Mahonia Alkaloids (MA) Ameliorate Depression Induced Gap Junction Dysfunction by miR-205/Cx43 Axis

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Abstract
Depression has become an important disease threatening human health. In recent years, the efficacy of Traditional Chinese Medicine (TCM) in treating the disease has become increasingly prominent, so it is meaningful to find new antidepressant TCM. Mahonia fortune (Lindl.) Fedde is a primary drug in traditional formulas for the treatment of depression, and alkaloids are the main components of it. However, the detailed mechanism of Mahonia alkaloids (MA) on depression remains unclear. This study aimed to investigate the effect of MA on gap junction function in depression via the miR-205/Cx43 axis. The antidepressant effects of MA were observed by a rat model of reserpine-induced depression and a model of corticosterone (CORT)-induced astrocytes. The concentrations of neurotransmitters were measured by ELISA, the expression of Connexin 43 (Cx43) protein was measured by Immunohistochemistry and western-blot, brain derived neurotrophic factor (BDNF), cAMP-response element binding protein (CREB) proteins were measured by western-blot, the pathological changes of prefrontal cortex were observed by hematoxylin–eosin (H&E) staining. Luciferase reporter assay was performed to verify the binding of miR-205 and Cx43. The regulation effect of Cx43 on CREB was verified by interference experiment. Gap junction dysfunction was detected by fluorescent yellow staining. The results confirmed that MA remarkably decreased miR-205 expression and increased Cx43, BDNF, CREB expression in depression rat and CORT-induced astrocytes. In addition, after overexpression of miR-205 in vitro, the decreased expression of Cx43, BDNF and CREB could be reversed by MA. Moreover, after interfering with Cx43, the decreased expression of CREB and BDNF could be reversed by MA. Thus, MA may ameliorate depressive behavior through CREB/BDNF pathway regulated by miR-205/Cx43 axis.
Introduction

Depression is a global disease with high incidence, high mortality and high cost [1]. At present, there are 340 million depression patients in the world, and more than 26 million in China [2]. Despite a series of psychological and drug treatments for depression, only 74% of depression patients showed improvement [3]. Meanwhile, these drugs exhibit slow onset, side effects and limited efficacy, which in turn limit clinical application. Recently, TCM has drawn great attention on its potent effect for the treatment of depression, which exhibited low toxicity and high efficiency. However, there is an unmet need for novel TCM antidepressants with high efficiency and low toxicity.

The Mahonia belongs to the angiosperm phylum Berberidaceae [Mahonia bealei (Fort.) Carr.] and is a traditional medicinal plant in China [4]. Studies have shown that the chemical components contained in the Mahonia are mainly berberine, berberamine, rhizobine and palmatine [5]. Pharmacological studies showed that Mahonia exerted antioxidant [6], anti-inflammatory [7] and antibacterial effects [8]. Our previous study showed that Mahonia have significant antidepressant effects, but the potential antidepressant mechanism is not yet clear.

MicroRNAs (miRNAs) are short RNA molecules ranging in size from 19 to 25 nucleotides [9, 10]. Many downstream target genes can be potentially regulate by miRNA through intracellular gene silencing mechanisms [11]. Studies have shown that miRNAs regulatory networks have an important role in neuronal development and brain function [12, 13], and the importance of miRNAs in the regulation of brain function has been well elucidated, while the pathological role of miRNAs in neuropsychiatric disorders still needed to be further understood.

A host of studies have revealed that Cx43 plays an important role in the process of depression, and lower expression of Cx43 was observed in depression patient or experimental models [14–16]. Besides, downregulation of Cx43 induced depression in rat and mice [17]. Cx43 has the function of linking gap junction channels (GJCs) with hemichannels (HCs) [18], and widely existed in nervous system. Furthermore, Cx43 is the main connexin of astrocyte [19]. Dysfunction of GJCs and HCs induced depressive behavior [20]. Therefore, the amelioration of the
function of gap junctions is important for the regulation of depression.

In this study, we firstly explored the antidepressants effect of MA. Then, the expressions of miR-20a-5p, miR-186-5p, miR-205, miR-206, miR-382-3p and miR-495 in the brain tissue of depressive rat were detected. These depression-related genes were acquired from the Gene Expression Omnibus (GEO) database. The results showed that MA significantly decreased the expression of miR-205. Furthermore, we predicted the potential targets of miR-205 through TargetScan database (https://www.targetscan.org/vert_72/), the result shown that Cx43 has binding site with miR-205, which was verified by Luciferase reporter gene. Next, after interfered with Cx43, MA effectively reversed the decreasing of CREB/BDNF. The above studies indicated that MA targeted Cx43 through miR-205/Cx43 axis, this may be a promising target for MA and other TCM in prevention or treatment of depression.

Method

Materials and Reagents

Dried Mahonia was purchased from Guangxi Xianju Chinese Medicine Technology Co. Acetonitrile (Nanning, Guangxi, China); methanol and formic acid were provided by Sigma (Sigma-Aldrich, USA); Fluoxetine was supplied by Lilly Suzhou Pharmaceutical Co., Ltd. (Suzhou, Jiangsu, China); The enzyme-linked immunosorbent assay (ELISA) kits for rat and human MAO, DA, NE, 5-HT were supplied by Shanghai Fanke Industrial Co., Ltd. (Shanghai, China); Lipofectamine 3000 and Trizol were provided by Invitrogen (USA); The PrimeScript RT Reagent Kit and SYBR Premix Dimer Eraser were provided by Vazyme Biotech Co., Ltd. (Nanjing, Jiangsu, China); The Mir-x miRNA First-Stand Synthesis Kit was purchased from TaKaRa (Japan); Anti-Cx43 and Anti-CREB were provided by Abcam (UK), Anti-p-CREB, Anti-BDNF, GAPDH were provided by Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, Hebei, China); Corticosterone (CORT) was provided by Aladdin (Shanghai, China).

Drug Preparation

Dried Mahonia slices (500 g) were extracted 3 times with 5 L of water in a reflux system. The filtrate was collected and concentrated into a paste. The thick paste was passed through silica gel column chromatography, recovered chloroform solution, concentrated, and obtained MA (3.22 g).

High-Performance Liquid Chromatography (HPLC)

A UHPLC Dionex Ultimate 3000 (Thermo Scientific, San Jose, USA) equipped with a column oven and cooling autosampler was used. The separation was performed in a Supersil ODS C18 column (5.0 µm, 4.6 mm × 250 mm, Dalian Elite, China) with a column temperature maintained at 25 °C. The binary mobile solvents consisted of water containing 0.1% formic acid (v/v) (A) and acetonitrile (B) with the following gradient elution program: 0–40 min, 15–20% A; 40–60 min, 25–90% A; 60–70 min, 90% A. The flow rate was 1.0 mL/min, the injection volume was 2 µL, and the split ratio was set at 1:1.

A Q-Exactive plus hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, San Jose, USA) with a heat electrospray ionization (HESI) was employed. The mass conditions were as follows: spray voltage: + 4.0 kV; capillary temperature: 320 °C; auxiliary gas heater temperature: 300 °C; S-lens RF level: 50 V; by full MS/dd-MS2 scan mode: Scan range: 75–1125 m/z; Resolution: 70,000; Automatic gain control (AGC) target: 1.0e5; Loop count: 5; Maximum injection time (IT): 50 ms; Isolation window: 2.0 m/z; Stepped NCE: 20, 40, 60; Apex trigger: 2–6 s; Dynamic exclusion: 10 s. Nitrogen was used for spray stabilization and as the collision gas in the C-trap. Q-Exactive 2.9 (Thermo Fisher Scientific, San Jose, USA) was used to control the mass spectrometer, Xcalibur 4.1 software (Thermo Fisher Scientific, San Jose, USA) was used to control the instrument and for data acquisition and analysis.

Animals

All of our experimental procedures conformed to internationally accepted principles for the use and care of experimental animals. The Animal Care Committee of the Institute of Chinese Medicine & Pharmaceutical Science approved the experiment. Sprague–Dawley (S–D) rats (180–220 g, 7–8 weeks), male, came from Changsha Tianqin Biotechnology Co., LTD. (Changsha, Hunan, China). All the animals
were fed on a 12 h daily/night cycle in an SPF level laboratory with a temperature of 25 ± 1 °C and humidity of 60 ± 10%.

**Reserpine-Induced Depression Model Protocol [21, 22]**

The experiment rats were separated into normal, model (reserpine), positive (fluoxetine), MA high dose (MA-H) and MA low dose (MA-L) groups, with 10 rats in each group. The normal group was intraperitoneally injected with normal saline, and the other groups were intraperitoneally injected with 0.5 mg·kg⁻¹·d⁻¹ reserpine for 10 days. The administration method is as follows when starting modeling: the positive group was given fluoxetine 1.8 mg·kg⁻¹, the MA-H group was assigned MA 2 mg·kg⁻¹, the MA-L group was assigned MA 1 mg·kg⁻¹, rats in normal group and model group were given an equal amount of distilled water intra-gastrically once a day.

**Sucrose Preference Test**

Water bottles of the same size, containing 1% sucrose water and drinking water respectively, were administered simultaneously to the rats, and the bottles were withdrawn after 48 h of pre-adaptation. After 12 h of fasting and water deprivation, the consumption of 1% sucrose water or drinking water within 1 h was measured. The ratio of sucrose water consumption was calculated.

Percentage of sucrose intake = \( \frac{\text{sugar water consumption}}{\text{sugar water consumption} + \text{drinking water consumption}} \) × 100%

**Motion Inhibition Experiment**

Animals were placed in the centre of a 40 cm diameter circular whiteboard and recorded the retention time in the circle within 30 s. The observed motion inhibition can be indicated by the reduced movement of animal.

**Cell Culture**

Human glioma cells U251 (Cell Bank of Chinese Academy of Sciences) were cultured in DMEM medium containing fetal bovine serum (10%), penicillin and 100 ng/mL streptomycin (100 U·mL⁻¹ and 100 ng·mL⁻¹). The cell lines were cultured in a 37 °C incubator with 5% CO₂.

CORT was dissolved with dimethyl sulfoxide (DMSO), and stored in a concentration of 50 mmol·L⁻¹. MA was dissolved with DMSO and stored in a concentration of 1 mg·mL⁻¹. U251 cells were treated with CORT for 48 h to mimic the astrocyte damage in depression. In brief, U251 cells were pre-incubated with 200 µmol·L⁻¹ CORT for 24 h, then together treated with MA-H (100 µg·mL⁻¹) and MA-L (50 µg·mL⁻¹) for another 24 h.

**Neurotransmitter Detection**

Rats were anesthetized with pentobarbital sodium (2%, 0.3 ml/100 g) intraperitoneally, blood was obtained from the abdominal aorta and centrifuged for 10 min to collect serum. Culture supernatant of U251 cells was collected and centrifuged. The concentrations of 5-HT, NE, DA were detected by ELISA kits.

**Histopathological Examination**

Prefrontal cortex tissues were obtained and fixed in 4% paraformaldehyde for 3 days, dehydrated and dewaxed, and then embedded in paraffin. The brain Sects. (3 µm) were stained with H&E and observed under a light microscope at 20×. All images were analyzed using the Image-Pro Plus 6.0 system.

**Immunohistochemistry**

The prefrontal cortex paraffin Sects. (3 µm) were baked in an oven for 4 h at 60 °C, dehydrated with xylene, followed by gradient ethanol. Then endogenous catalase was removed with 3% hydrogen peroxide, and blocked with 5% bovine serum albumin (BSA). Then the slices were incubated with primary antibody (Cx43, 1:100) at 4 °C overnight. After washing with phosphate-buffered saline (PBS) three times,
the secondary antibody was incubated at room temperature 1 h, stained with 3,3'-diaminobenzidine (DAB) and sealed. The analysis was performed using the image pro plus 6.0 system.

Relative Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNAs in U251 cells and hippocampus tissue were extracted by the TRIZol reagent. RNAs were reversed transcription by the PrimeScript RT Reagent Kit. miRNAs were reversed transcription by the Mir-x miRNA First- Stand Synthesis Kit. Quantitative RT-PCR was performed by the SYBR Premix Dimer Eraser on a LightCycler 480 II Real-Time PCR thermocycler (Roche, Switzerland). The primer of miR-205, miR-206-5p, miR-186, miR-186-5p, miR-20a-5p, miR-382-3p, miR-495, GJA1, GAPDH and U6 (Table 1) were synthesized by the biological company (Sangon, Shanghai, China). The relative expression of genes were normolized by 2−ΔΔCT relative quantitative method, and U6 and GAPDH were chosen as the internal control genes.

Western Blot Analysis

The proteins of rat hippocampus tissue and U251 cells were extracted by adding 150 μL mixed lysate, and BCA assay was performed to quantify the protein concentration. An equivalent denatured protein sample (100 μg) was added to each lane and separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes [23, 24]. Then the membranes were blocked with 5% milk powder solution at room temperature for 1 h, and then incubated with primary antibody at 4 °C overnight. Antibodies concentrations were Cx43 (1:1000), CREB (1:500), p-CREB (1:5000), BDNF (1:1000), GAPDH (1:5000) (Proteintech, Wuhan, China). The membrane was washed slowly with TRIS-buffer brine (TBST) on an oscillator 3 times for 5 min each time. Then, membranes were incubated in HRP-conjugated secondary antibody (1:5000) in room temperature for 1 h. Finally, the membranes were washed 3 times with TBST. Gray value of each band was analyzed by Image J software.

Cytotoxicity Assays

The viability of U251 cells was detected by the CCK-8 assay. Briefly, U251 cells were seeded into a 96-well plate with 1×10⁴ cell per well and treated by CORT or MA. After 24 or 48 h, 10 μL CCK-8 and 90 μL culture medium solution were added into each well. Then, the plate was placed at 37 °C in incubator for 1 h. At last, the absorbance of each well was measured at 450 nm by Microplate Reader (Bio- teck, USA).

Dual-Luciferase Reporter Assays

MiR-205 mimics, miR-NC and Luciferase reporter plasmids were co-transfected into HEK-293 T cells by using Lipo- fectamine 3000. At 24 h post-transfection, the double Luciferase Reporter Assay system was used to analyze the double Luciferase activities. Luciferase activity was normalized to Renilla Luciferase activity.

pMIR-REPORTER (Ambion, CA, USA) vector was used to insert the 3'-UTRs of GJA1, predicted miR-205 seed matching sites and corresponding mutant sites after they were synthesized and annealed. In HEK-293 T cells, a Dual-luciferase reporter assay was performed by co-transfected with wild-type plasmid or mutated reporter and miR-205 or miR-NC. 24 h after transfection, Luciferase activity was analyzed using a Dual-Luciferase Reporter Assay System (Promege, WI, USA).

MiRNA Mimics, SiRNA and Cell Transfection

The sequence of miRNA targeting miR-205 (miR-205 mimics) and the sequence of siRNA targeting Cx43 (si-Cx43) and scrambled negative control (NC) were purchased from RiboBio (Guangzhou, China). U251 cells were seeded in 6-well plates and transfected with miR-205 mimics, si-Cx43 or si-NC using Lipo-fectamine 3000 transfection reagent (Invitrogen, USA) for 24 h and then treated with MA for 32 h.

Lucifer Yellow Staining

U251 cells were seeded into a 24-well plate with 20×10⁴ cell per well and cultured for 24 h in a 37 °C incubator with 5% CO₂, 0.05% Lucifer yellow and 0.05% rhodamine mixed liquor 500 uL were added into per well, then, using a surgical blade, gently score the bottom of the plate with 3 parallel lines per well and continue incubation for 6 min, recycled dyes and added PBS 500 uL to cover. Take photograph under a fluorescent microscope (Leica, Germany).
Statistical Analysis

SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data expressed as mean ± SD. The data of each group were tested for normality. When the data of each group were normally distributed and the variances were homogeneous, one-way ANOVA was used for multiple group comparisons. P-values of less than 0.05 were considered significant, where *P < 0.05, **P < 0.01.

Results

Chemical Compounds in MA

As shown in Fig. 1, the chemical compounds of MA were identified by UHPLC/ESI Q-Orbitrap Mass Spectrometry. It can be seen from the figure that MA contains more than 13 chemicals, and 8 chemical substances were identified by first and second mass spectrometry as well as literature and database search, and the results are showed in Table 2.

Table 2  Identified chemical compounds in MA by UHPLC/ESI Q-Orbitrap Mass Spectrometry

| Peak No | Rt/min | Molecular formula | Product ions (m/z) | Identification       |
|---------|--------|-------------------|-------------------|----------------------|
| 1       | 29.89  | C_{20}H_{24}NO_{3}+ | 303.64600, 234.86003, 188.08308, 137.05991, 116.68137 | Unknown             |
| 2       | 32.16  | C_{20}H_{19}NO_{4} | 323.11469, 307.08347, 294.11118, 265.07343, 223.07343 | Dihydroberberine    |
| 3       | 32.90  | C_{21}H_{24}O_{5}N_{3}+ | 573.55127, 297.16238, 162.09108 | Unknown             |
| 4       | 33.68  | C_{20}H_{17}NO_{4} | 322.10672, 294.11191, 279.08853, 191.46196, 136.79010 | Columbamine         |
| 5       | 35.04  | C_{39}H_{45}N_{2}O_{7}+ | 545.07587, 441.31534, 282.16910, 227.10773, 174.09235 | Unknown             |
| 6       | 36.84  | C_{20}H_{17}NO_{4} | 323.11484, 307.08310, 279.08926, 250.08717, 210.13094 | Jatrohizine         |
| 7       | 37.51  | C_{39}H_{45}N_{2}O_{7}+ | 392.88751, 283.13419, 235.07533, 174.09126, 121.06539 | Unknown             |
| 8       | 40.17  | C_{19}H_{18}NO_{4}+ | 307.08353, 292.05716, 279.0893, 250.08569, 212.44121 | Berberrubine        |
| 9       | 43.51  | C_{19}H_{23}NO_{4}+ | 336.12238, 322.10746, 308.12723, 294.11139, 278.08139 | Palmation           |
| 10      | 43.56  | C_{20}H_{17}NO_{4} | 320.09131, 306.07565, 292.09647, 278.08051, 239.87100 | Berberine           |
| 11      | 45.14  | C_{22}H_{24}N_{2}O_{7}+ | 350.13821, 322.14337, 308.12747, 278.11804 | Dehydrocorydaline  |
| 12      | 47.17  | C_{21}H_{23}NO_{4}+ | 334.10681, 320.09042, 306.11203, 292.09637, 247.84787 | Unknown             |
| 13      | 53.99  | C_{20}H_{17}NO_{4} | 337.09375, 322.07043, 308.09174, 294.07568, 279.05252 | Oxyberberine        |
Fig. 2 Effects of MA on reserpine-induced depressive behaviors, neurotransmitters and inflammatory infiltration in the prefrontal cortex of depression rats. a: sucrose preference (%), b: in-circle retention (s), c: The concentration of 5-HT (pg/mL), d: The concentration of DA (nmol/L), e: The concentration of NE (ng/mL), f: HE staining (20×). a: normal group; b: model group; c: positive group; d: MA-H group; e: MA-L group. Data are expressed as the mean ± SD (n=6). #P<0.05, ##P<0.01 compared to the normal group, *P<0.05, **P<0.01 compared to the model group.
Effects of MA on Reserpine-Induced Depression in Rats

After reserpine administration, compared with normal group, the sugar water intake (Fig. 2A) was reduced \((P<0.01)\), yet the retention time in the circle (Fig. 2B) was increased \((P<0.01)\) in model group, indicating that reserpine treatment induced depression-related behavior. Compared with model group, rats treated with MA exhibited a significantly increase in the sugar water intake in MA-H \((P<0.05)\) and MA-L \((P<0.05)\) group; decreased retention time in the circle of MA-H \((P<0.01)\) and MA-L \((P<0.05)\).

Further, compared with normal group, the levels of 5-HT, DA and NE in model group were significantly decreased \((P<0.05, P<0.05, P<0.01)\), indicating that reserpine treatment induced a decrease in neurotransmitter. Compared with model group, levels of 5-HT, DA and NE in MA-H group were significantly increased \((P<0.05, P<0.05, P<0.05)\); but only 5-HT was significantly increased in MA-L group \((P<0.05)\). These data showed that treatment with MA improved depressive behaviors and neurotransmitter in rats.

Reserpine administration by intraperitoneal injection induced inflammation in different brain regions of rats, with partial nuclear pyknosis, nuclear marginalization, neuronal cells necrosis, and increased glial cells differentiation. While MA significantly improved these pathological changes (Fig. 2F), indicating that MA alleviated inflammation in the prefrontal cortex of depression rats.

MA Regulated miRNAs Expression in Depressive Rats

The underlying antidepressive mechanisms of MA remain elucidated. Here, we selected miRNAs (miR-20a-5p, miR-186-5p, miR-205, miR-382-3p and miR-205) through the GEO database that associated with depression to see whether MA has an effect on them. The qRT-PCR results showed that miR-20a-5p, miR-186-5p and miR-205 (Fig. 3A, B and C) in brain tissues of depressive rats were significantly downregulated by MA \((2 \text{ mg·kg}^{-1})\) \((P<0.01, P<0.05, P<0.01)\); miR-206 (Fig. 3D) was significantly upregulated by MA \((2 \text{ mg·kg}^{-1})\) \((P<0.01)\); however, miR-382-3p and miR-495 (Fig. 3E, F) showed no significant changes after MA.
Fig. 4 MA played an anti-depressive role in CORT-induced U251 cells. A Cell viability after 24 h of MA action (%), B Cell viability after 24 h of CORT action (%), C Cell viability after 48 h of CORT treatment (%), D Relative CREB mRNA expression after administration of different concentrations of CORT modeling and MA (100 μg/mL) treatment, E Relative BDNF mRNA expression after administration of different concentrations of CORT modeling and MA (100 μg/mL) treatment, F The concentration of 5-HT (pg/mL), G The concentration of DA (nmol/L), H The concentration of MAO (ng/mL), I Relative expression of miR-186-5p, J Relative expression of miR-205, K Relative expression of miR-20a-5p. Data are represented as the mean±SD [qRT-PCR (n=3), CCK8 (n=5) and Elisa (n=6)]. *P<0.05, **P<0.01 compared to the normal group, *P<0.05, **P<0.01 compared to the model group
treatment (P > .05). The results indicated that MA regulated some miRNAs expression in depressive rats.

**MA Played an Anti-Depressive Role in CORT-Induced U251 Cells**

Next, we investigated the antidepressant mechanism of MA in vitro. Firstly, the effects of MA and CORT alone at different concentrations on the U251 cells were measured using the CCK-8 assay. The results showed that 24 h after MA treatment, the viability of U251 cells was significantly inhibited, and its IC$_{50}$ was 200 µg·mL$^{-1}$ (Fig. 4A); however, the viability of U251 cells treated with individual CORT for 24 h was no significant change (Fig. 4B), while 48 h after CORT treatment, the cell viability was significantly inhibited, and its IC$_{50}$ was 200 µmol·L$^{-1}$ (Fig. 4C).

Then, in order to find the appropriate concentration of CORT in depression models, we examined the expression of depression-related proteins CREB and BDNF by qRT-PCR. The results (Fig. 4D, E) showed that after treatment with CORT for 48 h and MA 100 µg·mL$^{-1}$ for 24 h, CREB and BDNF levels were significantly decreased with 200 µmol·L$^{-1}$ CORT compared with normal group (P < 0.01, P < 0.01), while there was no significantly change of BDNF levels at 100 µmol·L$^{-1}$ CORT, indicating that CORT at 200 µmol·L$^{-1}$ could successfully establish an in vitro model of depression. Compared with model group, treated with MA (100 µg·mL$^{-1}$) significantly increased CREB and BDNF levels in CORT treated groups (P < 0.01, P < 0.01). Therefore, CORT at 200 µmol·L$^{-1}$ was chosen for modeling in subsequent experiments.

Further, the effect of MA on neurotransmitter in cell supernatant was measured by ELISA. In the results of Fig. 4F, G and H, compared with normal group, we observed decreased levels of 5-HT, DA and increased levels of MAO in CORT-induced U251 cells supernatant (P < 0.01,
Fig. 6 MA regulated Cx43 through miR-205 in CORT-induced U251 cells. A Western Blot strips, B Cx43/GAPDH, C BDNF/GAPDH, D p-CREB/CREB, E Western Blot strips (overexpress) F Cx43/GAPDH (overexpress), G BDNF/GAPDH (overexpress), H p-CREB/CREB (overexpress), I NC group, J miR-205 mimic group, K miR-205 mimic + MA-H group, L miR-205 mimic + MA-L group (10×). Data are represented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 compared to the normal/NC group, †P < 0.05, ††P < 0.01 compared to the model/miR-205 mimic group.
while MA reversed these changes. These results implied that MA could alleviate the depressive effects caused by CORT on U251 cells.

Last, we detected the expressions of miR-205-5p, miR-186-5p and miR-20a-5p in CORT-induced U251 cells, which shown a significant decrease in MA treated depression rats. The results (Fig. 4J, K) showed that the expression levels of miR-205-5p and miR-20a-5p in CORT-induced U251 cells were in consistent with that in depressive rats, moreover, miR-205-5p showed a higher expression.

\( P < 0.05, P < 0.01 \), while MA reversed these changes. These results implied that MA could alleviate the depressive effects caused by CORT on U251 cells.

GJA1 (Cx43) is a Target Gene of miR-205

To test whether miR-205 targets GJA1, GJA1 3′-UTR containing this seed sequence was cloned into pMIR-REPORTER vector (WT). Overexpression of miR-205 in HEK-293 T cells decreased Luciferase activities of wild type reporter to 70%, suggesting that miR-205 inhibits its 3′-UTR reporter activities of GJA1. To test whether miR-205 specifically inhibits the activity by binding its seed sequence, we also made mutant construct with the mutation of miR-205 binding site in the 3′-UTR of GJA1 (Mut). Forced expression of miR-205 did not affect...
the transcriptional activation of mutant GJA1 3′-UTR reporter activity (Fig. 5B), indicating that miR-205 directly targets GJA1 by binding to its seed sequence at 3′-UTR. In addition, overexpression of miR-205 in U251 cells significantly suppressed Cx43 protein expression (Fig. 5D). These results indicated that GJA1 is a target gene of miR-205.

MA Regulated Cx43 Through miR‑205 in CORT-Induced U251 Cells

Next, the effects of MA on Cx43 and CREB/BDNF pathway were detected by western blotting. Compared with normal group, the results showed that the protein expressions of Cx43, BDNF and p-CREB were significantly decreased in CORT-induced U251 cells (P<0.05, P<0.05, P<0.05). Compared with model group, MA-H group significantly increased the expressions of Cx43, BDNF and p-CREB (P<0.05, P<0.05, P<0.05) (Fig. 6B, C and D). These results demonstrated that MA ameliorated depression through Cx43.

In order to verify whether MA ameliorated depression through regulating Cx43 via miR-205, miR-205 was overexpressed with miR-205 mimics in CORT-induced U251 cells, and Cx43, CREB/BDNF pathway were detected by western blotting. Compared with NC group, the expression of Cx43, BDNF and p-CREB
were significantly decreased in miR-205 mimics group ($P < 0.01, P < 0.01, P < 0.01$), which were reversed by MA treatment (Fig. 6F, G and H). These results demonstrated that MA ameliorated depression by Cx43 through miR-205.

Further, we examined whether MA regulate miR-205 to improve gap junction function. Gap junction dysfunction is one of the manifestations of depression and can be measured by scrape-loading and dye transfer. As shown in Fig. 6I–L, gap junction function was impaired in the model group, and the fluorescent dye could not be delivered outward from scratch through the intercellular gap junctions. However, MA could enable the outward delivery of fluorescent dye, indicating that MA could improve CORT-induced intercellular gap junction dysfunction in astrocytes. All these results provided evidence that MA ameliorated gap junction function in depression by miR-205 regulating Cx43.

### MA regulated CREB/BDNF Pathway Through Cx43 in CORT-Induced U251 Cells

In addition, to verify whether MA ameliorated depression via Cx43 regulated CREB/BDNF pathway, Cx43 was inhibited by si-Cx43 in CORT-induced U251 cells, then the expressions of CREB and BDNF were detected by western blotting. Compared with si-NC group, the expressions of BDNF and p-CREB were significantly decreased in si-Cx43 group ($P < 0.01, P < 0.01$), which were reversed by MA treatment (Fig. 7B, C). These results demonstrated that MA ameliorated depression via Cx43 regulated CREB/BDNF pathway.

### Effect of MA on Cx43 in the Brain Tissue of Depressive Rats

Finally, we investigated the effect of MA on Cx43 expression in vivo. The western blotting results (hippocampus tissue) (Fig. 8A, B) showed that the expression of Cx43 was significantly decreased in model group compared with normal group ($P < 0.01$). However, compared with model group, the expression of Cx43 was significantly increased with the administration of MA-H and MA-L group ($P < 0.05, P < 0.01$). Similar result was observed in immunohistochemical detection (prefrontal cortex) (Fig. 8C–H). Evidently increase of Cx43 protein level was found in MA-H and MA-L group compared to the model group ($P < 0.01, P < 0.01$). The above results demonstrated that MA improved depression by increasing the level of Cx43 in hippocampus tissue and prefrontal cortex.

### Discussion

In this study, we found that MA improved depression in reserpine-induced rats via miR-205 through the methods of ELISA, H&E staining, qRT-PCR and western-blotting, further, the expression of Cx43 was increased after treatment with MA. Then, we overexpressed miR-205 in vitro and found that MA increased the expression of Cx43, while MA abolished the decrease of CREB and BDNF induced by Cx43 knockdown in vitro.

Studies have shown that Chinese herbal medicine can be used as supplements and substitutes for depression [25, 26]. There have been clinical trials to verify the effectiveness and safety of herbal therapies. The past few years, the mechanism of herbal medicines in treating depression has attracted significant attention [27]. Mahonia is a TCM herb, and in Anhui Herbal Medicine, it is described as clearing heat and detoxifying, detoxifying the liver and kidney; in Luchuan Materia Medica, it is described as cure febrile diseases, fever, upset, red eyes [28]. According to the TCM treatment of depression by draining the liver and relieving depression, warming the kidneys and strengthening the yang and the efficacy of Mahonia, we conducted an exploration of the antidepressant effects of its alkaloid components and found that it significantly increased sugar water preference and wilderness activity and improved depression-like behavior in rats.

Studies showed that miRNAs have an important regulatory role in the development of depression [29, 30]. In the present study, we found that MA downregulated miR-205 expression in depressed rat brain tissue and CORT-induced U251 cells, indicating that the antidepressant effect of MA is closely related to miRNA. MiR-205 has been reported to be associated with the development of cervical cancer [31] and gastric cancer [32]. However, a single miRNA can often play a role in multiple diseases. In this study, we reported for the first time the association between miR-205 and depression and demonstrate that down-regulation of miR-205 improves depression.

Further, we searched the target of miR-205 through Bioinformatics Website, and found that Cx43 has binding site with miR-205. Interesting, Cx43 is the predominant gap junction protein in astrocytes and has been reported by numerous of studies that was closely associated with depression [33–35], but the mechanism of antidepressive effect of miR-205 via Cx43 has not been reported. In the present study, CORT-induced astrocyte model was used to verify this result, which indicated that MA regulated miR-205/Cx43 axis to ameliorate depression. Studies have shown
that dysfunction of interstitial astrocyte gap junctions is an important pathogenesis of depression [36–38]. In this study, the result of fluorescent yellow dyeing confirmed of this view and revealed that MA via miR-205 improve gap junction dysfunction in astrocytes.

BDNF is synthesized in neurons and glial cells, transported to terminals and released, and it plays an important role in neuronal maturation, synapse formation and synaptic plasticity in the brain. Studies have shown that the synergistic interactions between BDNF in neuronal activity and synaptic plasticity make it an ideal and important regulator of cellular processes that underlie cognition and other complex behaviors [39]. Whereas the CREB is an important transcription factor regulating BDNF and memory formation [40], the transcription factor CREB has been shown to play a key role in cognition and initiating memory integration [41, 42]. Gene expression mediated by CREB plays an important role in long-term memory, hippocampal neuroplasticity, dendritic growth, and neurogenesis [43]. In the present study, we examined the expression of CREB/BDNF pathway after administration of MA and overexpression of miR-205, respectively, and found that the expression of BDNF and CREB increased after MA treatment, indicating that MA may has a role in regulating the CREB/BDNF pathway to improve depression. In addition, the expression of Cx43, BDNF and CREB increased after overexpression of miR-205 and combined with administration of MA treatment, suggesting that MA target Cx43 via miR-205 to improve depression. Last, we inhibited Cx43 and found that MA increased the expression of CREB and BDNF, indicating that MA has a role in regulating the CREB/BDNF pathway via Cx43 to improve depression.

Conclusions

In conclusion, the antidepressant effect of MA was associated with downregulation of miR-205 expression, and upregulation of Cx43, CREB and BDNF. MA improved depression by inhibiting miR-205, elevating Cx43 levels and activating CREB/BDNF pathway. MA could be investigated as a potential complementary drug for depressed patients. In addition, follow-up studies on the effects of MA on neuroplastic function and other molecular mechanisms of depression are currently underway to develop MA as an effective agent for the successful treatment of depression.

Supplementary Information

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Author Contributions

GN, NH, JH and DM designed all the experiments. JH, DM and J were responsible for the experiments, the analyses of data, and the first draft of the article. SF, Z, F, DM Wei, JX and Y assisted with the execution of the experiments. NH, GN, DM and KD assisted with the improvement of the article.

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Data Availability

Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest

The authors declare no conflict of interest associated with this study.

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