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# Akebia saponin D reverses corticosterone hypersecretion in an Alzheimer's disease rat model

Yuhui Wang[a,1], Jinyang Shen[b,1], Xiaolin Yang[c,f,1], Ye Jin[d], Zhonglin Yang[b], Rufeng Wang[e,f,*], Fuming Zhang[f], Robert J. Linhardt[f,g,h]

[a] Department of Pharmacology, Guilin Medical University, Guilin, China
[b] State Key laboratory of natural Medicines, China Pharmaceutical University, Nanjing, China
[c] Jiangsu Key Laboratory of Research and Development in Marine Bio-resource Pharmaceutics, Nanjing University of Chinese Medicine, Nanjing, China
[d] Pharmacy Department, Yangzhou Hospital of Traditional Chinese Medicine, Yangzhou, China
[e] Chinese Medicines and the SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Material Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China
[f] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA
[g] Department of Chemistry, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA
[h] Departments of Biology, Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies Rensselaer Polytechnic Institute, Troy, NY, USA

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ABSTRACT

*Background:* Glucocorticoid hormones are implicated in the pathogenesis of Alzheimer's disease (AD) and other diseases including diabetes, hyperlipidemia, and osteoporosis. Akebia saponin D (ASD) possesses numerous pharmacological activities, including as an anti-AD, anti-hyperlipidemia, anti-diabetes, and anti-osteoporosis agent. The anti-AD effect of ASD is possibly through its regulation of glucocorticoid levels.

*Purpose:* The present study was undertaken to investigate the neuroprotective effects of ASD on $A\beta_{25\text{-}35}$-induced cognitive deficits and to elucidate its underlying mechanism of action.

*Methods:* The AD rat model was established by an intracerebroventricular injection of $A\beta_{25\text{-}35}$ into the lateral ventricles. Spatial learning and anxiety state were assessed by Morris water maze task and elevated plus-maze assay, respectively. The degree of hypertrophy of adrenal gland was analyzed using the viscera coefficient. Corticosterone and ACTH concentrations in the plasm were measured using biochemical assay kits. The activity of 11β-hydroxysteroid dehydrogenase type-1 (11β-HSD1) in liver and groin fat pad was assessed by measuring cortisol production.

*Results:* Compared with the control group, AD rats displayed significant spatial learning and reference memory impairments, serious anxiety disorders, obvious hypertrophy of adrenal gland, elevated corticosterone and ACTH levels in the plasma, and increased 11β-HSD1 activity in liver and groin fat pad. ASD could significantly ameliorate the memory deficits and anxiety symptoms, markedly reduce the viscera coefficient of adrenal gland, observably decrease corticosterone and ACTH concentrations, and showed no effect on the activity of 11β-HSD1.

*Conclusions:* These results indicate that ASD might exert a significant neuroprotective effect on cognitive impairment, driven in part by reducing systemic corticosterone level by down-regulation of the hypothalamic-pituitary-adrenal (HPA) axis.

## 1. Introduction

Alzheimer's disease (AD) is an incapacitating neurodegenerative disease characterized by the presence in the brain of senile plaques and neurofibrillary tangles accompanied by synaptic and neuronal loss [1]. Up to now, approved medicines for AD, including acetylcholinesterase

*Abbreviations:* AD, Alzheimer's disease; ASD, Akebia saponin D; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type-1; ACTH, adrenocorticotropin hormone; Aβ, amyloid β-peptide; BDNF, brain-derived neurotrophic factor; APP, amyloid precursor protein; *icv*, intracerebroventricular; HPA, hypothalamic-pituitary-adrenal; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; BBB, blood brain barrier; NFTs, neurofibrillary tangles

* Corresponding author at: 1200 Cailun Road, Zhangjiang Hi-Tech Park, Shanghai 201203, China.
*E-mail address:* sjy898989@sina.com (R. Wang).

[1] These authors contributed equally to this work.

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inhibitors (donepezil) and *N*-methyl-D-aspartic receptor antagonist (memantine), all have only a short-lived symptomatic benefit to cognition and some of these agents show side effects [2,3]. Therefore, new drugs with improved efficacy and safety profiles and different mechanisms of action are needed for the treatment of AD.

Glucocorticoid hormones (cortisol in primates, corticosterone in mice and rats [4] endogenous substances are mainly released from adrenal cortex and regenerated from cortisone by 11β-hydroxysteroid dehydrogenase type-1 (11β-HSD1). In recent years, glucocorticoid hormones have been shown to strongly correlate with AD. Incresed plasma glucocorticoid and cerebrospinal fluid cortisol levels are described in patients with AD [4–7]. Higher mean 24 h plasma glucocorticoid levels are related to increased cognitive decline in AD [8]. Stress-level cortisol treatment can decrease memory performance even in healthy humans [9,10]. Elevated glucocorticoid levels had multiple pathological effects in the hippocampus, a key region for learning and memory, including altered dendritic morphology [11], long-term potentiation impairment [12], neurogenesis suppression [13], increased levels of excitotoxic amino acids [14] and decreased expression of the brain-derived neurotrophic factor (BDNF) [15]. Stress-level glucocorticoid administration increases amyloid β-peptide (Aβ) formation (possibly through the expression regulation of amyloid precursor protein (APP) and β-APP cleaving enzyme) and accumulated tau protein in neurofibrillary tangles [16,17], both of which are hallmarks of AD. Acute inhibition of 11β-HSD1 improved memory in rodent models of cognition [18]. These findings suggest that high levels of glucocorticoids play a key role in the development and progression of AD, and reducing the systemic glucocorticoid levels may be an attractive target for developing innovative AD therapeutics.

Akebia saponin D (ASD, Fig. 1), a triterpenoid saponin isolated from the rhizome of *Dipsacus asper* Wall, has showed anti-AD efficacy [19–21], but its mechanism is unclear. Meanwhile, ASD also has other pharmacological effects including anti-osteoporosis [22–24], anti-diabetes (unpublished results), and anti-hyperlipidemia (unpublished results) activities. Elevated glucocorticoids are also thought to be involved in many aging-related diseases, including AD, osteoporosis, obesity, insulin resistance, and hyperlipidemia [25–27]. Therefore, it is reasonable to hypothesize that ASD attenuates amyloid β-induced cognitive deficits by regulating glucocorticoid levels. The present study was undertaken to test this hypothesis in an AD rat model.

## 2. Experimental procedures

### 2.1. Chemicals and reagents

Cortisone was from Aladdin (China). Corticosterone enzyme-linked immunosorbent assay (ELISA) kit was from Abcam (UK). Cortisol ELISA kit was from Elabscience Biotechnology Co., Ltd. (China). Rat adrenocorticotropic hormone (ACTH) ELISA kit was from Nanjing Jiancheng Bioengineering Institute (China).

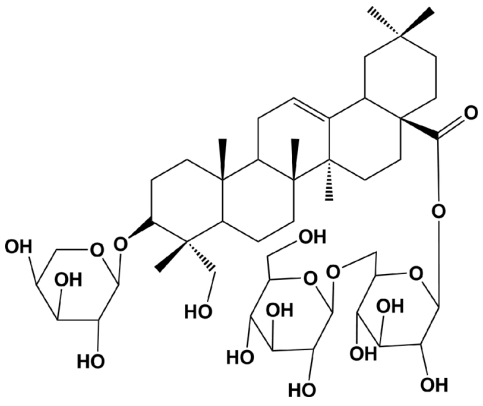


**Fig. 1.** Chemical structure of ASD.

### 2.2. Drug and Aβ peptide

ASD (95.23%, HPLC purity) was a pilot product produced by our laboratory. ASD purity was determined based on the standard substance purchased from the National Institutes for Food and Drug Control (Beijing, China). Donepezil (5 mg/tablet) was obtained from Eisai China Inc. (Suzhou, China).

Commercially available $A\beta_{25\text{-}35}$ (Sigma, USA) was solubilized in sterile water at 1 μg/μL concentration and then was aggregated by *in vitro* incubation at 37 °C for 4 days [28].

### 2.3. Animals

Male Sprague-Dawley rats weighing 260–280 g (6–7 weeks old) (Zhejiang Province Laboratory Animal Center (China) were housed in a standard animal facility (23 ± 2 °C, humidity of 50 ± 10%, 12 h light/dark cycle, food and water *ad libitum*). All animal care and experimentation was approved by the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.4. Experimental procedures

The rats were randomly divided into six groups of 12 rats each. These six groups included control, model ($A\beta_{25\text{-}35}$), Donepezil ($A\beta_{25\text{-}35}$ + 0.5 mg/kg donepezil) as a positive control, ASD-H ($A\beta_{25\text{-}35}$ + 270 mg/kg ASD), ASD-M ($A\beta_{25\text{-}35}$ + 90 mg/kg ASD) and ASD-L ($A\beta_{25\text{-}35}$ + 30 mg/kg ASD).

The intracerebroventricular (*icv*) injection into the lateral ventricles was performed in anesthetized animals with an *intraperitoneal* injection of pentobarbital sodium (50 mg/kg), and using a stereotaxic apparatus (coordinates: anterior-posterior, −1 mm; lateral, ± 1.5 mm; dorsal-ventral, −3.5 mm) [28]. Animals were injected with 5 μL sterile doubly distiled water (control group) or 5 μL aggregated $A\beta_{25\text{-}35}$ solution (other groups) into each lateral ventricle.

The surgery took place on day 0. Donepezil and ASD at the different dose were intragastrically administered once per day for consecutive 30 days starting on day 5. Control and model groups received the same volume of vehicle (0.5% sodium carboxyl methyl cellulose). Morris water maze task took place from day 27 to day 32, and elevated plus-maze assay took place from day 33 to day 34. The rats were decapitated on day 35 for gravimetric and biochemical examination. All experiments were performed between 9 a.m. and 2 p.m. (*i.e.*, during the diurnal portion of their hypothalamic-pituitary-adrenal (HPA) axis rhythm) [28].

### 2.5. Behavioral procedures

Spatial learning and reference memory ability of rats was measured using Morris water maze task from day 27 to day 32 as described [29]. The water maze was a black circular tank (180 cm in diameter, 50 cm deep, water temperature 21 ± 1 °C) divided into 4 equal imaginary quadrants (I-II-III-IV) for data analysis and was placed in a room with several visual cues hanging on the walls. Spatial acquisition phase was from day 27 to day 30. In this phase, a platform (10 cm in diameter) was placed in the middle of the IV quadrant and 2 cm below the surface of the water. Every day, the animals received four consecutive trials. Animals were placed into the tank from each of the four quadrants, and those not finding the platform within 90 s were guided to it by the experimenter. The time latency to reach the platform and the distance traveled were recorded. On day 32, a 90 s probe trial with the platform removed from the tank was given to assess reference memory, all rats started from the same position in II quadrant. The time spent on

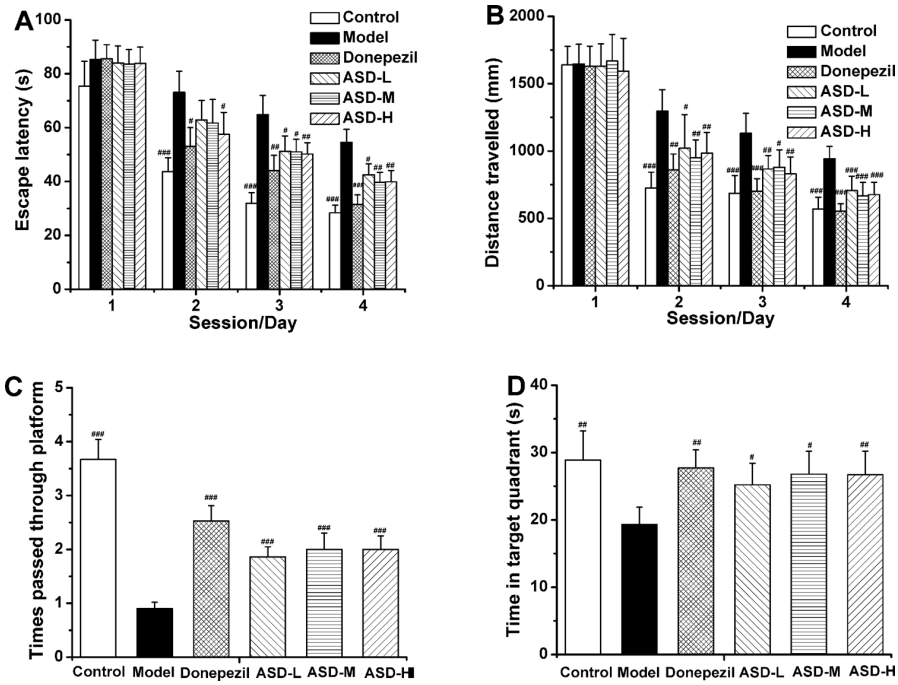


**Fig. 2.** Effects of ASD on $A\beta_{25\text{-}35}$ - induced cognitive impairment of rats in Morris water maze task. (A) The latency to escape onto the submerged platform in acquisition trials. (B) Distance traveled during the 4-day acquisition trials. (C) The times passed through the platform position in probe trials. (D) The time spent in target quadrant of probe trials. The data were expressed as mean ± SEM ($n$ = 10). #$p$ < 0.05, ##$p$ < 0.01, ### $p$ < 0.001 *vs.* model group.

swimming in the IV quadrant and the times of crossing through the previous platform position were measured.

Anxiety state of rats was measured using an elevated plus-maze assay according to the reported literature [30] from day 33 to day 34. The apparatus consisted of two open arms (50'10 cm) and two enclosed arms (50'10'45 cm high), extending from a central platform (10'10 cm) and placed 50 cm above the floor. Each rat was placed at the center facing open arm and its exploration behavior was recorded for 5 min. The time spent in all arms and number of entries into open and enclosed arms were recorded. The apparatus was cleaned between animals with diluted ethanol (75%).

### 2.6. Determination of viscera coefficient

On day 35, the rats were weighed and then anesthetized with an *intraperitoneal* injection of pentobarbital sodium (50 mg/kg). Whole adrenal gland was carefully separated and residual liquids on the surfaces of these glands were removed using suction papers. Viscera coefficient = the weight of adrenal gland / the weight of corresponding rat.

### 2.7. Corticosterone and ACTH concentrations

Blood samples were collected from the eye ground veins of rats between 9 a.m. and 9:30 am on day 35. Plasma corticosterone and ACTH were assayed using Corticosterone ELISA kit (Abcam, UK) and ACTH ELISA kit (Nanjing Jiancheng Bioengineering Institute, China), respectively.

### 2.8. Determination of 11β-HSD1 activity

The liver and groin fat pad were collected from the anesthetized rats and stored at −20 °C until use. The 11β-HSD1 activity was measured using methods as previously described [31,32]. Snap-frozen livers and groin fat pads were lysed with RIPA buffer, then treated with 1% Triton X-100 in assay buffer containing 1 mM EGTA, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, and 20 mM Tris HCl. Enzyme assays were conducted using 50 mg sample protein incubated for 1 h at 37 °C in 600 μL of assay buffer containing NADPH (500 μM) and cortisone (2000 nM). Immediately following incubation, the samples were placed in a boiling water bath for 5 min. 11β-HSD1 activity was then assessed by measuring cortisol production using a cortisol ELISA kit Elabscience Biotechnology Co., Ltd., (China), and enzyme activity was expressed in units of pg of cortisol produced per mg of protein per min.

### 2.9. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). All data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test. In all cases, $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. ASD ameliorates memory deficits and anxiety states induced by $A\beta_{25\text{-}35}$ in Morris water-maze and elevated plus-maze tasks

Cognitive impairment and emotional disturbances (such as anxiety, depression, agitation and psychosis) are the major clinical symptoms of AD patients [33,35]. The AD rat model established by an *icv* injection of $A\beta_{25\text{-}35}$ into the lateral ventricles could simulate the symptoms of memory deficits and anxiety [28]. In this part, Morris water-maze and elevated plus-maze tasks were used to study the effects of ASD on memory deficits and anxiety symptoms of AD rats, respectively.

During the acquisition trials in the Morris water-maze test, the escape latency and distance travelled gradually decreased over the course of four training days for all groups (Fig. 2A and B). The decrease extent of escape latency and distance travelled of Model group was smaller than that of Control group ($p < 0.05$), which indicated that $A\beta_{25\text{-}35}$ treatment significantly slowed the learning speed of rats. The $A\beta_{25\text{-}35}$ - induced cognitive deficits were reversed by chronic treatment with ASD and donepezil. ASD (30, 90 and 270 mg/kg) and donepezil (0.5 mg/kg) all significantly reduced the escape latency and distance travelled on the fourth day compared to $A\beta_{25\text{-}35}$ treatment ($p < 0.05$). In probe trial, the times passed through platform and distance travelled in the target quadrant of model group were markedly less than that of control group

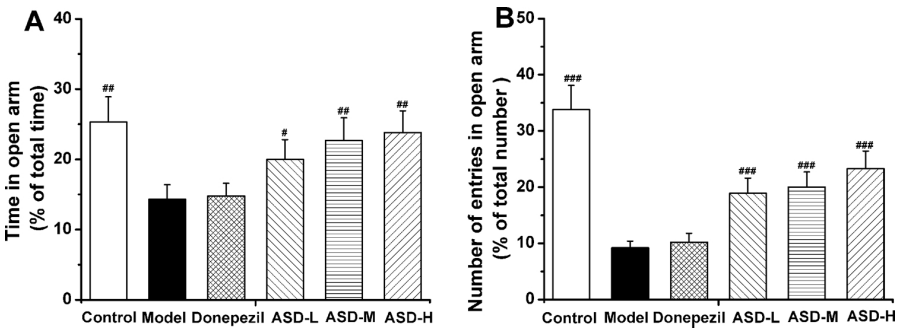


**Fig. 3.** Effects of ASD on $A\beta_{25\text{-}35}$ - induced depression state of rats in the elevated plus-maze assay. (A) The ratio of time spent in the open arm and time spent in all arms. (B) The ratio of number of entries in open arm and number of entries in all arms. The data were expressed as mean ± SEM ($n$ = 10). #$p$ < 0.05, ##$p$ < 0.01, ###$p$ < 0.001 *vs.* model group.

($p < 0.05$) (Fig. 2C and D), indicating that $A\beta_{25\text{-}35}$ treatment deteriorated retention of spatial memory. ASD (30, 90 and 270 mg/kg) and donepezil (0.5 mg/kg) all significantly increased the times passed through platform and distance travelled in the target quadrant compared to model group ($p < 0.05$). All the above results showed that ASD significantly ameliorated the cognitive deficits of AD rats.

In the elevated plus-maze test, the time spent in the open arms and the total number of entries in open arms of model group were smaller than that of control group ($p < 0.05$) (Fig. 3A and B), indicating that $A\beta_{25\text{-}35}$ could cause anxiety symptoms of AD rats. ASD (30, 90 and 270 mg/kg) significantly increased the time spent in the open arms and the total number of entries in open arms ($p < 0.05$). The finds suggested that ASD could reverse $A\beta_{25\text{-}35}$ - induced anxiety.

### 3.2. ASD changes the viscera coefficient of adrenal gland

Adrenal gland is a part of HPA axis. Hyperactivity of the HPA axis will induce corticosterone hypersecretion, causing a significant increase in adrenal gland size [36]. In this section, the influence of ASD on the viscera coefficient of adrenal gland was studied. The results indicated the effect of ASD on hyperactivity of the HPA axis and secretion of glucocorticoid hormones.

The viscera coefficient of adrenal gland of rats in model group was larger than that in control group ($p < 0.05$) (Fig. 4). This indicated that $A\beta_{25\text{-}35}$ treatment could result in hypertrophy of adrenal gland in rats. ASD (30, 90 and 270 mg/kg) could significantly decreased the viscera coefficient of adrenal gland compared to model group ($p < 0.05$). The results suggested that ASD might inhibit the hyperactivity of the HPA axis and reduce adrenal gland secretion of glucocorticoid hormones.

### 3.3. ASD reverses $A\beta_{25\text{-}35}$-induced corticosterone increasement

As previously suggested, down-regulation of the systemic glucocorticoid levels may be a feasible strategy for treating AD. The effect of ASD on the level of corticosterone in AD rats was illustrated in Fig. 5A. $A\beta_{25\text{-}35}$ treatment induced a significant increasement of corticosteron in model group compared to control group ($p < 0.05$). ASD (30, 90 and 270 mg/kg) effectively prevented $A\beta_{25\text{-}35}$ induced increasement of plasma corticosterone ($p < 0.01$). Therefore, lowering the systemic glucocorticoid levels may be the mechanism by which ASD affects AD.

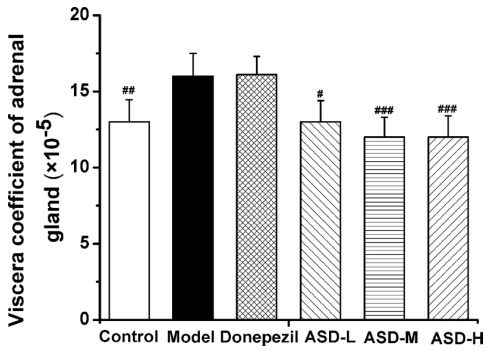


**Fig. 4.** Effects of ASD on $A\beta_{25\text{-}35}$ - induced hypertrophy of adrenal gland in rats. The data were expressed as mean ± SEM ($n$ = 10). #$p$ < 0.05, ##$p$ < 0.01, ###$p$ < 0.001 *vs.* model group.

Glucocorticoid hormones are mainly released from adrenal cortex (controlled by HPA axis) and regenerated from cortisone by 11β-HSD1. The influence of ASD on the activities of HPA axis and 11β-HSD1 was studied to illuminate the mechanism leading to lowering the plasma corticosterone levels.

### 3.4. ASD attenuates $A\beta_{25\text{-}35}$-induced hyperactivity of hypothalamic-pituitary-adrenal (HPA) axis

Plasma adrenocorticotropin hormone (ACTH) level is a critical index of activity of the HPA axis [28]. $A\beta_{25\text{-}35}$-induced an obvious increase in plasma ACTH ($p < 0.05$) (Fig. 5B), indicating that $A\beta_{25\text{-}35}$ treatment resulted in hyperactivity of HPA axis. ASD (30, 90 and 270 mg/kg) reversed the hyperactivity of HPA axis ($p < 0.05$). The results showed that attenuating $A\beta_{25\text{-}35}$-induced hyperactivity of HPA axis was one way for ASD to lower the plasma corticosterone levels.

### 3.5. ASD has no effect on $A\beta_{25\text{-}35}$-induced hyperactivity of 11β-HSD1

11β-HSD1 catalyzes the reduction of inactive cortisone (11-dehydrocorticosterone in rat) to cortisol (corticosterone in rat). 11β-HSD1 is mainly present in peripheral tissues, notably liver and adipose tissue [37]. The effect of ASD on activity of 11β-HSD1 in liver and groin fat pad was next studied.

$A\beta_{25\text{-}35}$ induced a significant hyperactivity of 11β-HSD1 of model group compared to control group in both liver and groin fat pad ($p < 0.05$) (Fig. 6A and B). However, different dose of ASD didn't change the hyperactivity of 11β-HSD1 induced by $A\beta_{25\text{-}35}$.

## 4. Discussion

AD is one of the largest global public health challenges facing our generation. According to epidemiological surveys, about 7–10% of those over 65 and 50–60% over 85 suffer from AD [38,39], involving approximately 46.8 million people worldwide in 2015 and an estimated 131.5 million in 2050. The current anti-AD medicines, while active at ameliorating AD symptoms, are unable to modify disease progression, providing the driving force behind the ongoing research for new and potent anti-AD compounds [40]. Targeting at glucocorticoid hormones to find the new anti-AD drugs might be a promising strategy.

Glucocorticoid levels are mainly controlled by the HPA axis and 11β-HSD1. Inhibition of 11β-HSD1 or inactivation of the HPA axis is the two main methods to reduce the systemic glucocorticoid levels of AD patients. Some natural and synthetic inhibitors have been discovered and developed [32,41], and preclinical experiments showed

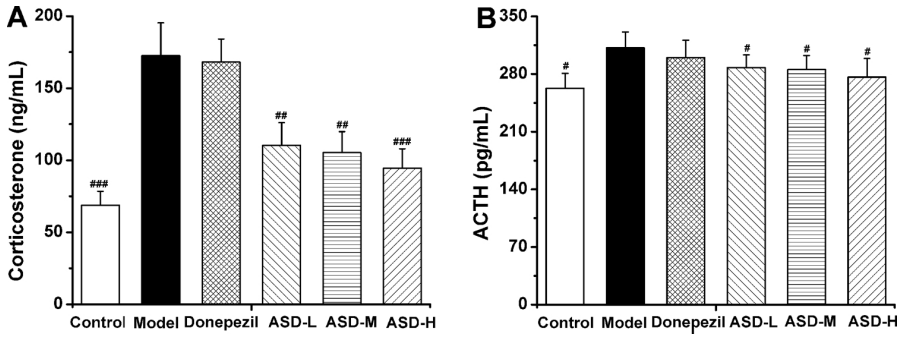


**Fig. 5.** Effects of ASD on $A\beta_{25\text{-}35}$ - induced increase of corticosterone (A) and ACTH (B) in rats. The data were expressed as mean ± SEM ($n$ = 10). $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ *vs.* model group.

that 11β-HSD1 inhibitors could improve memory in rodent models of cognition [42]. However, a phase II study showed that ABT-384 (a 11β-HSD1 inhibitor) could fully inhibit intracellular cortisol regeneration, but did not improve cognitive symptoms of mild-to-moderate AD patients [2]. Glucocorticoid concentrations are a balance between production under the negative feedback control and diurnal rhythm of the HPA axis and peripheral production by 11β-HSD1 [43]. The HPA axis will be over activated and adrenal gland will produce extra cortisol to compensate the reduced cortisol levels due to inhibition of 11β-HSD1 gateway [32,43]. Therefore, long-term sole using 11β-HSD1 inhibitors might not lower the body's cortisol levels in AD patients. Although down-regulation of the HPA axis could decrease the secretion of adrenal cortisol, the systemic cortisol of AD patient might keep at a high level due to the peripheral production by 11β-HSD1. Thus, only potent down-regulation of the HPA axis, associated with inhibition of 11β-HSD1, might represent a thorough and effective measure to control the body's cortisol levels. In this study, ASD reversed $A\beta_{25\text{-}35}$ - induced corticosterone increase by down-regulation of the HPA axis, but had no effect on the activity of 11β-HSD1 (illustrated in Fig. 7). There is a strong likelihood that ASD combined with a 11β-HSD1 inhibitor could control the glucocorticoid levels and attenuate amyloid β-induced cognitive deficits more better.

It has been reported that $A\beta_{25\text{-}35}$ induced the dysregulation of the HPA axis and increase of corticosterone level in rats [28]. However, there was no published literature about the influence of amyloid peptide on the activity of 11β-HSD1 in rats. The present study showed that the activity of 11β-HSD1 in liver and adipose tissue was enhanced. Previous studies showed that 11β-HSD1 expression and activity was upregulated by physiological concentrations of glucocorticoids in a dose-dependent manner [44,45]. Therefore, the hyperactivity of 11β-HSD1 in tissues of rats might be caused by $A\beta_{25\text{-}35}$ via the up-regulation of corticosterone.

Anxiety occurs with a high prevalence of up to 25–75% in AD patients and increases great social, medical, and economic burden [33,34]. Some research indicated that anxiety was also closely related with glucocorticoids. Overproduction of glucocorticoids is known to cause Cushing syndrome and patients with Cushing syndrome display psychiatric abnormalities including anxiety [46]. Chronic glucocorticoid administration in mice resultd in increased anxiety - like behavior [47]. Thus, glucocorticoids were probably related to the anxiety symptoms in AD patients and ameliorating anxious disorders induced by $A\beta_{25\text{-}35}$ possibly correlated with down-regulation of corticosterone levels for ASD.

In summary, the present study demonstrated that ASD could ameliorate memory deficits and anxiety states induced by $A\beta_{25\text{-}35}$ in rats. The anti-AD effect of ASD might be taken by reducing the level of corticosterone via down-regulation of the HPA axis. Further study was in need to study the effect of ASD combined with a 11β-HSD1 inhibitor on controlling the glucocorticoid levels and attenuating amyloid β-induced cognitive deficits.

## Declarations of interest

none

## Acknowledgments

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## Conflict of interest

The authors have declared that there is no conflict of interest.

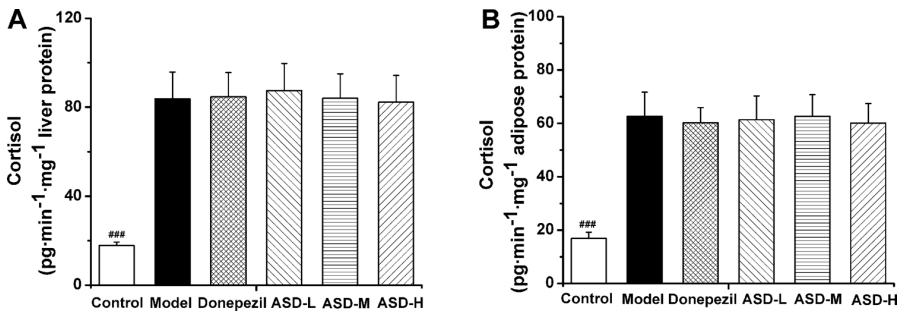


**Fig. 6.** Effects of ASD on $A\beta_{25\text{-}35}$ - induced hyperactivity of 11β-HSD1 in liver (A) and groin fat pad (B) in rats. The data were expressed as mean ± SEM ($n$ = 10). $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ *vs.* model group.

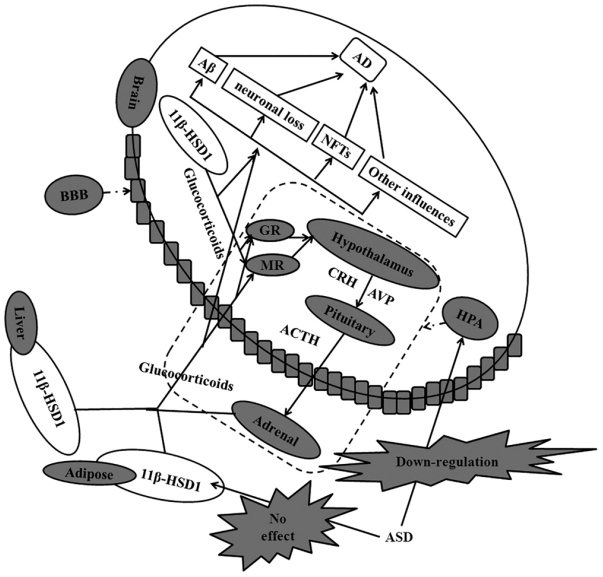


**Fig. 7.** Proposed anti-AD mechanisms of ASD in the $A\beta_{25\text{-}35}$-induced AD rat model. The *icv* injection $A\beta_{25\text{-}35}$ resulted in the dysregulation of the HPA axis and an increased adrenal corticosterone [28]. Long-term exposure to physiological concentrations of glucocorticoids might induce the hyperactivity of 11β-HSD1 in peripheral and brain tissues. More corticosterone were regenerated from 11-dehydrocorticosterone by 11β-HSD1 and passed through the blood brain barrier (BBB) into the brain. High levels of glucocorticoids increased Aβ and neurofibrillary tangles (NFTs) formation [16,17], neuronal loss [48], other neuropathies [5], cognitive impairment and emotional disturbances. ASD could reverse $A\beta_{25\text{-}35}$-induced corticosterone increase and cognitive deficits via down-regulation of the HPA axis. **Abbreviations**: GR: glucocorticoid receptor; MR, mineralocorticoid receptor; CRH: corticotropin-releasing hormone; AVP: arginine vasopressin; BBB: blood brain barrier; NFTs: neurofibrillary tangles; ASD: Akebia saponin D; AD: Alzheimer's disease; HPA: hypothalamopituitary-adrenal axis; Aβ: amyloid β-peptide; 11β-HSD1: 11β-hydroxysteroid dehydrogenase type-1.

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