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# Effect of edaravone on synaptic damage in Alzheimer's disease via Rho/ROCK signaling

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**Abstract**: **Background**: Edaravone can reduce damage to brain cells in a variety of ways. Rho/ROCK signaling is involved in Alzheimer's disease. **Objectives**: This study uses AD cell models to explore edaravone's effect on AD nerve synapse damage. **Methods**: The ROCK2 promoter luciferase reporter gene was constructed, Aβ25-35 was used as a processing factor and transfected into PC12 cells to construct an AD cell model which was then treated with edaravone, followed by analysis of 95 antibody (PSD95), synapse-related mRNA synapsin 1 (SYN1) level to observe its effect on Rho/ROCK signaling. **Results**: Edaravone can effectively inhibit the transcriptional activity of ROCK2 promoter in AD cell model, upregulate SYN1 and GAP43 protein and downregulate ROCK2. After ROCK2 overexpression, edaravone can affect SYN1 expression in AD cell model, while SYN1 expression did not change significantly after ROCK2 was silenced. **Conclusion**: Edaravone has a protective effect on AD nerve synapse damage and plays a role in repairing nerve synapse damage through ROCK2 signaling pathway.

**Keywords**: Alzheimer's disease; Edaravone; PC12 cells; Rho/ROCK pathway

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## INTRODUCTION

Alzheimer's disease is a progressively developing neurodegenerative disease, clinically characterized by memory loss, intellectual disability, behavioral changes and emotional abnormalities (Zhang *et al.*, 2020). In recent years, with the aging process, the incidence of AD has increased. The research and development of AD drugs is a current research hotspot and it is also an important help for social health (Sheu *et al.*, 2020). The treatment mechanism is not yet fully understood. The precipitation of Aβ protein in the brain of senile plaques is the main case feature. Aβ protein is an important apoptotic product of precursor protein (Shamsi *et al.*, 2020). There are also decreased synapses and neuron loss in the brains of AD patients. It is directly related to clinical symptoms and an important pathological basis of AD (Mehri *et al.*, 2020). The Rho/ROCK pathway is closely related to Aβ protein precipitation and synapse reduction, which can directly inhibit neuronal interaction and the activation of Rho/ROCK pathway can also increase Aβ protein precipitation. Studies have shown that the Rho/ROCK signaling pathway is involved in a variety of neurological diseases of the brain and statins are helpful in the treatment. Edaravone can reduce brain cell damage, play a role by reducing oxygen free radicals in the brain and perform well in anti-oxidation and can reduce reperfusion injury. Current research also shows that edaravone has a protective effect on nerve synapses. Therefore, consider Rho/ROCK as a targeted pathway for AD treatment to explore its in-depth effects (Lu *et al.*, 2020). Existing studies have shown that Rho/ROCK is related to cell proliferation, migration and cytoskeleton formation (Liu *et al.*, 2020b). The Rho/ROCK pathway plays an important role in the process of inflammation and cerebral hemodynamic disorders and can directly target neurons to play a role (Liu *et al.*, 2020a). In recent years, studies have found that certain non-steroidal anti-inflammatory drugs can reduce the incidence of AD. The reason is that these drugs can significantly reduce the content of Aβ42 and the mechanism is related to the inhibition of ROCK activity (Gao *et al.*, 2020, Hassan *et al.*, 2020). Statins have been used clinically to prevent AD. By affecting APP metabolism, reducing Aβ deposition content, the mechanism is closely related to the Rho/ROCK signal pathway (Bharani *et al.*, 2020). The mechanism of edaravone in the Rho/ROCK signal remains to be explored (Arnold *et al.*, 2020). In order to further study the protective effect of edaravone on AD nerve synapse damage, this study intends to establish an AD cell model to verify the biological effects of edaravone on AD nerves.

## MATERIALS AND METHODS

### *Experimental materials*

PC12 cells (Shanghai Shenggong); primary antibodies: Rho, ROCK; SYBR Premix Ex Taq kit (Wuhan Kehaoja Co., Ltd.); Real-time PCR system (Shanghai Shanpu Co., Ltd.).

### *Main experimental reagents and instruments*

Edaravone (from the Department of Pharmacy, The Affiliated Hospital of Hebei Engineering University; MTT). SYN1, ROCK (Beijing Boaosheng), DMEM high glucose complete medium; Rho (abclonal, USA); Aβ

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protein fragment 25-35 preparation (Wuhan Boster), P62, GAP43, β-actin antibodies (Tianjin Best).

***Experimental grouping and the rationale behind it***

Grouping: high-dose group (Lu *et al.*, 2024), low-dose group (Zhao *et al.*, 2022), model group, normal group; transfection group: ROCK2-siRNA12h group, ROCK2-siRNA6h group, control group; joint intervention experiment group: ROCK2-siRNA, ROCK2-overexpression, ROCK2-siRNA+according to Daravone, ROCK2-overexpression+edaravone.

In this study, the administration concentration of edaravone was determined based on previously effective *in vivo* doses. Research has shown that in a rat model of autism, oral administration of edaravone at a dose of 30 mg/kg effectively alleviated behavioral deficits and acted on the oxidative stress pathway (Lu *et al.,* 2024). Through pharmacological extrapolation, this high *in vivo* dose provided the basis for selecting 30μg/mL as the high concentration in our study. Furthermore, a study on a middle cerebral artery occlusion model confirmed that oral edaravone, at a dose as low as 10 mg/kg (representing the lower limit of its effective dose range), could produce significant neuroprotective effects, with the efficacy being dose-dependent (Zhao *et al.,* 2022). Therefore, these effective *in vivo* doses support the rationale for our dose selection.

***MTT assay***

PC12 cells were inoculated into a 96-well plate and Aβ aging solution was diluted with 1640 complete medium to different concentrations to treat cells. After gently washing, MTT was added for 4 hours and then DMSO was added to measure OD value by a microplate reader after crystallization (Ghasemi *et al.*, 2021).

***Flow cytometry detection***

Cells were collected, washed with pre-cooled PBS and then resuspended in 1× binding buffer. Annexin V-FITC and propidium iodide (PI) staining solutions were added, followed by incubation at room temperature for 15 minutes in the dark. After the incubation, cell apoptosis was analyzed by flow cytometry (Lakshmanan and Batra, 2013).

***qRT-PCR d method***

Total RNA was extracted from the cells. Amplification was performed using the SYBR Premix Ex Taq kit on a real-time quantitative PCR instrument. The reaction protocol was as follows: Initial denaturation at 95°C for 30 seconds; followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 34 seconds. The relative mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Rao *et al.*, 2013), with GAPDH used as the internal reference. The primer sequences were: SYN1: Forward 5'- ATCACGACCTCAA CGACCTG -3', Reverse 5'- GTCGTTGAGGTCGT GATGGT -3';GAPDH: Forward 5'- TGGAGTCTACTGG CGTCTTC -3', Reverse 5'- GTCATCATACTTGGC AGGTT -3'.

***Statistical analysis***

Statistical analysis was performed using SPSS software (version 23.0). All continuous data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. Upon confirmation that the data met the assumptions for parametric tests, one-way analysis of variance (ANOVA) was employed for comparisons among multiple groups, followed by post-hoc pairwise comparisons using the Bonferroni correction. If the data failed to meet the assumptions for parametric tests, the Kruskal-Wallis H test was used for comparisons among multiple groups. A P-value of less than 0.05 was considered statistically significant.

## RESULTS

***Detection of Aβ25-35 on PC12 cell activity***

The effect of Aβ25-35 on the viability of PC12 cells was evaluated by MTT assay. ANOVA results indicated that the effects of different doses of Aβ25-35 on cell viability were statistically significant at different treatment times (7 days and 14 days) (all $P<0.05$). Subsequent Bonferroni post-hoc analysis revealed that after 14 days of treatment, cell viability in all Aβ25-35 treatment groups (1-30 μM) was significantly lower than that in the control group at the same time point (all $P<0.05$). In contrast, after 7 days of treatment, no statistically significant differences were observed between any treatment group and the control group (all $P>0.05$) (Table 1).

***The transcriptional active expression of ROCK2 gene promoter***

The promoter activity of the PGL3-basic group was lower than that of the PGL3-ROCK2 group, indicating that the expression of the transfected ROCK2 gene promoter was increased significantly in PC12 cells (Fig. 1).

***Edaravone reduces the expression of ROCK2 in PC12 cells induced by Aβ25-35***

Compared with normal group, Aβ25-35 can significantly promote the expression of ROCK2 in model group. The low-dose group and high-dose group edaravone treatment can effectively inhibit ROCK2 level with higher effect for high-dose group (30μg/ml) ($P<0.05$) (Figs. 2a and 2b).

***ROCK2-siRNA can effectively silence the expression of ROCK2 mRNA in cells***

PC12 cells were transfected with ROCK2-siRNA at 6h and 12h and PCR analysis showed that compared with normal group, a significantly reduced ROCK2 protein level was found at 6h ($P<0.05$) (Fig. 3).

**Table 1**: The effect of Aβ25-35 on PC12 cell activity.

| Aβ25-35 dose | Relative cell viability (ratio to control) | |
|---|---|---|
| | 7d | 14d |
| 0(control) | 1.000±0.023 | 1.000±0.044 |
| 1μM | 0.794±0.034 | 0.498±0.042* |
| 10μM | 0.692±0.045 | 0.463±0.054* |
| 20μM | 0.637±0.046 | 0.476±0.035* |
| 30μM | 0.624±0.023 | 0.446±0.048* |
| F | F(4,20)=4.068 | F(4,20)=4.584 |
| P | 0.018 | 0.013 |

Note: Data are presented as mean ± standard deviation (n=5, representing five independent biological replicates). According to ANOVA with Bonferroni post-hoc test, * P<0.05 versus the control group at the same time point.

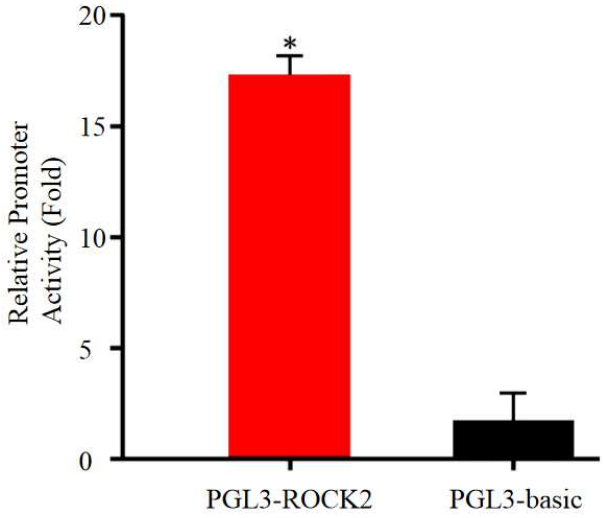


**Fig. 1**: The transcriptional activity of ROCK2 gene promoter in PC12 cells.
Note: Data are presented as mean ± standard deviation (n=4). According to the t-test, P<0.05 versus the PGL3-basic group. PGL3-basic: Empty plasmid, negative control. PGL3-ROCK2: Reporter plasmid containing the ROCK2 promoter.

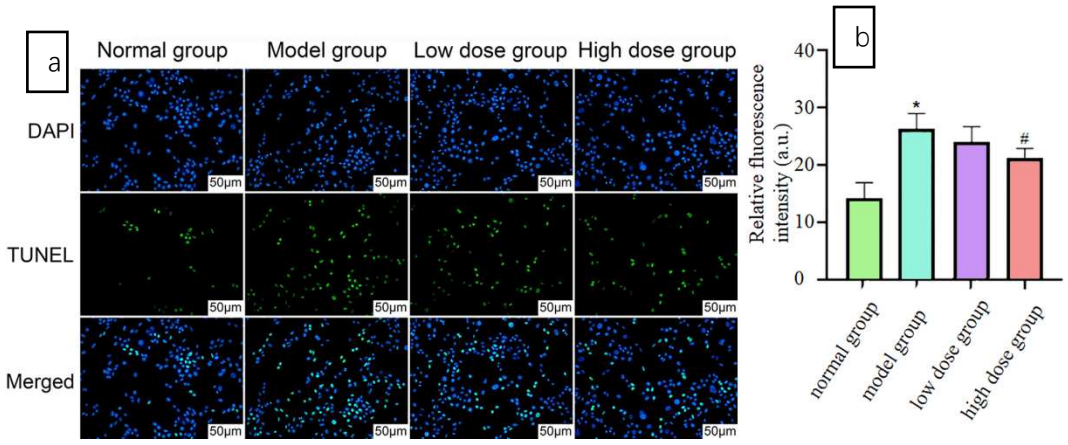


**Fig. 2**: Edaravone reduces ROCK2 expression in Aβ25-35-injured PC12 cells (a) after different treatment and (b) quantitative analysis.
Note: Scale bar in fluorescence images=100μm. Data are presented as mean±standard deviation (n=3 independent experiments). According to ANOVA with Bonferroni post-hoc test, #P<0.05 versus the Normal group; #P<0.05 versus the Low-dose group.

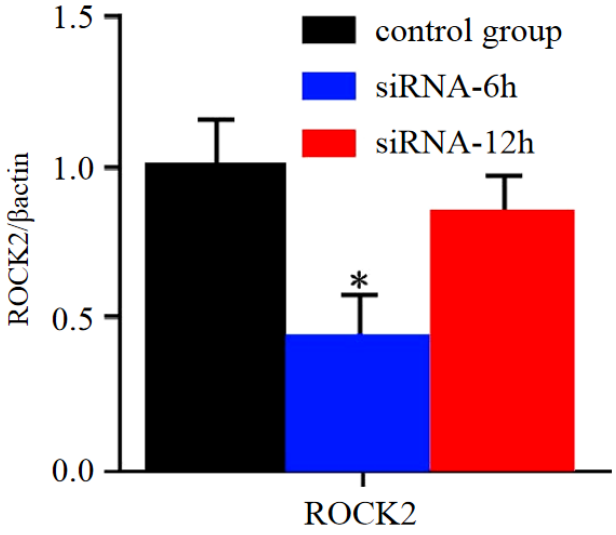


**Fig. 3**: ROCK2-siRNA can effectively silence the expression of ROCK2 mRNA in cells.
Note: Data are presented as mean ±SD (n=5). According to ANOVA with Bonferroni post-hoc test, *P<0.05 versus the control group. siRNA: Small interfering RNA. si-NC: Negative control siRNA. si-ROCK2: ROCK2-targeting siRNA.

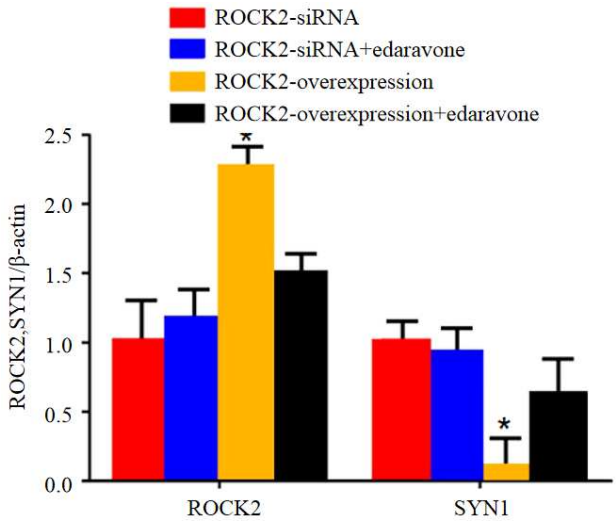


**Fig. 4**: The protective effect of edaravone targeting ROCK2 on nerve synapses.
Note: Data are presented as mean ±SD (n=5). According to ANOVA with Bonferroni post-hoc test, *P<0.05 versus the ROCK2-siRNA group.. ROCK2-siRNA: ROCK2-targeting siRNA. ROCK2-overexpression: ROCK2 overexpression. SYN1: Synapsin I. ROCK2: Rho-associated coiled-coil-containing protein kinase 2.

***The protective effect of edaravone on nerve synapses***

In the case of ROCK2 silencing (siRNA) and overexpression (overexpression), it was found that there was no significant difference between the ROCK2 inhibition and the Aβ25-35 treatment group after silencing alone. The SYN1mRNA expression in the edaravone treatment group was weakened. In the case of ROCK2 expression, compared with simple overexpression, SYN1 in treatment group increased more significantly, indicating that edaravone may directly interact with nerve synapses by targeting ROCK2 (Fig. 4).

## DISCUSSION

AD, as a systemic disease of the elderly, progresses rapidly, has a long course with cognitive impairment, which affects the quality of life of the elderly. The development of AD drugs is of positive significance for the prevention and treatment of the disease (Arnold et al., 2020). This study established a cell model for AD. By inducing the aging of PC12 cells, cells present a polygonal, adherent cell aging morphology and have neuroendocrine characteristics, which can well simulate the pathogenesis of AD. Because of its stable cell line passage and long survival time, it was selected as one of the AD cell models in this experiment. In this experiment, Aβ25-35 was used as the cell modeling reagent. In order to confirm the appropriate AD cell model, Aβ25-35 with different aging times was applied to PC12 cells. After preliminary screening by MTT, Aβ25-35 can cause cell aging. At 14 days, the activity is significantly lower than that at 7 days. In order to study the protective effect of edaravone on nerve synapses, in cell experiments, it is necessary to consider the state of the cell model and maintain a certain activity, but it is better that no obvious apoptosis occurs. Therefore, the selection of this cell model is feasible (Abe *et al.*, 2020).

In order to better study the protective effect of edaravone on AD nerve synapse damage, the ROCK2 promoter reporter gene assay was constructed. In order to elucidate the relationship between ROCK2 and the protective effects of edaravone against synaptic damage, the ROCK2 promoter reporter gene was transfected into AD cell model. The results show that edaravone can reduce the transcriptional activity of the ROCK2 promoter in AD cell models, which indicates that edaravone has a good protective effect on AD cell models and can reduce synaptic damage (Calvo and Einstein, 2023, Zegarra-Valdivia *et al.*, 2023). To further verify the synaptic protective effect of edaravone on cell aging, edaravone was used to intervene in AD cell models and the changes in ROCK2 promoter transcriptional activity were observed. The results showed that the expression of synaptic-associated proteins SYN1 and GAP43 was significantly reduced. After edaravone treatment, the expression of synapse-associated protein was significantly increased and the expression of ROCK2 protein was significantly weakened. The results suggest that edaravone has a good synaptic protective effect on AD cell models, which can effectively inhibit the expression level of Rho/ROCK and exert neuroprotective effects (Liu et al., 2020a, Liu et al., 2020b).

It remains to be investigated whether Rho/ROCK is involved in this regulatory process. We use exogenous to construct AD cell model, transfect the interfering plasmid ROCK2-siRNA into the cell model and detect the expression level of Rho/ROCK pathway. In order to understand the mechanism of edaravone, the ROCK2-overexpression group was set up at the same time and the control observation was carried out.

The positive and negative proofs of the effect of edaravone on the ROCK2 promoter reporter gene. After the successful silence of ROCK2, there was no significant difference in the expression of SYN1 in AD model cells after edaravone treatment. After overexpression of ROCK2, the cell damage was severe and the edaravone treatment group could significantly increase the expression of SYN1, suggesting the protective effect of edaravone on nerve synapse damage. The protective effect of AD cell model is closely related to ROCK2 (Aksnes *et al.*, 2022, Quiroz *et al.*, 2020, Vignoli *et al.*, 2025). Although this study, utilizing the PC12 cell model, has effectively revealed the potential mechanism of action of edaravone, this model still possesses certain limitations in simulating the complex pathology of human Alzheimer's disease. PC12 cells inadequately recapitulate the chronic deposition of Aβ, tau protein pathology and the intricate neuroinflammatory environment found in the human body. Consequently, this research primarily elucidates the direct effects of edaravone at the cellular level. To more comprehensively evaluate its therapeutic potential, subsequent in vivo experiments should be conducted in AD transgenic mouse models. This would allow for a thorough assessment of the long-term effects of edaravone on learning and memory behavioral deficits, Aβ plaque deposition, tau pathology and synaptic ultrastructure, thereby providing more compelling evidence for the feasibility of edaravone as a candidate therapeutic agent for AD.

## CONCLUSION

In summary, this study demonstrates that the protective effect of edaravone against synaptic damage in Alzheimer's disease is achieved through inhibition of ROCK2, a core molecule of the Rho/ROCK signaling pathway. This finding not only provides a novel mechanistic explanation for the neuroprotective effect of edaravone but also positions ROCK as a highly potential therapeutic target for AD. Future studies will further validate this mechanism in animal models and delve deeper into the precise molecular pathways by which ROCK2 regulates the expression and function of downstream synaptic proteins.

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***Authors' contribution***
Yixie Fan: Methodology and conceptualization; Qiuyue Lai: Investigation and formal analysis; Qiong Li: Writing - original draft; Yuejun Li: Writing - review and editing. All authors have read and agreed to the published version of the manuscript.

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***Data availability statement***
Data are available from the corresponding authors.

***Ethical approval***
This study utilized only commercially available cell lines and did not involve any experiments on humans or animals.

***Conflicts of interest***
All authors of this study declare no conflicts of interest.

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