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Resveratrol promotes hUC-MSCs engraftment and neural repair in a mouse model of Alzheimer's disease

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Abstract

Mesenchymal stem cell transplantation is a promising therapeutic approach for Alzheimer's disease (AD). However, poor engraftment and limited survival rates are major obstacles for its clinical application. Resveratrol, an activator of silent information regulator 2, homolog 1 (SIRT1), regulates cell destiny and is beneficial for neurodegenerative disorders. The present study is designed to explore whether resveratrol regulates the fate of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) and whether hUC-MSCs combined with resveratrol would be efficacious in the treatment of neurodegeneration in a mouse model of AD through SIRT1 signaling. Herein, we report that resveratrol facilitates hUC-MSCs engraftment in the hippocampus of AD mice and resveratrol enhances the therapeutic effects of hUC-MSCs in this model as demonstrated by improved learning and memory in the Morris water maze, enhanced neurogenesis and alleviated neural apoptosis in the hippocampus of the AD mice. Moreover, hUC-MSCs and resveratrol jointly regulate expression of hippocampal SIRT1, PCNA, p53, ac-p53, p21,

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Author's contributions

BY, FXG and XXW conceived and designed the experiments, and revised the final version of the paper. XXW, NM, TJH, LX and JWW performed the experiments. XXW, QX and SSM analyzed the data. XXW, SSM, KZ, QHL, YTZ and JWW wrote the paper, TZ, KC prepared reagents and materials and performed part of the experiments. All authors reviewed the manuscript prior to submission.

Disclosure statement

The authors have no competing interests.

and p16. These data strongly suggests that hUC-MSCs transplantation combined with resveratrol may be an effective therapy for AD.

Keywords

Alzheimer's disease; Transgenic mice; Human umbilical cord-derived mesenchymal stem cells; Resveratrol, SIRT1

1. Introduction

Alzheimer's disease (AD) is characterized by age-dependent degeneration of the central nervous system and decline of cognitive and behavioral function. There is no efficient treatment for AD currently. Emerging evidence have shown that mesenchymal stem cells (MSCs) transplantation are proposed to be an appealing therapeutic strategy for AD treatment. MSCs are immune privileged, they are negative for CD40, CD80, CD86, HLA-DR, and are able to suppress T-cell proliferation, accounting for their tolerogenicity. Besides, MSCs express key markers of immunomodulation (indoleamine 2,3-dioxygenase and HLA-G) and retain the safety aspect of reducing telomere length with increasing passage number. These data strongly suggests that MSCs are unique for allogeneic cell based therapy [1]. Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) are promising candidate for stem cell based therapy for AD because of their potential role in neural-differentiation and paracrine release of neurotrophins to support endogenous neurogenesis [2]. MSCs transplantation is demonstrated to improve learning and memory and attenuate A β deposition in an AD mouse model [3]. Additionally, they can be easily obtained while posing limited risks to the patient [4].

However, the percentage of transplanted MSCs that survived, migrated, and differentiated into neural-like cells in AD mice is low [3], likely due to the chronic underlying AD pathology which has been shown to negatively affect the survival and function of MSCs [5]. Therefore, it is necessary to identify strategies to enhance the ability of transplanted hUC-MSCs to survive and differentiate into neurons, in order to improve potential efficacy of hUC-MSCs in the treatment of AD.

Resveratrol (3, 5, 4, -trihydroxystilbene) is a natural polyphenol found in a variety of plants, including grapes, peanuts, and blueberries. Studies have shown that resveratrol can mimic certain aspects of caloric restriction and it exerts a wide range of biological effects that can protect against age-related diseases including cardiovascular and neurodegenerative diseases [6]. It has been speculated that the beneficial effects of resveratrol in the brain and other tissues may involve rejuvenation of resident stem cell function. Indeed, resveratrol was previously shown to enhance the survival, homing, engraftment, and differentiation of cardiac stem cells, and implantation of these cells improved cardiac function [7]. Resveratrol also augmented the therapeutic efficiency of MSCs in a mouse model of autoimmune encephalomyelitis [8]. Resveratrol and MSCs exerted synergistic effects on liver regeneration and homing in partially hepatectomized rats [9]. However, whether resveratrol could promote the survival and engraftment of hUC-MSCs in the central nervous system and contribute additively to neuroprotection in AD has yet to be elucidated.

Resveratrol is an activator of SIRT1 (a member of the sirtuins family), which is an NAD⁺-dependent deacetylase that regulates various genes and proteins involved in cell proliferation, apoptosis, senescence, and differentiation [10–12]. In addition, SIRT1 activation with resveratrol has been linked to protection against oxidative stress, metabolic decline and inflammation, while conferring cardiovascular and neural protection against nutrient stressors in rodents and non-human primates [13]. Furthermore, SIRT1 is shown to attenuate AD pathogenesis, synaptic plasticity, and learning and memory [14,15], and a reduction in SIRT1 activity in neurons is suggested as an early prognostic marker for AD [16,17]. However, it is unclear whether SIRT1 signaling provides the mechanism basis by which hUC-MSCs and resveratrol offer neuroprotection in the setting of AD. Therefore, we explored the combined effects of hUC-MSCs and resveratrol on behavior, pathology, as well as the SIRT1 signaling in the hippocampus of a mouse model of AD. Herein, we demonstrate that combination of hUC-MSCs and resveratrol as an effective approach for AD treatment via SIRT1 signaling.

2. Materials and methods

2.1. Isolation, culture, and identification of hUC-MSCs

hUC-MSCs were isolated, cultured, and identified as previously described [18]. Cells at passage 4 (P4) were collected for experiments. The study was approved by the Ethics Committees of the First affiliated hospital of Zhengzhou University, and written informed consent was obtained from hUC-MSC donors. All procedures were performed in strict accordance with the guidelines for using the human subjects of Zhengzhou University.

2.2. Animals and groups

This study was approved by the Institutional Animal Care and Use Committee of Zhengzhou University, China. All procedures were performed in strict accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. The transgenic AD mice used in this study were described as previously described [19]. Mice were housed at 25 °C under a 12 h light/dark cycle, with free access to standard food and water. Finally, at the end of 3 months of age, 21.03 ± 1.7 g, the AD mice and their wild-type littermates were randomly assigned into five experimental groups (15 mice/group) as shown in Table 1

hUC-MSCs were transplanted intravenously into the AD mice every 2 weeks for 2 months. As suggested by previous study [20], approximately 1×10^6 cells suspended in 200 μ l of normal saline were transplanted into each mouse through the tail vein. For those mice that received resveratrol directly, resveratrol was administered by oral gavage (200 mg/kg-per day) for 8 weeks.

2.3. Morris water maze (MWM)

The MWM test was carried out as previously described [21]. Each mouse underwent four trials a day for 6 consecutive days. The latency to find the hidden platform was recorded. The probe trial was performed on day 7. The number of crosses over the platform location (crosses), the time to reach the platform location (latency), the time spent in the target

quadrant (T%), and the speed were recorded by a video tracking system (Chengdu Taimeng Tech. Co. LTD, China).

2.4. Tissue preparation

Mice were sacrificed after MWM test. Tissue was prepared as previously described [22]. Mice were anesthetized and brains were immediately removed onto ice. The hippocampus was separated for Western blotting and PCR. For immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), mice were perfused intracardially with 4% paraformaldehyde in PBS. Then the brains were removed and post-fixed in the same medium at 4 °C overnight before being embedded in paraffin and cut into serial coronal sections at thickness of 5 µm. Sections were stored at -20 °C until further processing.

2.5. Human-specific DNA assay

Total DNA from hippocampus was obtained tissue samples using a Tissue DNA kit (Life feng Biotechnology Co., Shanghai, China), and quantified using a NanoDrop 2000C spectrophotometer (ThermoFisher, Wilmington, DE, USA). The presence of human-specific DNA in the hippocampus was detected by PCR amplification of a 479-bp fragment of a highly repetitive a-satellite DNA sequence of the centromere region of human chromosome 17, with primers: 5'-GGG ATA ATT TCA GCT GACTAA ACA G-3' and 5'-AAA CGT CCA CTT GCA GTT CTA G-3', as previously described [23].

2.6. Immunofluorescence

Sections were deparaffinized and hydrated before boiled in high pressure in 0.01 mol/L sodium citrate buffer (pH 6) for 2 min, cooled to room temperature, washed in PBS (3 × 5 min), incubated in permeabilized solution containing 0.1% Triton X-100 at room temperature for 10 min and blocked for 20 min with 5% bovine serum albumin, then incubated overnight at 4 °C with anti-human nuclei antibody (MAB1281, 1:500 dilution, Millipore, Billerica, MA, USA), anti-nestin (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA), anti III -tubulin (1:1000 dilution, Abcam, Cambridge, UK). Negative controls were incubated with PBS instead of primary antibody. The slices were rinsed in PBS for three times, and then incubated in Cy3/FITC-conjugated anti-mouse/rabbit anti IgG (1:1000, Molecular Probes, USA) at room temperature for 1 h, followed by DAPI (Biotech, China) staining for 10 min. Images were observed under microscopy (OLYMPUS, Japan) and captured by EC300 software. Positive cells in four random fields were counted and normalized as percentage of the total number of the cells.

2.7. TUNEL assay

To assess the apoptosis levels in the brain, we performed TUNEL staining using the In Situ Apoptosis Detection Kit (Jiancheng, China) according to manufacturer's protocol. Briefly, paraffin-embedded brain sections were deparaffinized, hydrated, permeabilized, incubated with proteinase K at room temperature for 10 min, and washed three times with PBS before antigen retrieval. Sections were then incubated with TdT enzyme in reaction buffer containing FITC-labeled dUTP at 37 °C for 60 min. Positive controls were incubated with

5000U of DNase at 37 °C for 10 min and then with TdT enzyme, whereas negative controls were incubated in reaction buffer without TdT enzyme. After three washes in PBS, sections were stained with DAPI for 10 min and washed three times in PBS, TUNEL-positive cells were detected and counted.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from hippocampus with Trizol reagent (Invitrogen, Grand Island, NY, USA), and mRNA was reverse transcribed with Prime ScriptTMRT-PCR Kit (TaKaRa, Japan) according to the instruction [24]. qRT-PCR was carried out on an ABI 7500 real-time PCR system (Thermo Fisher Scientific, Grand Island, NY, USA) using SYBR Premix Ex TaqTM (Perfect Real Time, Takara, Japan). Glycer-aldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal standard. Relative expression levels of different genes were calculated by the 2^{-C_t} method, and the histogram for fold comparison of different samples was generated by GraphPad Prism 5 (Roche, Switzerland). Experiments were carried out in triplicate three times. The sequences of primers for qRT-PCR are shown in Table 2.

2.9. Western blotting

Total protein of tissue samples were harvested and Western blotting was performed as previously described [25]. Equal quantities of each protein sample were resolved in SDS-PAGE and transferred to PVDF membranes (Sigma, USA). Membranes were blocked with 5% BSA in TBST for at least 1 h followed by incubation with primary antibodies to SIRT1 (1:1000, Cell Signaling Technology, USA), proliferating cell nuclear antigen (PCNA; 1:1000, Cell Signaling Technology, USA), p53 (1:200, Thermo fisher, USA), ac382-p53 (1:200, Thermo fisher, USA), p21 (1:100, Santa cruz, USA), and p16 (1:500, Cell Signaling Technology, USA), overnight at 4 °C and then with the respective horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:3000, Sango, China) for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence Western blotting detection reagents. Image J software was used to quantify the relative expression level of target proteins, which were normalized to each internal control (β -actin). Three independent experiments were carried out.

2.10. Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Analysis of variance (ANOVA) followed by LSD test was used to determine significance between different groups. $p < 0.05$ was considered statistically significant. Data shown were representative of at least three independent experiments. All data analysis was carried out with SPSS 18.0 statistical software.

3. Results

3.1. Resveratrol enhanced the engraftment of hUC-MSCs in the hippocampus

Previous studies demonstrated that intravenously transplanted MSCs were able to cross the blood- brain barrier (BBB) [26]. We found that hUC-MSCs could successfully migrate into the hippocampus of AD mouse 2 months after transplantation via tail vein. Moreover, in

vivo administration of resveratrol to AD mice led to more integration of MSCs in the brain ($p < 0.05$, Fig. 1A–D).

3.2. hUC-MSCs and resveratrol improved cognition of AD mice

We assessed effects of the treatments on the learning and memory in AD mice by Morris water maze by (MWM) test. All the enrolled mice completed the evaluation with improved learning ability over time during the six-days training phase as evidenced by reduced escape latencies (Fig. 2A). Compared to the WT mice, AD mice exhibited significantly longer escape latency ($p < 0.05$, Fig. 2A). When subjected to MSCs or/and RES, the escape latency was significantly reduced ($p < 0.05$, Fig. 2A). Moreover, results indicated that deteriorated learning and memory of AD mice was rescued in the treated groups, as evidenced by the swimming path during the acquisitional test (Fig. 2B), as well as significantly increased number of crosses and time spent in the target quadrant, and decreased escape latency ($p < 0.05$, Fig. 2C–E) in the probe test. The most effective improvement in MWM was detected in the group treated both with MSCs and resveratrol.

3.3. hUC-MSCs and resveratrol attenuated neural apoptosis in the hippocampus

Neural apoptosis is one of the major pathologies in AD. To determine the effects of hUC-MSCs and resveratrol on neural apoptosis, we performed TUNEL assay in this study. As shown in Fig. 3A and B, the number of apoptotic cells in the hippocampus was significantly increased in the AD group compared to the WT group, while reduced in the MSCs and RES group compared to that in the AD group ($p < 0.05$). Moreover, the combination of MSCs and resveratrol achieved better effect ($p < 0.05$).

3.4. hUC-MSCs and resveratrol enhanced neurogenesis in the hippocampus

The effect of resveratrol and hUC-MSCs on hippocampal neurogenesis was examined by immunostaining of nestin (Fig. 4A) and β III-tubulin (Fig. 4B), markers for neural precursors, in the dentate gyrus. Neurogenesis was significantly compromised in AD mice but improved by treatment with hUC-MSCs transplantation and resveratrol ($p < 0.05$).

3.5. hUC-MSCs and resveratrol enhanced neurotrophins in the hippocampus

In the molecular level, we found that gene expression of hippocampal BDNF, NGF, and NT-3 were reduced in the AD mice compared to the WT mice, however, MSCs and resveratrol treatment significantly upregulated the BDNF, NGF, and NT-3 ($p < 0.05$, Fig. 5). This may be the underlying mechanism of the enhanced neurogenesis and ameliorated neural apoptosis after MSCs and resveratrol treatment.

3.6. hUC-MSCs and resveratrol regulated SIRT1 signaling in the hippocampus

Western blotting revealed that the protein levels of SIRT1 and PCNA were reduced, whereas levels of p53, ac-p53, p21, and p16 were increased in the hippocampus of AD mice compared with those in the hippocampus of WT mice. These differences were reversed by the aforementioned treatments, and particularly resveratrol combined with hUC-MSCs transplantation ($p < 0.05$, Fig. 6A–B).

4. Discussion

AD is a leading cause of dementia and poses an ever-increasing threat to the aging population. However, to date, no effective treatment for AD has been identified. MSCs transplantation provides a promising therapeutic strategy for neurodegenerative disorders based on its multifunctional benefits in anti-aging, neural protection, and neuroregeneration [27]. In the present study, we confirmed that learning and memory were significantly improved by transplanting hUC-MSCs biweekly. The hippocampus was the hub for learning and memory, yet vulnerable to the effects of aging [28]. We found that neurogenesis was enhanced while apoptosis was ameliorated in the hippocampus of AD mice after hUC-MSCs transplantation. Moreover, the beneficial effects were enhanced by combining hUC-MSCs transplantation with resveratrol.

To explore the mechanisms by which MSCs plus resveratrol enhanced neural repair, we examined the number of hUC-MSCs presented in the brain of AD mice. Consistent with previous studies [29], we found the distribution of the MSCs to the hippocampus after intravenous injection was poor. However, resveratrol promoted the survival and engraftment of hUC-MSCs in the hippocampus of AD mice. In combination with resveratrol, hUC-MSCs were more resistant to pathologic environment of AD and were maintained longer in the brain. Moreover, the results of our in vitro study revealed that resveratrol enhanced the viability and proliferation, reduced the senescence and apoptosis of hUC-MSCs [30]. Taken together, we proposed that the fate of hUC-MSCs could be regulated by resveratrol both in vivo and in vitro.

Some researchers suggested that the number of transplanted MSCs within a specific brain area might not be fundamental for the neuroprotection [31]. The low engraftment of these cells, reported in previous studies, indicated that the paracrine effects of cells but not the number of cells was essential [32]. Mesenchymal stem cells have been demonstrated to secrete several factors, BDNF, bFGF, CNTF, FGF-2, GDNF, HGF, IGF, NGF, NT-3, VEGF, to regulate the microenvironment in the brain [33]. We explored neurotropic factors, BDNF, NGF, and NT-3, which were known to be important for neurogenesis [34], learning and memory [35]. We found that hUC-MSCs upregulated the expression of BDNF, NGF, and NT-3 in the hippocampus, and the effects were further enhanced when hUC-MSCs was combined with resveratrol, therefore, hippocampal neurogenesis and cognition were enhanced.

On the molecular level, we examined the expression of the age-related gene SIRT1 and its downstream substrates PCNA, p53, ac-p53, p21, and p16 in hippocampus of the mice. Two months after treatment, compared to levels in the AD group, the expression of SIRT1 and PCNA were increased, whereas, p53, ac-p53, p21, and p16 were decreased in the treated groups.

SIRT1 played a critical role in the management of AD pathogenesis. Recent reports indicated that SIRT1 protein level and deacetylase activity decreased with age [36]. Upregulation of SIRT1 promoted dendritic development and neurogenesis [37], enhanced hippocampal neuronal synaptic plasticity, facilitated learning and memory and inhibited

neurodegeneration [38]. However, SIRT1 expression varied in different regions of the brain and might be regulated by resveratrol in a tissue- and region-specific way [39]. The nucleocytoplasmic shuttling, which was important for the function of SIRT1 [40], should be examined in future studies. The increased level of PCNA, a cell cycle-related gene, was consistent with the enhanced neurogenesis in the hippocampus. p53 was a major deacetylative substrate of SIRT1. It was the cellular gatekeeper for growth and division, and controlled the death of neurons in hippocampus [41]. p53 was deposited in the brain of AD mice and patients, resulting in the degeneration [42] and apoptosis of neurons [43]. The increased level of SIRT1 accompanied with decreased level of ac-p53 and p53 was consistent with the reduced apoptosis in the hippocampus. p21 could be induced by p53, and increased levels of p21 also led to neural death [44]. The upregulation of p16 signified the aging of neurons [45]. In the present study, we demonstrated that hippocampal expression of SIRT1, PCNA, p53, ac-p53, p21, and p16 were regulated by the treatments, particularly hUC-MSCs transplantation combined with resveratrol. However, gene expression is regulated by many factors in a complex manner and contingent on the response of different cells and tissues to different protocols of treatment, thus, in-depth mechanism has to be further explored.

Resveratrol and hUC-MSCs are promising, but challenging for AD treatment. The poor bioavailability, solubility and stability of resveratrol remain major obstacles for its higher efficiency in vivo, in spite of the high bioactivity. In addition, resveratrol has many effects besides activation of SIRT1, the complex interaction and joint effects of resveratrol activated pathways warrant further exploration. In respect to hUC-MSCs transplantation, it is influenced by the passage, approach, dose, timing, and individual conditions. Optimizing the aforementioned factors may further improve therapeutic efficacy. Moreover, hUC-MSCs are not a panacea and cautions should be taken before translating these techniques into humans. Although no detectable immune rejection or tumor have been observed in any of the transplanted animals, long-term monitoring would further reveal the safety and efficacy of the transplanted hUC-MSCs in vivo.

5. Conclusion

Herein, we report that resveratrol facilitates the engraftment of hUC-MSCs in the hippocampus of AD mice. hUC-MSCs and resveratrol produce additive effects on neurotrophin secretion, neurogenesis, neural cell survival and apoptosis as well as SIRT1 signaling in the hippocampus of AD mice. Combining hUC-MSCs transplantation with resveratrol contribute to neuroprotection in AD mice than any individual treatment. Although the pathogenesis of AD is complex and the effects of resveratrol and hUC-MSCs are multi-faceted, requiring further exploration on the underlying molecular mechanism, our study suggests that hUC-MSCs transplantation combined with resveratrol treatment might be a promising therapy for AD and possibly other neurodegenerative disorders and that the benefits may be mediated, at least in part, by SIRT1 signaling.

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Fig. 1.

Resveratrol enhanced the engraftment of hUC-MSCs in the brain of AD mice. (A) Representative pictures of immunofluorescence with human nuclei antibody MAB1281 to identify the migration and survival of hUC-MSCs in the hippocampus of different groups. Scale bar = 50 μ m. (B) Quantitative analysis of MAB1281⁺ cells. Data were from three mice of each group and three slides of each mice. (C) PCR results of the human-specific DNA in the hippocampus of the mice. (D) Quantification of human-specific DNA in different groups. Data were from three mice of each group and expressed as mean values $\pm$ SEM. # $p < 0.05$ vs MSCs + RES.

Fig. 2. hUC-MSCs and resveratrol reversed cognitive impairments in AD mice. The escape latency during the training (A) and representative swimming path at day 6 (B) During the probe trial, the number of crosses (C), the percentage of time spent in target quadrant (T1%) (D), and the latency to find the location of the previous platform (E) were recorded for mice at the end of 5-month-old. The data were collected from fifteen mice in each group, and expressed as mean values $\pm$ SEM. * $p < 0.05$ vs AD, # $p < 0.05$ vs MSCs + RES.

Fig. 3. hUC-MSCs and resveratrol attenuated apoptosis in the hippocampus of AD mice. (A) Apoptotic neurons (green) in the hippocampus detected by TUNEL. Scale bar = 100 μ m. (B) The percentages of apoptotic cells in each group. Data were from three mice of each group and three slides of each mice and results were expressed as mean values $\pm$ SEM. * p < 0.05 vs AD, # p < 0.05 vs MSCs + RES. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. hUC-MSCs and resveratrol increased neurogenesis in the dentate gyrus of the hippocampus in AD mice. Representative immunofluorescent images of nestin⁺ cells (A) and β III-tubulin⁺ cells (B) in the dentate gyrus of hippocampus in each field. (C) nestin⁺ cell counts and β III-tubulin⁺ cell counts in the dentate gyrus of hippocampus in each field. Scale bar = 100 μ m. Data were from three mice of each group and three slides of each mice and results are expressed as mean values $\pm$ SEM. * p < 0.05 vs AD, # p < 0.05 vs MSCs + RES.

Fig. 5. qRT-PCR results of the relative expression of neurotrophic factors BDNF, NGF, and NT-3 in the hippocampus of the mice. Data were from three mice of each group and three independent experiments for each mice, and normalized to the corresponding expression of housekeeping gene GAPDH and quantified relative to that of the AD mice. * $p < 0.05$ vs AD, # $p < 0.05$ vs MSCs + RES.

Fig. 6. hUC-MSCs and resveratrol regulated SIRT1 signaling in the hippocampus of AD mice. **(A)** Representative images of Western blotting results. **(B)** Grey value analysis of Western blotting were from three mice of each group and three independent experiment for each mice. Relative expression of SIRT1, PCNA, p53, ac-p53, p21, and p16 in the hippocampus of mice from the six groups was normalized by the corresponding expression of β -actin and quantified relative to that in the AD group. Results were expressed as mean values $\pm$ SEM from three repeats. * $p < 0.05$ vs AD, # $p < 0.05$ vs MSCs + RES.

Table 1

Experimental groups and treatment.

Group	Treatment
WT	No treatment
AD	No treatment
MSCs	hUC-MSCs transplantation
RES	Gavage with resveratrol
MSCs + RES	hUC-MSCs transplantation and gavage with resveratrol

Table 2

Sequences of primers for qRT-PCR.

gene	sequence	bp
BDNF	5'-CATAGACAAAAGGCACTGGAAGTC-3' (F)	62
	5'-TAAGGGCCCGAACATACGAT-3' (R)	
NGF	5'-TACAGGCAGAACCGTACACAGATAG-3' (F)	64
	5'-CAGTGGGCTTCAGGGACAGA-3' (R)	
NT-3	5'-GGTGGGCGAGACTGAATGA-3' (F)	59
	5'-AGGGACGTCGACATGAAGAGAA-3' (R)	
GAPDH	5'-GGTGAAGGTCGGTGTGAAC-3' (F)	191
	5'-CCTTGACTGTGCCGTTGAA-3' (R)	