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Reduces depression symptoms brought on by long-term stress with Sanggenon C

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Abstract

The goal of this study is to determine if Sanggenon C alleviates depression in Wistar rats subjected to chronic unexpected mild stress (CUMS).
Methods: A forced swimming test and a sucrose preference experiment were used to evaluate Sanggenon C's anti-depressant impact. Histological investigations were conducted on the cortex and hippocampus using hematoxylin and eosin (H & E) stains, while an open-field test was used to quantify the locomotor change produced by CUMS. To assess cell death, researchers used TUNEL staining, which stands for terminal deoxynucleotidyl transferase dUTP nick end labeling. We also used Western blotting to assess the levels of AMP-activated protein kinase (AMPK) phosphorylation and the expressions of brain-derived neurotrophic factor (BDNF), Bax, Bcl-2, cleaved caspase-3, LC3, Beclin, and P62.
Sanggenon C had a substantial impact on open-field CUMS rats, increasing their preference for sucrose, decreasing their immobility time in the forced swimming test, and increasing the size of the crossing squares and rearing periods ($p < 0.05$).
Nuclear shrinkage and damage in the cortex and hippocampus were both alleviated by sanggenon C. Additionally, Sanggenon C controlled the expression of autophagy-associated molecules (LC3, Beclin, and p62), proteins linked with apoptosis (Bax, Bcl-2, and cleaved caspase-3), and other proteins. There was an increase in BDNF expression and AMPK phosphorylation that Sanggenon C showed.
Results: Sanggenon C activates the AMPK pathway in CUMS rats, which enhances neuroprotection and depressed behavior while inhibiting apoptosis and inducing neuronal autophagy. To completely comprehend the therapeutic importance of Sanggenon C-mediated AMPK activation in various cellular settings as prospective therapeutic targets, more study is necessary.
Apoptosis, Sanggenon C., Depression, Autophagy, and the AMPK pathway

INTRODUCTION

Depression is a widespread neuropsychiatric disorder, and it is implicated in neuronal damage in specific brain regions. The most common pathological characteristics of depression are neuronal damage and apoptosis [1]. It has been reported that increased apoptosis suppresses the renewal of dendritic spines and hinders restoration of normal neuronal function, whereas inhibition of neuronal apoptosis accelerates neuronal regeneration and facilitates recovery of depressive symptoms [2,3]. In addition, brain cell inflammation, oxidative stress, and neuronal autophagy are also causes of depression [4].

Sanggenon C is isolated from the traditional Chinese medicine Morus alba, which is traditionally used for anti-inflammatory, analgesic, and blood stasis-dissipating treatments [5]. Sanggenon C also possesses over pharmacological activity. For example, under hypoxic conditions, sanggenon C reduces pro-inflammatory factors, reactive oxygen species (ROS) and apoptosis. Sanggenon C regulates Ras homolog gene A/Rho-dependent coiled-coil kinases (RhoA/ROCK) signaling to inhibit inflammation and oxidative

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stress, consequently improving brain ischemiareperfusion (I/R) injury [6]. Furthermore, sanggenon C promotes autophagy of cardiomyocytes through activating AMPactivated protein kinase (AMPK), leading to a cytoprotective effect on cardiomyocytes' hypoxic injury [7]. It also inhibits gastric cancer tumorigenesis by blocking ERK-Dynamin-related protein 1 (Drp1)-mediated mitochondrial fission [8]. Whether sanggenon C relieves depressive symptoms remains to be elucidated. This research investigated the neuroprotective effect of sanggenon C on autophagy and apoptosis in the brains of chronic unpredictable mild stress (CUMS) rats to relieve depression-like symptoms.

EXPERIMENTAL

Animal housing and materials

Wistar rats aged 7 - 8 weeks and weighing approximately 140 g were obtained from Shanghai Laboratory Animal Center (Shanghai, China). Rats were raised in specific pathogenfree (SPF) space provided with adequate feed, drink and 12-h light/dark cycle. Sanggenon C (SC) was purchased from Winherb Medical S & T Development Co. Ltd. (Shanghai, China).

Ethics statement

Animal experiments were approved by Experimental Animals Ethics Committee of the Sichuan Institute of Traditional Chinese Medicine (approval no. SYLL (2023)-033). All experiments were performed according to the Guidelines and Rules of Animal Care [9].

Stress induction, treatment and investigations

Rats were induced following a chronic unpredictable mild stress program [10]. A total of 24 Wistar rats were divided into four groups, namely; control, CUMS, CUMS + 10 mg/kg SC, and CUMS + 20 mg/kg SC. Treatment timeline was structured as follows: CUMS treatment occurred from 1st to 20th day; Sanggenon C (SC) oral administration spanned from 21st to 27th day; animal behavior experiments were conducted on 28th day, and histological analysis was performed on 35th day.

Sucrose preference test

All rats were habituated to two drinking bottles for more than 3 days in cages, and water intake was measured daily in both bottles. Rats were separated into single cages and provided with two drinking bottles: one containing 1 % sucrose and another with water only. The weight of each bottle was recorded daily to measure water (W) and sucrose solution intake (S). Sucrose preference rate (Sp) was calculated using Eq 1.

$$Sp = (S/S+W)100 \quad \text{.....................} \quad (1)$$

Forced swimming test

A cylindrical transparent plastic container (60 cm height, 25 cm diameter) was used for forced swimming test. The container was filled with water at 25 ℃ at a depth of 30 cm where rats could not touch the floor. Each rat was kept in the cylinder for 6 min, and their behavior was digitally recorded and automatically analyzed using video tracking software (BioBserve, Bonn, Germany).

Open field test

Each rat was placed in a 96 × 96 × 50 cm box with a black background for 5 min. Thereafter, distance and mean velocity were recorded. Line crossing, time of freezing, rearing, and grooming episodes were recorded and analyzed with ANYmaze behavioral tracking software [11]. The presence of fecal bolus and frequency of urination was noted.

Haematoxylin and eosin (H & E) staining

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Haematoxylin and eosin stainings were performed using hematoxylin-eosin stain kit (E607318, Sangon Biotech, Shanghai, China). All rats were sacrificed on 35th day, and the hippocampus or cortex was fixed with 4 % paraformaldehyde, embedded into paraffin wax, sectioned into 5 µm slices, and dewaxed. Tissue samples were stained with hematoxylin and incubated with a differentiation solution for 30 s. After being washed twice, samples were subjected to eosin stain for 1 min. Tissue slices were dehydrated using gradient ethanol, soaked in xylene, and sealed with neutral gum and sections were photographed under a Nikon Eclipse E400 microscope (Nikon, Japan).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

The hippocampus or cortex of rats was analyzed using TUNEL Assay Kit (ab66110, Abcam, MA, USA). Tissues were fixed with formaldehyde, incubated with proteinase K solution for 5 min and refixed with formaldehyde. Then, samples were treated with DNA labeling solution for 1 h at 37 ℃ and incubated with antibody reagent for 30 min. Finally, RNase A solution was used to incubate samples for 30 min, and signal was observed using fluorescent microscopy.

Western blot assay

Total protein in brain tissues was extracted using lysis buffer (25 mM Tris-HCl pH 7.4, 250 mM NaCl, 50 mM KCl, 10 % Glycerol, 0.5 % NP-40) and centrifuged at 10,000 g for 10 min. The supernatant was collected and protein concentration was determined using a BCA protein assay kit (23225, ThermoFisher). Lysates were subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane, followed by incubation with primary antibodies (Table 1) at 4 ℃ overnight. After incubation with horseradish peroxidaselabeled goat anti-rabbit IgG (B900210, ProteinTech; 1:5000), target bands were visualized with ECL reagents (Solarbio, Beijing, China), and the relative intensity of each band was quantified using ImageJ software, and normalized to β-actin [12].

Statistical analysis

Data are presented as mean ± standard deviation (SD) from three biological replicates, and differences between any two groups were calculated using unpaired t-tests. Multiple group comparisons were analyzed with ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Sanggenon C relieved depressive symptoms and provided neuroprotection for CUMS rats

To investigate the effect of Sanggenon C (SC) on depressive behavior, phenotypes of CUMS rats were evaluated and treated with SC. In comparison to control group, CUMS group exhibited a significant decrease in average weight. When the dose of SC was increased from 10 mg/kg to 20 mg/kg, body weight of CUMS rats correspondingly increased, but was still lower than that of control group (Figure 1 A). Sucrose intake of CUMS rats was significantly reduced compared to control group, indicating that CUMS rats were prone to depression ($p < 0.05$). However, sucrose intake was increased by SC treatment, which indicated that SC relieved depressive symptoms (Figure 1 B). In the forced swimming test, the immobility time of CUMS rats in water was significantly prolonged ($p < 0.05$). This indicated that CUMS rats were slow in action and easily prone to depression. Immobility time was significantly shortened ($p < 0.05$) by SC treatment, which indicated that SC ameliorated depression symptoms (Figure 1 C). Crossing, grooming, and rearing were measured to assess spontaneous locomotor capacity. Results showed that motor ability reflected by crossing, grooming, and rearing times was significantly reduced in CUMS rats ($p < 0.05$).

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Table 1: Primary antibodies

Protein	Cat. no.	Manufacturer	Dilution
BAX	50599-2-Ig	ProteinTech, IL, USA	1:8000
BCL-2	12789-1-AP	ProteinTech, IL, USA	1:9000
Caspase3	19677-1-AP	ProteinTech, IL, USA	1:10000
LC3	14600-1-AP	ProteinTech, IL, USA	1:2000
Beclin-1	11306-1-AP	ProteinTech, IL, USA	1:1000
P62	18420-1-AP	ProteinTech, IL, USA	1:10000
p-AMPK	ab133448	Abcam, Cambridge, MA, USA	1:2000
AMPK	ab32047	Abcam, Cambridge, MA, USA	1:1000
BDNF	Ab9794	Abcam, Cambridge, MA, USA	1:2000
β-actin	20536-1-AP	ProteinTech, IL, USA	1:5000

This indicated that CUMS rats were sluggish and easily tended to depression, while SC treatment significantly promoted exercise capacity and relieved depression symptoms of CUMS rats (Figure 1 D). When rat hippocampi and cortices were subjected to H & E staining, the results revealed observable nuclear shrinkage and neuronal damage in CUMS group. Notably, SC administration alleviated these symptoms (Figure 1 E) and consequently exhibited neuroprotective effect in CUMS rats.

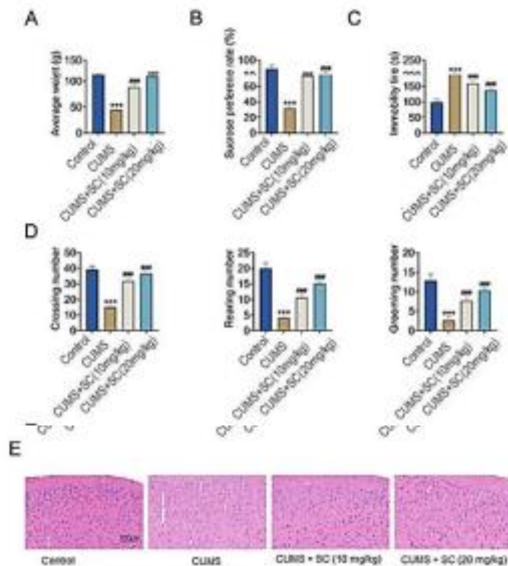

Figure 1: Sanggenon C improves depressive behavior in CUMS rats. (A) Average body weight of CUMS rats. (B) Sucrose intake of CUMS rats. (C) Immobility time of CUMS rats in water. (D) Times of crossing, grooming, and rearing actions of CUMS rats. (E) H & E staining of hippocampus or cortex of CUMS rats. Note: Error bar indicate mean ± SD; ***$p < 0.001$ vs. Sham, ###$p < 0.001$ vs. CUMS

Sanggenon C inhibited apoptosis in CUMS rats

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to assess neuronal apoptosis in the hippocampus and cortex. Findings revealed a higher number of positive signals for dUTP Nick-End Labeling in CUMS rats compared to control group. Notably, administration of SC at doses of 10 and 20 mg/kg reduced these signals, indicating a suppression of apoptosis induced by CUMS (Figure 2 A). Expression of three apoptosisrelated molecules, Bax, Bcl-2, and Cleaved caspase-3, was evaluated using western blot. In CUMS rats, protein levels of Bax and Cleaved caspase-3 were significantly increased ($p < 0.05$) and were mitigated by SC administration (Figure 2 B). In contrast, expression of Bcl-2 was diminished in CUMS rats, but SC treatment significantly promoted its production ($p < 0.05$; Figure 2 C). Thus, SC administration suppressed apoptosis in CUMS rats.

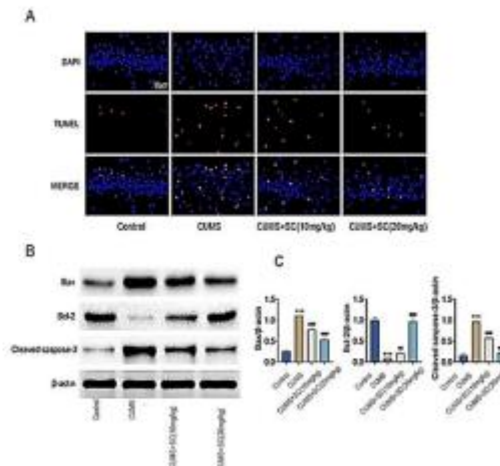

Figure 2: Sanggenon C inhibited apoptosis in CUMS rats. (A) Apoptosis in the hippocampus or cortex of CUMS rats. (B) Expression of Bax, Bcl-2 and Cleaved caspase-3. Note: ***$P < 0.001$ vs. control, ##$p < 0.01$ and ###$p < 0.001$ vs. CUMS

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Sanggenon C-induced neuronal autophagy in CUMS rats

Results revealed that LC3-II/LC3-I ratio and Beclin expression were reduced in neurons of CUMS (Figure 3 A) rats, while their protein levels were increased in SC-treated CUMS rats (Figure 3 B and C). Expression of p62 was increased in CUMS rats but significantly decreased in SCtreated rats ($p < 0.05$; Figure 3 D). Thus, Sanggenon C facilitated the autophagy of neurons of CUMS rats.

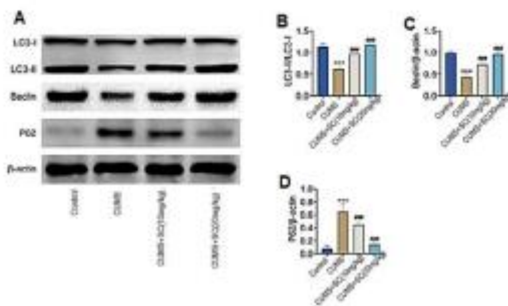

Figure 3: Sanggenon C induces neuronal autophagy in CUMS rats. Western blots analysis on the expression of autophagy-associated proteins LC3, Beclin and p62. ***P < 0.001 vs. control, ###p < 0.001 vs. CUMS Sanggenon C activated AMPK pathway

Despite the fact that AMPK expression was not affected by SC (Figure 4 A), its phosphorylation level was reduced in CUMS rats, while SC increased AMPK phosphorylation, which is necessary for activating the AMPK pathway (Figure 4 B). Moreover, BDNF was upregulated by SC administration in CUMS rats (Figure 4 C). Thus, sanggenon C initiated activation of AMPK pathway in CUMS rats.

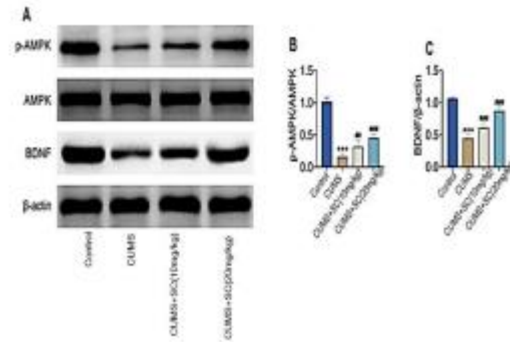

Figure 4: Sanggenon C activated the AMPK pathway. Western bot analysis of AMPK phosphorylation level and BDNF expression in CUMS rats. Note: ***P < 0.001 vs. control, ##p < 0.01, ###p < 0.001 vs. CUMS

DISCUSSION

Depression, a widespread neuropsychiatric disorder, is linked to neuronal damage in specific regions of the brain. At present, there is no effective curative therapy [2]. Sanggenon C has been reported to have widespread medicinal effects against inflammatory, analgesic and dissipative blood stasis. In this study, the depressive behavior of CUMS rats was evaluated and treated with sanggenon C. Average body weight, sucrose preference, and spontaneous locomotor activity which reflect depression of rats was recorded and analyzed, and results revealed that sanggenon C at doses of 10 and 20 mg/kg significantly improved depressive actions of CUMS rats. This indicated that sanggenon C plays a role in relieving depression. Inhibition of neuronal apoptosis promoted neuronal renewal and restoration of depressive symptoms in a depressed rat model [2,13]. So, it is necessary to clarify the effect of sanggenon C on the apoptosis of CUMS rats. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (most common method for detecting apoptosis), was performed to evaluate apoptosis in the hippocampus or cortex.

The results showed there were more positive signals of dUTP nick-end labeling in CUMS rats, meaning

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that apoptosis was enhanced in CUMS rats. Apoptosis produced in CUMS rats was repressed by sanggenon C administration. Apoptosis-associated molecules Bax, Bcl-2, and cleaved caspase-3 were also consistently regulated by Sanggenon C. Apoptosis is a natural process of programmed cell death. It plays a crucial role in mental health disorders such as depression [14]. Thus, sanggenon C relieves depression by regulating apoptosis. Autophagy is a cellular process of degradation and recycling of cellular components [15]. It maintains cellular homeostasis and regulates various physiological and pathological conditions, including neurodegenerative disorders. Autophagy dysregulation has been implicated in certain neurodegenerative disorders [16].

For instance, sanggenon C promoted autophagy of cardiomyocytes by activating AMPK, leading to cytoprotective effect on cardiomyocytes hypoxic injury [7]. Effect of Sanggenon C on autophagy in CUMS cells was also investigated in this research. Western blot analysis was performed to assess expression levels of autophagyassociated proteins, LC3, Beclin, and p62, and results indicated that sanggenon C promoted neuronal autophagy in rats subjected to chronic unpredictable mild stress. Sanggenon C has been reported to activate AMPK pathway which is an important cellular energy sensor and regulator in maintaining energy balance within cells. It is closely associated with glucose and lipid metabolism [17]. Previous studies have investigated effects of sanggenon C on AMPK pathway. Aqueous extracts of Mori ramulus, enriched with sanggenon C, were discovered to enhance AMPK phosphorylation and subsequently activate acetyl-CoA carboxylase, effectively mitigating oxidative damage induced by hydrogen peroxide [18]. In addition, Sanggenon C increased AMPK phosphorylation and subsequently enhanced autophagy in cardiomyocytes [7]. Autophagy as a cellular process for recycling and degradation of damaged cellular components, plays a crucial role in cellular homeostasis and adaptation to stress [15]. Activation of AMPK pathway and subsequent induction of autophagy have been implicated in various physiological and pathological conditions, including metabolic disorders, neurodegenerative diseases, and cancer. These findings suggest potential therapeutic implications of sanggenon C.

CONCLUSION

Sanggenon C inhibits apoptosis, induces neuronal autophagy, relieves depressive behavior and provides neuroprotection by activating AMPK pathway in CUMS rats. Thus, Sanggenon C might be a potential therapeutic candidate for depression treatment. However, further research is needed to fully understand the clinical significance of sanggenon C-mediated AMPK activation in different cellular contexts as potential drug targets.

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