Ginsenoside Rd promotes non-amyloidogenic pathway of amyloid precursor protein processing by regulating phosphorylation of estrogen receptor alpha

Xiaodong Yan a,1, Gengyao Hu b,1, Weiming Yan c,1, Tao Chen c,e, Feng Yang a,d, Xiao Zhang b, Gang Zhao b, Juanfang Liu c,*

a Department of Orthopaedics, Tangdu Hospital, The Fourth Military Medical University, Xi’an 710032, China
b Department of Neurology, Xijing Hospital, The Fourth Military Medical University, Xi’an 710032, China
c Department of Clinical Aerospace Medicine, The Fourth Military Medical University, Xi’an 710032, China
d Diagnosis, Treatment and Rehabilitation Center of Neurological Diseases, Second Sanatorium, Qingdao 266071, China
e Department of Health Service, Faculty of Aerospace, The Fourth Military Medical University, Xi’an 710032, China

Aims: Previous study demonstrated that Ginsenoside Rd, (GS-Rd) could improve cognitive and memory function in animal model of Alzheimer’s disease. This study was aimed to investigate whether GS-Rd could improve non-amyloidogenic pathway by activating estrogen receptor (ER).

Main methods: 10 mg/kg GS-Rd in ovariectomy (OVX) + GS-Rd group and equivalent volume of saline in sham operated group and OVX group were administrated intraperitoneally for two months, respectively. The Morris Water Maze was used to examine cognitive function of rats, with sAPPα and Aβ levels in the hippocampi measured. The culture medium of HT22 hippocampal neuronal cells were incubated with GS-Rd, ER antagonist ICI182.780, MAPK inhibitor PD98059, or PI3Kinhibitor LY294002, respectively. sAPPα levels was measured, and expression of α-secretase, sAPPα, β-secretase, Aβ, phosphorylation form of AKT (p-AKT), total AKT, p-ERK, total ERK, p-ERα, total ERα, p-ERβ and total ERβ were examined by Western blot to explore the estrogen-like activity of GS-Rd.

Key findings: GS-Rd attenuate cognitive and memory impairment, increased levels of sAPPα and reduced extracellular Aβ of OVX rats. In HT22, GS-Rd could upregulate sAPPα level, which can be inhibited by inhibitor of MAPK and PI3K pathway. In addition, inhibitor of estrogen receptor prevented GS-Rd triggered release of sAPPα and activation of MAPK and PI3K pathways. GS-Rd could increase expression of α-secretase and sAPPα, while decrease expression of β-secretase and Aβ. Besides, GS-Rd promoted phosphorylation of estrogen receptor alpha at Ser118 residue.

Significance: Our findings show that GS-Rd enhances learning and memory function of OVX rats by activating estrogen-like activity.

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1. Introduction

A number of lines of evidence suggest that plummeting levels of estrogen after menopause may play a role in the increased prevalence of Alzheimer’s disease (AD) in women [1–3] and cognitive deficits in elderly people [3–5]. Moreover, an animal model using ovariectomy and ovarioectomy with estradiol replacement in female Sprague-Dawley rats was developed to support the role of estrogen in promoting learning and memory function impaired by OVX treatment [6–8].

Aberrant amyloid precursor protein processing and amyloid Aβ aggregation are main characteristics of AD. APP can produce sAPPα via α-secretase mediated non-amyloidogenic pathway, and produce Aβ via β-secretase and γ-secretase mediated amyloidogenic pathway. More recently, attention has been paid to the α-secretase pathway of APP metabolism [9], because α-secretase cleaves APP without Aβ peptide domain and thus precludes Aβ peptide generation [10]. Researches in vivo and in vitro showed that estrogen increased the secretory metabolism of APP [11] and reduced Aβ generation [12], in part, due to the increased activity of α-secretase. It has been reported that various cell signaling pathways are closely related to activation of α-secretase, such as Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide-3 Kinase (PI3K) pathway [13,14].
Panax ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), is a famous traditional herb medicine popular in Asia such as China, Korea in the past, and considered as dietary supplement in US and Europe nowadays [15]. Saponins (Ginsenosides), the major active components in the extracts of Panax ginseng, are responsible for the pharmacological effects of the treatment of cardiovascular diseases, cerebrovascular diseases, cancers, and other diseases [15,16]. There are >30 ginsenosides identified, and ginsenoside Rd (GS-Rd) is one of the most active and abundant components and contributing to many of the effects of ginsenosides. Abundant studies have demonstrated that GS-Rd could exert a protective effect on cerebrovascular sufferings [17–19]. Our recent studies showed that GS-Rd could attenuate cognitive dysfunction in a rat model of AD [20] and improve learning and memory ability in APP transgenic mice (unpublished). But the mechanism involved is still unknown.

Based on the evidence above, in this study we tested whether GS-Rd could act through estrogen-like activity and subsequently affected APP processing by regulating estrogen receptor activation. Using hippocampal neuronal cell line HT22 and OVX model, we showed that GS-Rd could promote memory ability of OVX rats by activating estrogen-like activity.

2. Method

2.1. Reagents

Dulbecco’s modification of Eagle’s Minimum Essential Medium (DMEM) and antibody to p-ERK (Ser105) were purchased from Sigma-Aldrich (St. Louis, MO, USA, Catalog # D5030 and SAB2100713). Neurobasal-A medium (phenol red-free), N-2 supplement, fetal bovine serum, penicillin, streptomycin were from Invitrogen (Grand Island, NY, USA, Catalog # 12349015, 17502001, 16000044, 15140163, respectively). GS-Rd with a purity of 98% was obtained from TaiHe Biopharmaceutical Co. Ltd. (Guangzhou, China). IC1182.780 was from Cayman (Michigan, USA), LY294002 and PD98059 were from Abcam (USA, Catalog # ab120243 and ab120234). sAPPα and Aβ ELISA kit were purchased from IBL International (Hamburg, Germany, Catalog # JP27734 and RES9651). Antibodies to α-secretase, sAPPα, β-secretase, Aβ were from Abcam (USA, Catalog # ab1997, ab32136, ab2077 and ab32136 respectively). Antibodies to p-Serine/threonine Kinase (AKT) (Ser473), total AKT, p-Extracellular-Signal Regulated Kinase (ERK) (Thr202/Tyr204) and total ERK were from Cell signaling technology (MA, USA, Catalog # 3787, 37882, 3510 and 3552). Antibodies to ERα, p-ERα (Ser118) and ERα, were from Abcam (USA, Catalog # ab3577, ab32396 and ab32063 respectively). GAPDH antibody was purchased from CWBIO (Beijing, China, Catalog # CW0100M).

2.2. Experimental animals and drug administration

Thirty adult female Sprague-Dawley (SD) rats (280–300 g) were from the animal center of Fourth Military Medical University. Rats were randomly divided into three groups: sham operated, OVX and OVX + GS-Rd groups (n = 10). For OVX and OVX + GS-Rd groups, the bilateral ovariectomy was performed as previously described [21]. For sham group, there were no ovaries removed during the procedure. According to our previous study, 10 mg/kg dose of GS-Rd in OVX + GS-Rd groups and equivalent volume of saline in sham operated group and OVX group were administrated intraperitoneally for two months from the first day after surgery, respectively. All animal protocols were approved by the Ethics Committee for Animal Experimentation of The Fourth Military Medical University.

2.3. Behavioral testing

The Morris Water Maze test is a widely accepted method for examining cognitive function and was used in the present study as previously described [22]. Briefly, a circular plastic pool (height 60 cm and diameter 150 cm) was filled with water maintained at 22–26 °C. An escape platform was submerged 1.5 cm below the surface of the water infiltrated with 80 ml Chinese black ink. The pool was divided into four quadrants with four starting locations, north (N), east (E), south (S), and west (W). Each rat’s swimming track was recorded by a camera tracking system. The test was performed 3 times a day for continuous 5 days, with three randomized starting points, and the platform stay fixed in the south. The rats need to find the submerged platform within 60 s, if not, they are placed on the platform for 10–15 s. The latency to find the platform for each group from 2 to 5 days of the test were recorded and averaged into blocks. On the last day of test, the platform was removed and the rats are allowed to swim for 120 s. The percentage of time the rat spent exploring the quadrant that had contained the platform in relation to the total time spent exploring the entire pool was recorded and calculated for each rat.

2.4. Cell culture and drug treatment

HT22 hippocampal neuronal cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 g/ml streptomycin in a 5% CO2 humidified atmosphere at 37 °C. Before being used, cells were differentiated in Neurobasal-A medium containing 1 × N-2 supplement for 24 h. During experiments, culture solution was replaced by fresh DMEM without fetal bovine serum. GS-Rd, ER antagonist IC1182.780, MAPK inhibitor PD98059, and PI3K inhibitor LY294002 were prepared in Dimethyl Sulfoxide (DMSO) and added to the culture medium at the indicated concentrations. In all the experiments, an equivalent volume of vehicle was added to the control cultures. DMSO used at the final concentrations (~0.1%) was found to have no effects on any of the parameters tested.

2.5. sAPPα level

sAPPα levels in the culture medium of HT22 and in the rats hippocampi of all the three groups were measured by the sAPPα ELISA kit according to the manufacturer’s protocol. Before measurement of sAPPα released into the culture medium, the medium was replaced by fresh serum-free DMEM. The inhibitors (IC1182.780, PD98059, and LY294002) were added 30 min before GS-Rd did respectively, and indicators were detected 24 h later. sAPPα levels in the collected medium were measured by sAPPα ELISA kit using the Biotek ELx808 at a wavelength of 450 nm. For measurement of sAPPα level in hippocampal tissues, the hippocampus were homogenized and centrifuged as previously reported [13], and a 100 μl sample of the supernatant was needed for sAPPα measurement every time. The protein concentration of samples was determined with protein quantification kit, and data were obtained using Biotek ELx808 at a wavelength of 562 nm. sAPPα levels were corrected for the protein contents.

2.6. Aβ level

Aβ levels in the hippocampi of rats were measured using the Mouse/Rat Aβ (1–42) ELISA kit according to the manufacturer’s protocol. The hippocampal tissues were homogenized and then centrifuged, and a 100 μl sample of the supernatant was needed for the Aβ measurement. Spectrophotometric data were then gained using Biotek ELx808 at a wavelength of 450 nm. The protein concentration was measured as described above. Aβ levels were corrected for the protein contents.

2.7. Western blot

Levels of α-secretase, sAPPα, β-secretase, Aβ, p-AKT (Ser473), total AKT, pERK, total ERK, ERα, p-ERα, and total ERβ expression were examined by Western blot analysis. After treated as previously described, cells were trypsinized and collected by centrifugation at
400 × g for 3 min. The pellets were lysed and centrifuged at 12,000 rpm for 15 min to obtain the supernatant. The protein concentration of the supernatant was determined with protein quantification kit. The protein extracts in each group were boiled for 5 min and then centrifuged at 5000 rpm for 5 min. The proteins were separated by 10% SDS–PAGE and transferred onto NC membranes by an electron transfer device. After incubated in blocking buffer (0.1% Tween-20 and 5% nonfat dry milk in Tris-buffered saline, pH 7.4) at room temperature for 60 min, following primary antibodies: α-secretase (1:1000), sAPPαx (1:1000), β-secretase (1:1000), Aβ (1:1000), p-AKT (Ser473) (1:1000), total AKT (1:1000), p-ERK1/2 (Thr202/Tyr204) (1:1000), total ERK1/2 (1:1000), p-ERα (Ser118) (1:1000), total ERα (1:1000), p-ERβ (Ser105) (1:1000), total ERβ (1:1000) or GAPDH (1:3000), and corresponding HRP conjugated secondary antibodies were used. Detection was performed using an electrochemiluminescence (ECL) procedure (ALPHA, GDS8000, Ultra-Violet Products, UK). All band signals were quantified using ImageJ (NIH). The data acquired were normalized to the GAPDH expression. In each experiment, three plates of cell samples of 150 mm were used.

2.8. Statistical analysis

All the data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test using SPSS10.0 software. A probability of < 0.05 was considered statistically significant.

3. Results

3.1. Effect of GS-Rd treatment on memory performance of the OVX rats

The OVX female animals were proved to develop progressive memory deficits, and central cholinergic nerve system degeneration. This model has been widely used as an in-vivo model to mimic estrogen deprivation in postmenopausal women. With this model, a number of researchers demonstrated that OVX in rats resulted in an estrogen-reversible impairment of memory [8].

To investigate whether GS-Rd treatment has a similar role like estrogen in improving the learning and memory ability of the OVX rats, we tested rats in the Morris Water Maze experiment. Firstly, the memory ability of rats was indicated by the escape latency to find the platform. As shown in Fig. 1A, in the acquisition experiment, path length in OVX treated rats was increased than did sham operated rats (P < 0.05), which is consistent with reports previously. The path length in OVX + GS-Rd group was significantly decreased compared with OVX group (P < 0.05), which suggested that GS-Rd could improve spatial memory ability of OVX rats.

In the spatial probe trials, the percentage of time the rat spent exploring the quadrant that had contained the platform in relation to the total time spent exploring the entire pool was used to evaluate memory ability (Fig. 1B). Rats of OVX group spent significantly less time in the target quadrant than those of control group (P < 0.05). But, rats in OVX + GS-Rd group increased the time spent in target quadrant (P < 0.05). According to one-way ANOVA, 10 mg/kg GS-Rd for 2 months significantly improved the spatial memory ability of OVX rats.

3.2. Effect of GS-Rd on non-amyloidogenic cleavage of APP in the hippocampi of the OVX rats

Since GS-Rd could improve the learning and memory performance of the OVX rats, the sAPPαx and Aβ ELISA kits were used to observe molecular changes in hippocampus of rats treated with GS-Rd or not (Fig. 2A, B). Compared with sham group, OVX treatment resulted in an increased Aβ level (P < 0.05), without affecting sAPPαx level. In OVX + GS-Rd group, GS-Rd not only led to a significant decrease in level of Aβ (P < 0.05) formation upregulated by OVX, but also provoked an increase in sAPPαx level (P < 0.05).

3.3. Effect of GS-Rd on APP processing in HT22 cells

In order to further discuss the mechanism involved, we used HT22, a hippocampal neuronal cell line expressing estrogen receptor, to test the secretory soluble sAPPαx level in the presence and absence of GS-Rd for 24 h. In Fig. 3A, results showed that sAPPαx released into the media was significantly increased in a concentration-dependent manner with ranges from 5 μM to 20 μM of GS-Rd doses, and the peak is at 10 μM (P < 0.05, vs. control group).

We then investigated the interaction of GS-Rd with APP metabolism associated signaling pathways, including MAPK and PI3K pathways. We showed that inhibitors of MAPK and PI3K reversed the increase of sAPPαx by GS-Rd treatment (Fig. 3B, P < 0.05), suggesting MAPK and PI3K pathway might be closely associated with GS-Rd-induced sAPPαx release.

It has been indicated that estrogen could induce the accumulation of sAPPαx in the medium of cultured cells, and it’s involved in multiple signaling pathways, such as MAPK and PI3K pathways. To explore whether the effect of GS-Rd on sAPPαx was mediated by estrogen-like activity, MAPK inhibitor PD98059, PI3K inhibitor LY294002, ER inhibitor ICI182.70 were added to culture media 30 min before GS-Rd treatment. Compared with GS-Rd group, the levels of sAPPαx released into the medium were decreased at the presence of PD98059, LY294002 and ICI182.780 (Fig. 3B, P < 0.05). These data suggested that the effect of GS-Rd on APP processing might be mediated by MAPK/ERK and PI3K/
AKT signal pathways and endogenous ER, in accorded with the information revealed in vivo.

3.4. Effect of GS-Rd on the expression levels of $\alpha$-secretase and $\beta$-secretase of APP processing in HT22 cells

The above results showed that GS-Rd could decrease the level of $A_\beta$, while it could increase the level of sAPP$\alpha$ both in vivo and in vitro. Since sAPP$\alpha$ is produced via the $\alpha$-secretase mediated non-amyloidogenic pathway, while $A_\beta$ via the $\beta$-secretase and $\gamma$-secretase mediated amyloidogenic pathway, we then investigated the effect of GS-Rd on the expression levels of $\alpha$-secretase and $\beta$-secretase of APP processing in HT22 cells. Compared with control group, GS-Rd treatment resulted in increased expression levels of $\alpha$-secretase ADAM and sAPP$\alpha$ ($P < 0.01$), while it decreased expression levels of $\beta$-secretase BACE1 and $A_\beta$ ($P < 0.01$).

To further explore whether the effect of GS-Rd on $\alpha$-secretase and $\beta$-secretase was mediated by estrogen-like activity, MAPK inhibitor PD98059, PI3K inhibitor LY294002, ER inhibitor ICI182.70 were added to culture media 30 min before GS-Rd treatment. Compared with GS-Rd group, the expression levels of $\alpha$-secretase ADAM and sAPP$\alpha$ were decreased at the presence of PD98059, LY294002 and ICI182.780 (Fig. 4A, B, $P < 0.01$), while the expression levels of $\beta$-secretase BACE1 and $A_\beta$ were increased at the presence of PD98059, LY294002 and ICI182.780 (Fig. 4C, D, $P < 0.01$). These data suggested that the effect of GS-Rd on $\alpha$-secretase and $\beta$-secretase of APP processing might be mediated by MAPK/ERK and PI3K/AKT signal pathways and endogenous ER. (See Fig. 5.)

3.5. Effect of GS-Rd on levels of p-ERK and p-AKT in HT22 cells

Since APP metabolism processing can be modulated by MAPK and PI3K pathways, next we investigated whether GS-Rd regulated the expression and activity of key factors in these pathways (Fig. 4A, B). ERK and AKT are considered as a representative of MAPK/ERK and PI3K/AKT, thus first we examined the expression of ERK, AKT, and their corresponding phosphorylated active form in cells treated with 10 $\mu$M GS-Rd at a series of time points. It was found that GS-Rd provoked a significant increase in level of p-ERK and p-AKT protein in HT22 cells at 12 h ($P < 0.05$) and 24 h ($P < 0.05$), compared with that of control.
However, total ERK and AKT protein levels were not altered by GS-Rd administration. To further test if GS-Rd activated ERK and AKT signaling pathways were mediated by estrogenic activity, cells were treated with GS-Rd for 24 h in the absence or presence of ER antagonist ICI182.780. Compared with GS-Rd group, the upregulated expression of p-ERK and p-AKT proteins by GS-Rd were blocked by ICI182.780 (Fig. 4).

**Fig. 4.** Effect of GS-Rd treatment on expression of α-secretase and β-secretase of APP processing in HT22 cells. (A) α-secretase ADAM, (B) sAPPα, (C) β-secretase BACE1, (D) Aβ were assessed and analyzed in the collected lysates by western blot from HT22 cells incubated with 10 μM GS-Rd in the absence or presence of MAPK inhibitor PD98059, PI3K inhibitor LY294002, ER inhibitor ICI182.70, respectively. All experiments were repeated at least 3 times for each plate’s sample (n = 3). The data were analyzed by a repeated-measures ANOVA followed by contrast analysis. *P < 0.01 versus control group; #P < 0.05 versus Rd group.
4C, D. *P < 0.05). No changes of total ERK and AKT expression were detected among those three groups. These data suggested that the effect of GS-Rd on MARK and PI3K signaling pathways might be mediated by endogenous ER.

3.6. Effect of GS-Rd on phosphorylation of ERα at Ser118 residue

All data above indicated that estrogen receptor might be involved in the effect of GS-Rd on APP metabolism and thus improving learning and
memory ability of OVX rats. To investigate whether GS-Rd has a positive effect on estrogen receptor, we measured expression of estrogen receptor subtypes and their phosphorylation forms (Fig. 6A). Results showed that GS-Rd upregulated the p-ERα at Ser118 residue compared to the control group (Fig. 6B, *P* < 0.05). But no obvious changes were observed in the expression of total ERα, total ERβ, and p-ERβ at Ser105. It indicated that the GS-Rd triggered activation of ER may be mediated by upregulating phosphorylation of ERα at Ser118 residue.

### 4. Discussion

The main finding of the present study was that GS-Rd could improve memory deficits impaired by estrogen deprivation in female OVX rats, which may probably due to accelerated non-amyloidogenic cleavage of APP processing via modulating the activity of α-secretase and β-secretase through the estrogen receptor alpha mediated MAPK/ERK and PI3K/AKT pathway.

Epidemiologic studies show that postmenopausal women have higher incidence of AD than men, on average, which suggests that dropping estrogen level associated with menopausal may be a risk factor of AD in some female elderly [23]. As progress moves, an experimental estrogen deprivation model of OVX in animals was used widely to mimic estrogen loss after menopause in female elderly. Studies based this model showed that ovariectomy impaired while estrogen treatment improved learning and memory ability of OVX rats by Morris Water Maze test. As it is known, Aβ is a key factor in AD development. What our study focused on is what factors are involved in this progress.

Ablent proteolytical processing of APP gives rise to Aβ overproduction, which is characteristic for the brains of AD patients and may be followed by the progression of late-onset AD in some elderly individuals. So, understanding the mechanism in the process of APP metabolism and Aβ aggregation is very critical for cognition about AD, as well as its prevention and treatment. It is well-known that APP can produce sAPPα via α-secretase mediated non-amyloidogenic pathway, and produce Aβ via β-secretase [24] and γ-secretase mediated amyloidogenic pathway. In fact, these two processes don’t always come into play at the same time, but one suppressed, the other enhanced. Intensive studies have been focused for decades on the current leading doctrine that Aβ overproduction is the product of the “overactivated” β- and γ-secretases [25,26]. Researchers recently have then targeted α-secretase [13,27] and a new controversial view came up: an inefficient α-secretase in the normal processing of APP is primarily responsible for Aβ overproduction, and is also the rational drug target for intervention [28]. Chen M [29] argued that the putative α-secretase may be a calcium-dependent protease, and may play a primary role in the regulation of APP processing. What’s more, a series of related researches dissected that an inefficient α-secretase led to “overactivated” β- and γ-secretases, and thus Aβ aggregation in elderly body [29-33]. Drugs aimed APP processing via α-secretase pathways were discussed, and Rg1 and epigallocatechin-3-gallate (EGCG) (a main constituent in green tea extract) have been shown to have a protective effect by promoting non-amyloidogenic cleavage of APP, respectively [21,25]. ADAM 10 is a member of α-secretase and can cleaves APP in a specific target. Therefore, it plays a key role in preventing Aβ generation [34]. Researchers showed that ADAM 10 levels are lower in AD patients compared with normal people [35]. Moreover, AD-like neuropathy can be induced by down-regulating ADAM 10 or inhibiting its activity, with an increase of Aβ aggregated production [36]. Besides, ADAM 10 expression was found to increase in many therapies of AD directly or indirectly [37]. β-secretase BACE1 cleaves APP to form Aβ and thus exhibits opposed functions to α-secretase. It was reported that BACE1 is exclusively expressed in neurons and its level is increased in the remaining neurons in AD brains [38,39]. Besides, BACE1 overexpression in AD was found to partly associated with estrogen deprivation [40].

In this study, we found that GS-Rd could upregulate sAPPα generation and downregulate Aβ production in model of OVX rats, which were proved in HT22 hippocampal neuronal cell line. At the same time, we found that GS-Rd can increase ADAM 10 expression in HT22 cells, indicating that ADAM 10 can be an effective target of GS-Rd for AD therapy. Besides, we also discovered that GS-Rd treatment also decreased BACE1 expression in HT22 cells. These results revealed a possible mechanism through which GS-Rd functions on AD, and further suggested potential targets of ADAM 10 and BACE1 in AD therapy.

Numerous studies reported that some signal transduction pathways are able to activate α-secretase mediated cleavage of APP, which is in most instances accompanied by a corresponding reduction of Aβ generation. Receptor mediated and direct activation of Protein Kinase C (PKC) are linked to the regulation of APP α-secretase cleavage [41–46], not by phosphorylation but through the downstream MAPK cascades [42]. Muscarinic stimulation activates two transduction pathways that leads to secretion of the soluble form of APP in PC12 cells: PKC-dependent and MAPK-dependent pathways [47]. Recently, our previous studies have demonstrated that GS-Rd increased the levels of p-AKT and p-ERK in SD model and astrocyte culture after ischemia insults [48]. So, in this study, these two pathways have been investigated, and we observed a significant increase in levels of p-ERK and p-AKT proteins in GS-Rd–treated HT22 cells at a series of time points, but no change in total ERK and AKT protein expression. Interestingly, this effect can be blocked by inhibitor of estrogen receptor IC182 780, which suggested an estrogen-like effect of GS-Rd. Estrogen receptor is the upstream of MARK and PI3K, and the latter signal transduction pathways have been proved to promote non-amyloidogenic APP metabolism. Classical ERs compromise ERα and ERβ, and their activity or phosphorylation form can mediate intracellular signal transduction of estrogen. Our results showed that GS-Rd treatment could induce ERα phosphorylation activity, which indicated that GS-Rd comprised estrogen-like activity in promoting regulation of secretory APP processing.

In conclusion, our study presented indicated at least that GS-Rd can accelerate non-amyloidogenic cleavage of APP processing by modulating the activity of α-secretase and β-secretase through upregulating ERα expression, which further affirmed the importance of non-amyloidogenic cleavage in APP metabolism. However, the exact way how GS-Rd regulates ERα activity still needs further investigation in the future work.

**Conflict of interest statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

**Author Contribution to Study**

The specific contribution of each author and signed by all authors as follows:

Xiaodong Yan, Gengyao Hu and Weiming Yan, contributed equally to conceiving the study, drafting the manuscript and participating in data acquisition, and should be considered as co-first authors; Tao Chen, made a contribution to interpretation of data; Feng Yang, conducted analysis of data from animal experiments; Xiao Zhang, conducted analysis of data from cell experiments; Gang Zhao, revised the language and grammar of the manuscript, co-corresponding author; Juanfang Liu, supervised the experiment performance and data analyzing, corresponding author.

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