
A toxic effect of A β on AD neurons has been confirmed [4]. A β 25-35 is a short toxic fragment with high cytotoxicity and neurotoxicity, that has been widely used to establish AD models in vitro [18]. JAT has been proven to have the effect of scavenging oxygen free radicals and reducing neuronal stress response, and its therapeutic effect on AD has been preliminarily reported [16]. Compared with the control group, JAT treatment of SH-SY5Y cells stimulated by A β 25-35 can improve cell proliferation and

Jatrorrhizine improves nerve cell damage induced by A β 25-35



Jatrorrhizine improves nerve cell damage induced by A β 25-35

Figure 4. miR-223-3p inhibitor can partially reverse JAT's protective effect on SH-SY5Y cells. A, B: Relative expression of miR-223-3p in each group of cells; C: Cell proliferation is tested by MTT assay; D, E: Apoptosis is tested by flow cytometry; F-H: Detection results of OS parameters (MDA, LDH and SOD). Compared with control group, *P<0.05; compared with AD group, #P<0.05; compared with AD+JAT+miR-NC group, #P<0.05. JAT: Jatrorrhizine; MDA: malondialdehyde; LDH: lactate dehydrogenase; SOD: superoxide dismutase.



Jatrorrhizine improves nerve cell damage induced by A β 25-35

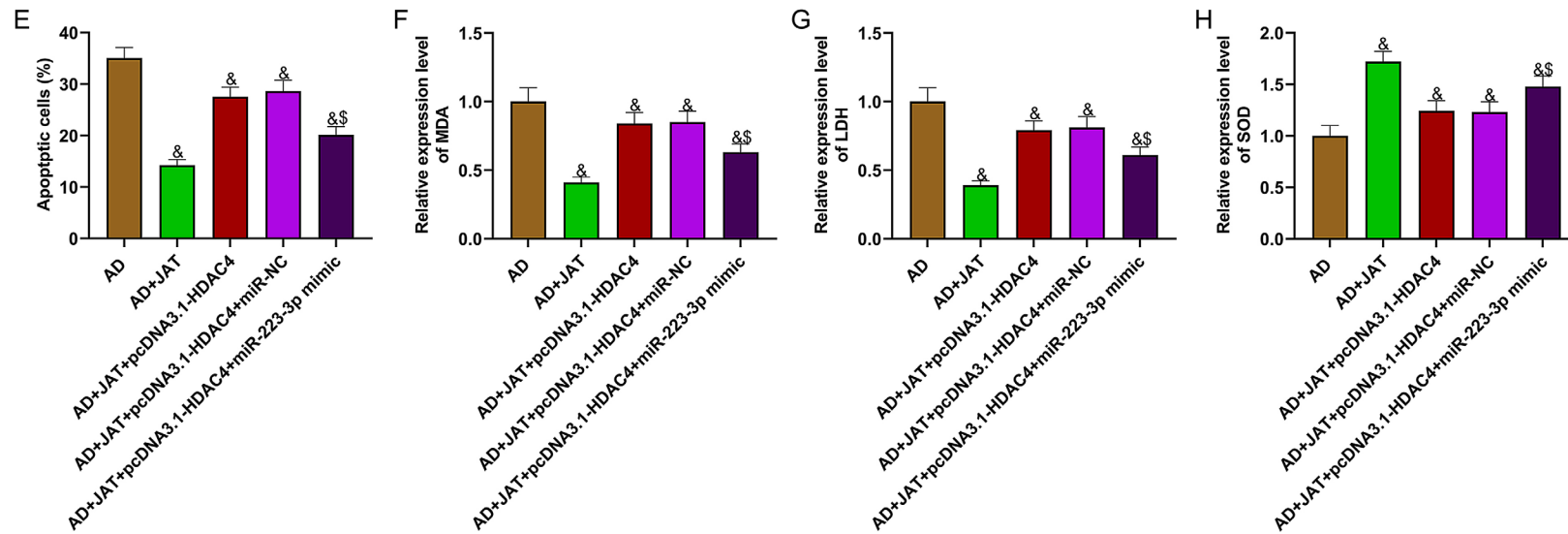


Figure 5. JAT regulates miR-223-3p/HDAC4 to reduce SH-SY5Y cell injury stimulated by A β 25-35. A: Relative expression of HDAC4 in each group of cells; B: Relative expression of miR-223-3p in each group of cells; C: Cell proliferation is tested by MTT assay; D, E: Apoptosis is tested by flow cytometry; F-H: Detection results of OS parameters (MDA, LDH and SOD). Compared with AD group, ^{&}P<0.05; compared with AD+JAT+pcDNA3.1-HDAC4+miR-NC group, ^{&}P<0.05. JAT: Jatrorrhizine; MDA: malondialdehyde; LDH: lactate dehydrogenase; SOD: superoxide dismutase.

Jatrorrhizine improves nerve cell damage induced by A β 25-35

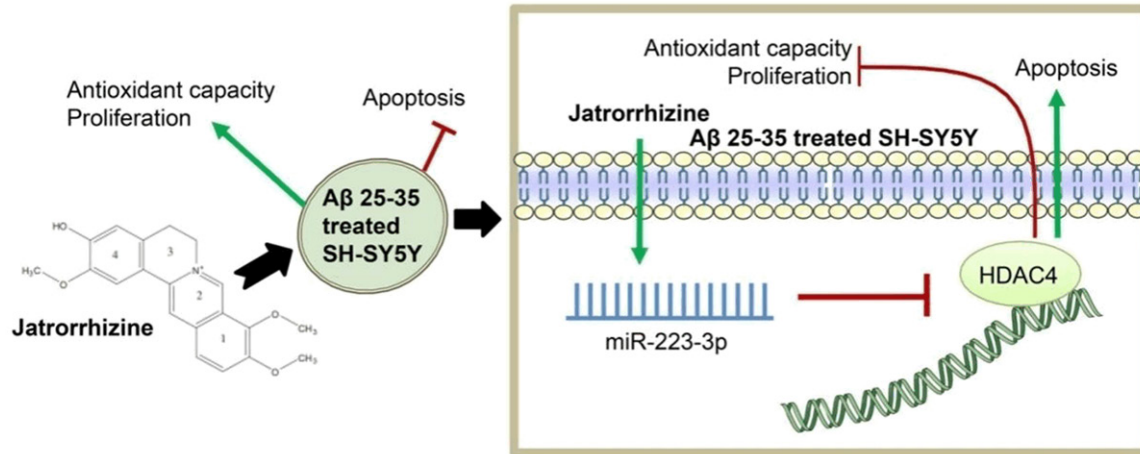


Figure 6. Jatrorrhizine ameliorates A β 25-35-induced neuronal injury through the miR-223-3p/HDAC4 axis.

inhibit apoptosis, inflammation and OS, suggesting the value of JAT in AD treatment. Thus, we studied the mechanism of JAT in AD with molecular biology.

Next, we explored HDAC4's effects on SH-SY5Y cells stimulated by A β 25-35. Our results manifested that the HDAC4 expression increased in SH-SY5Y cells treated with A β 25-35, while JAT could decrease the expression. Overexpressing HDAC4 can aggravate apoptosis and OS stimulated by A β 25-35, inhibit cell proliferation, and partially reverse the therapeutic effect of JAT on nerve cells in AD. HDAC4 mainly exists in the cytoplasm of neurons and shuttles between cytoplasm and nucleus induced by incoming and outgoing signals [19]. HDAC has been reported to regulate the survival of neurons. Some studies have found that ApoE4 exerts its harmful effects by affecting the deposition of A β and neurofibrillary tangles in the brain, and it reduces the expression of brain-derived neurotrophic factors by up-regulating the expression of HDAC4 [20]. These results indicate that JAT regulates HDAC4 to participate in the occurrence and development of AD, which further proves the previous studies on the nerve damage function of HDAC4.

Many miRNAs are effective in AD progression [21]. In this research, miR-223-3p was selected as HDAC4's target miRNA and the targeted relationship between them was verified. Previous research manifested that the serum miR-223-3p expression could be used as an important diagnostic index for AD [22]. miR-

223-3p is a latent therapeutic target in AD progression, but its mechanism is vague. This research found that the interaction between miR-223-3p and HDAC4 was verified by dual luciferase reporter experiment. In addition, JAT can up-regulate the down-regulated miR-223-3p level in AD cell models. miR-223-3p inhibitor can partially reverse JAT's efficacy on AD, and overexpressing miR-223-3p can partially reverse the nerve cell injury aggravated by overexpression of HDAC4. These results reveal that JAT can protect nerve cells in AD progression by up-regulating miR-223-3p and inhibiting HDAC4. However, in vivo experiments have not been discussed in this experiment, and animal experiments are needed for further verification.

In general, this research confirmed that JAT regulated the miR-223-3p/HDAC4 axis to protect neurons induced by A β 25-35. JAT/miR-223-3p/HDAC4 may be an effective target for AD.

Disclosure of conflict of interest

None.

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