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Bisdemethoxycurcumin exerts a cell-protective effect *via* JAK2/STAT3 signaling in a rotenone-induced Parkinson's disease model *in vitro*

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Abstract

Introduction. Oxidative stress and cell apoptosis have both been suggested to be closely associated with the pathogenesis of Parkinson's disease (PD). Previously, bisdemethoxycurcumin (BDMC) has been shown to exhibit several desirable characteristics as a candidate neuroprotective agent, including antioxidant and anti-inflammatory activities in the nervous system. However, whether BDMC can exert cell-protective roles in an *in vitro* model of PD remains unknown.

Material and methods. SH-SY5Y cells were pretreated with BDMC, with or without AG490 and SI-201, for 30 min, followed by a co-incubation with rotenone for 24 h. Subsequently, a cell viability assay and western blotting was performed, and SOD and GSH activities were analyzed.

Results. The results revealed that the pretreatment with BDMC enhanced the cell survival, antioxidative stress capacity and the phosphorylation levels of JAK/STAT3 in SH-SY5Y cells treated with rotenone. However, following the incubation with AG490 and SI-201, inhibitors of the JAK/STAT3 signaling pathway, BDMC was unable to exert cell-protective roles in SH-SY5Y cells treated with rotenone.

Conclusions. In conclusion, the results suggested that BDMC may exert a cell-protective role in SH-SY5Y cells *in vitro* via JAK2/STAT3 signaling, thus suggesting the possible application of BDMC for the treatment of neurodegenerative diseases related to JAK2/STAT3 signaling. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 2, 127–134)*

Key words: oxidative stress; rotenone; SH-SY5Y cells; bisdemethoxycurcumin (BDMC); JAK/STAT3

Introduction

Parkinson's disease (PD), a common neurodegenerative disease [1], is mainly characterized by a substantial loss of dopaminergic neurons in the substantia nigra, which causes a reduction of striata dopamine, and promotes cognitive impairment and functional defects [2]. Both motor and non-motor symptoms are observed in patients with PD, who require assistance in performing all activities [3, 4]. It has been suggested that the excessive generation of reactive oxygen species (ROS), oxidative stress, neuroinflammation and mitochondrial dysfunction may all account for the loss of dopaminergic neurons and neuronal apoptosis [5–7]. However, the available current therapeutic strategies only provide symptomatic improvements due to the complex pathogenesis of PD. As a consequence,

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there remains an urgent requirement to determine the pathological mechanisms of PD to discover novel potential therapeutic targets to effectively treat PD.

Bisdemethoxycurcumin (BDMC), a natural derivative of curcumin, has demonstrated higher polarity, hydrophilicity and water solubility compared with the parent compound curcumin [8]. It was also reported to exert considerable antioxidant, anti-inflammatory and antiproliferative activities [9], and it demonstrated more effective pharmacological properties than curcumin [10], which suggested that it may be a more promising drug for clinical use [11].

Oxidative stress is considered to be the main cause of dopaminergic neuron degeneration in PD [12, 13]. Attempts to target signaling pathways that may be associated with the pathological process of PD have been made to counteract oxidative stress and cell apoptosis, of which one of the signaling pathways is the Janus-activated kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway. Briefly, the signaling pathway consists of ligands binding to the membrane receptor and inducing the formation of Janus-activated kinase 2 (JAK2) binding sites in the cytoplasm to phosphorylate JAK2. Subsequently, the exposure of the STAT3 anchor point leads to its phosphorylation and activation, resulting in the translocation of STAT3 from the cytoplasm to the nucleus, to promote the transcription and expression of target genes [11]. Phosphorylated STAT3 has been discovered to promote cell proliferation, anti-inflammatory properties and the inhibition of apoptosis [14], and it also exerted neurotrophic effects on newly generated neurons and synapses [15].

The aim of the present study was to investigate the effects and underlying mechanisms of BDMC in SH-SY5Y cells treated with rotenone, an agent used to mimic the behavioral and neuropathological conditions of PD. The results indicated that BDMC may exert a cell-protective role against rotenone-induced neurotoxicity in SH-SY5Y cells *via* JAK2/STAT3 signaling.

Materials and methods

BDMC. BDMC, which contains > 80% curcumin and > 94% curcuminoid content purity, was purchased from Sigma-Aldrich (St. Louis, MO, USA). For cell incubations, BDMC was dissolved in 0.2% DMSO.

Cell culture and treatments. SH-SY5Y cells were cultured as previously described [16]. It was previously reported that BDMC exerted protective effects against cytotoxicity in retinal pigment epithelial cells at a concentration of 15 μM [17]. Also, 100 nM rotenone induced neuronal loss, neuroinflammation, oxidative stress and NF-κB activity [18]. Therefore, the present study selected 15 μM BMDC (the protective concentration) and 100 nM rotenone (the neurotoxic concentration) for subsequent experiments. A total of 1×10^4 SH-SY5Y cells were seeded into 96-well cell culture plates (for the cell viability assay) or 24-well cell culture plates (for SOD, GSH and western blotting assays), and treated as follows: 1) Cells were pretreated with 15 μM BDMC for 30 min prior to a 24-h co-culture with 100 nM rotenone; 2) cells were pretreated with 10 μM AG490 and 15 μM BDMC for 30 min prior to a 24-h co-culture with rotenone; or 3) cells were pretreated with 100 nM S3I-201 and 15 μM BDMC for 30 min prior to a 24-h co-culture with rotenone. At the end of the treatment duration, a cell viability assay and western blotting were performed, and SOD and GSH activities were analyzed.

Cell viability assay. Cell viability assay was performed as previously described [19–21], with minor modifications. Briefly, at the indicated time points, SH-SY5Y cells were cultured in DMEM, supplemented with 10 μl 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl- 2-H-tetrazolium bromide (MTT; 500 μg/ml; cat. no. M1020; Beijing Solarbio Science & Technology, Co., Ltd., Shanghai, China) for 4 h. After aspirating the culture medium, 100 μl dimethyl sulfoxide (DMSO) was added to each well in the culture plates and the cells were incubated at 37°C for 30 min. Finally, the optical density was measured with a spectrophotometer at a wavelength of 410 nm.

Measurement of SOD activity. The WST-1 Cell Proliferation assay kit was used to detect SOD activity, according to the manufacturer's instructions (cat. no. A001-3-2; Jiancheng Biotech Ltd., Nanjing, China) [22, 23]. The xanthine-xanthine oxidase system was applied to produce superoxide ions, which can react with 2-(4-iodophenyl)-3-(4-nitrophenol-5-phenyltetrazolium chloride) to form a red formazan dye. The absorbance was determined at the wavelength of 550 nm. The protein concentration was determined using a BCA protein assay kit (QPBCA, Sigma-Aldrich; Merck KGaA, Saint Louis, MO, USA). The values were expressed as units/mg protein, with one unit of SOD defined as the amount of SOD inhibiting the rate of reaction by 50% at 25°C.

Measurement of GSH. Measurement of GSH content was performed according to a previously described method [24], with minor modifications. Briefly, SH-SY5Y cells in the culture medium were centrifuged at 500 × *g* for 10 min and washed with PBS twice. The collected SH-SY5Y cells were resuspended in protein removal reagent and vigorously vortexed. Subsequently, the samples were frozen rapidly and thawed with liquid nitrogen twice at 37°C, and then incubated at 4°C for 5 min. Following centrifugation at 10,000 × *g* for 10 min, the supernatants were collected. The

determination of GSH levels was performed using a GSH and GSSG assay kit (cat. no. S0053; Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's instructions.

Western blotting. Western blotting was performed as previously described [25–27], with minor modifications. Briefly, whole cell lysates were obtained using a RIPA lysis buffer mixture (Beijing Solarbio Science & Technology Co., Ltd.), supplemented with PMSF (1:200; Beijing Solarbio Science & Technology Co., Ltd.). The lysates were then combined with 25% LDS Sample buffer (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) and heated at 95°C for 15 min. Protein samples were separated via 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) for 3 h at 300 mA. The membranes were blocked with 5% nonfat dry milk or BSA dissolved in Tris-HCl saline buffer containing 0.1% Tween-20 (TBST; pH 7.4). Subsequently, the blots were incubated overnight at 4°C with the following antibodies: Rabbit anti-p-STAT3 (1:500; cat. no. S2690; Sigma-Aldrich; Merck KGaA) and rabbit anti-p-JAK2 (1:500; cat. no. SAB4300124; Sigma-Aldrich; Merck KGaA). Following the primary antibody incubation, the membranes were washed three times for 5 min each in TBST and incubated with HRP-conjugated goat anti-rabbit secondary antibodies (1:1,000; Boster Biological Technology, Wuhan, China) diluted in TBST for 1 h. The membranes were washed three times in TBST for 5 min each at room temperature and proteins bands were visualized with an enhanced chemiluminescence (ECL) solution (Bio-Rad Laboratories, Inc., Hercules, CA , USA). The expression levels were semi-quantified using ImageJ 5.0 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± SEM. Statistical comparisons between groups were performed using a Student's t test for independent samples on SPSS 18.0 software (SPSS, Inc., New York, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BDMC treatment enhances cell survival and the antioxidative stress ability in SH-SY5Y cells treated with rotenone

To investigate the effects of BDMC treatment on rotenone-induced SH-SY5Y cells, a cell viability assay was performed, and SOD and GSH activities were analyzed, following the pretreatment of cells with BDMC and the co-culture with rotenone.

The results of the cell viability assay revealed that compared with the control group, the cell survival rate was decreased in response to the treatment with 100 nM rotenone. Notably, compared with the rotenone-treated cells, BDMC treatment significantly increased the cell survival rate in a dose-dependent manner (Fig. 1A).

It was further discovered that compared with the control group, BDMC treatment did not increase the SOD levels (Fig. 1B), while the SOD levels were decreased in response to the treatment with rotenone. Moreover, compared with the rotenone-induced group, BDMC treatment significantly increased the SOD levels (Fig. 1B). A similar trend was observed for the GSH levels (Fig. 1C).

BDMC treatment enhances the phosphorylation levels of JAK/STAT3 in SH-SY5Y cells treated with rotenone

To investigate the effects of BDMC treatment on rotenone-induced SH-SY5Y cells, the phosphorylation levels of JAK2/STAT3 were evaluated following the pretreatment of cells with BDMC and the co-culture with rotenone.

The western blotting results revealed that compared with the control group (*i.e.* rotenone-treated cells), BDMC treatment did not upregulate the JAK2 and STAT3 phosphorylation levels. The JAK2 and STAT3 phosphorylation levels were downregulated in response to the treatment with rotenone. Moreover, compared with the rotenone-induced group, BDMC treatment significantly upregulated the JAK2 and STAT3 phosphorylation levels (Fig. 2A–C).

These results suggested that BDMC may inhibit the toxicity induced by rotenone in SH-SY5Y cells.

AG490, an inhibitor of the JAK/STAT3 signaling pathway, reverses the cell-protective effects of BDMC treatment in SH-SY5Y cells treated with rotenone

To investigate the effects of the JAK/STAT3 signaling pathway on the cell-protective role of BDMC in rotenone-induced SH-SY5Y cells, STAT3 phosphorylation levels, cell survival, and SOD and GSH activities were evaluated following the pretreatment with AG490 and BDMC, and the co-culture with rotenone.

The results of the cell viability assay demonstrated that BDMC treatment did not increase the cell survival of SH-SY5Y cells induced by rotenone following the inhibition of JAK/STAT3 signaling pathway (Fig. 3A).

In addition, it was observed that BDMC treatment did not increase the SOD levels in SH-SY5Y cells induced by rotenone following the inhibition of JAK/ /STAT3 signaling pathway (Fig. 3B). A similar trend for GSH levels was observed (Fig. 3C).

These results suggested that BDMC treatment may not inhibit the toxicity induced by rotenone in

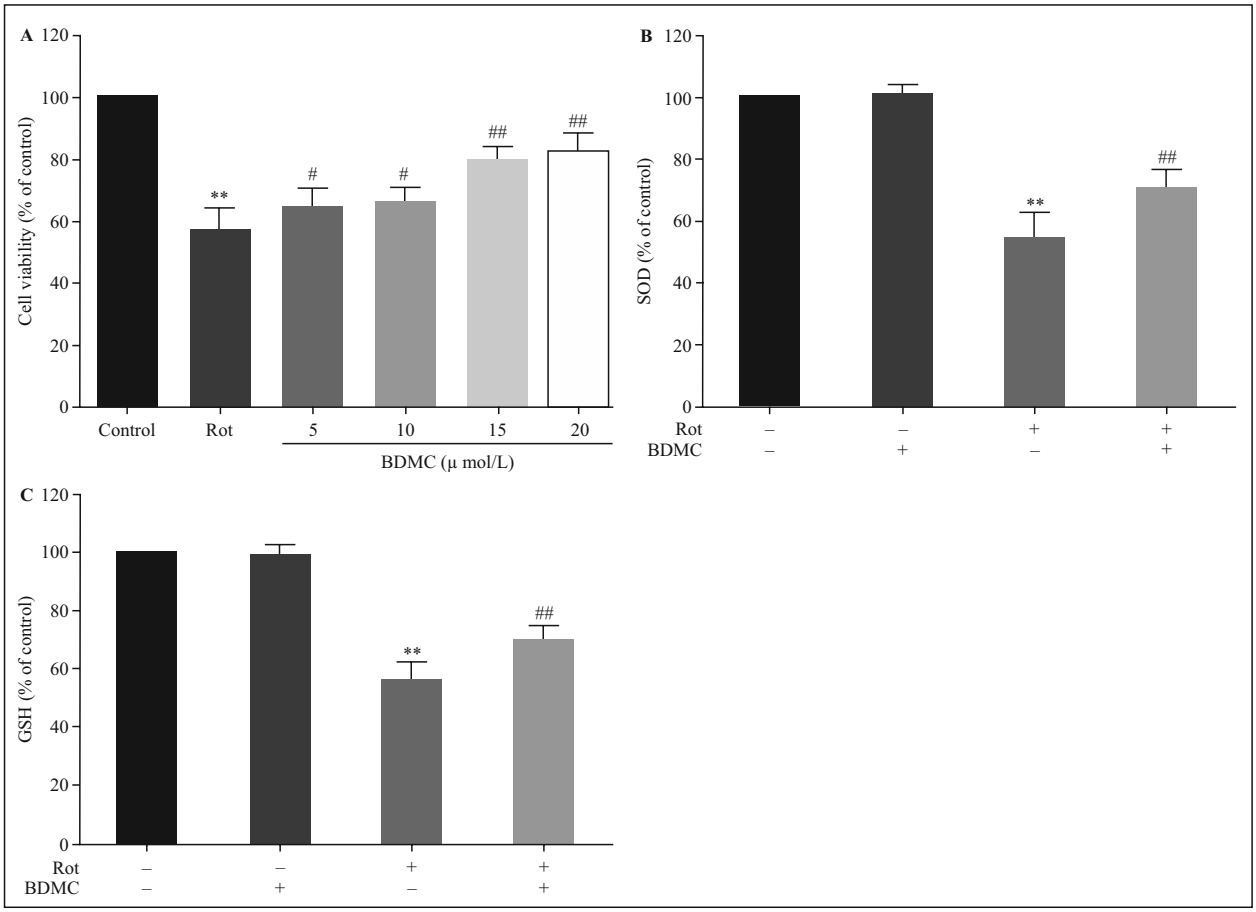

Figure 1. Determination of the effects of BDMC treatment on cell survival and oxidative stress in SH-SY5Y cells treated with rotenone. (**A**) A total of 1×10^4 SH-SY5Y cells were pretreated with BDMC (0, 5, 10, 15 or 20 μM) for 30 min prior to a 24-h co-culture with 100 nM rotenone, and the cell survival rate was calculated. A total of 1×10^4 SH-SY5Y cells were pretreated with 15 μM BDMC for 30 min prior to a 24-h co-culture with 100 nM rotenone, and (**B**) SOD and (**C**) GSH activities were measured. $^{**}P < 0.01$ *vs.* control; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ *vs.* rotenone-induced group from 5 independent experiments.

SH-SY5Y cells upon the inhibition of the JAK/STAT3 signaling pathway.

S3I-201, an inhibitor of the STAT3 signaling pathway, reverses the cell-protective effects of BDMC treatment in SH-SY5Y cells treated with rotenone

To investigate the effects of the JAK signaling pathway on the cell-protective role of BDMC in SH-SY5Y cells induced by rotenone, the phosphorylation levels of JAK, cell survival, and SOD and GSH activities were analyzed following the pretreatment of cells with S3I-201 and BDMC and the co-culture with rotenone.

The results of the cell viability assay revealed that BDMC treatment did not increase the cell survival of SH-SY5Y cells induced by rotenone following the inhibition of the JAK signaling pathway (Fig. 4A).

Furthermore, BDMC treatment did not increase the SOD levels in rotenone-induced SH-SY5Y cells following the inhibition of the JAK signaling pathway (Fig. 4B). A similar pattern was observed for GSH levels (Fig. 4C).

These results indicated that BDMC treatment may not inhibit the toxicity induced by rotenone in SH-SY5Y cells upon the inhibition of the JAK signaling pathway.

Discussion

In the present study, BDMC treatment was revealed to enhance the cell survival, antioxidative stress ability and the phosphorylation levels of JAK/STAT3 in SH-SY5Y cells treated with rotenone. Moreover, following the inhibition of the JAK/STAT3 signaling pathway, BDMC was unable to exert these cell-protective roles. Thus, the present study suggested that BDMC treatment may protect SH-SY5Y cells from the neurotoxicity induced by rotenone treatment via the JAK/STAT3 signaling pathway.

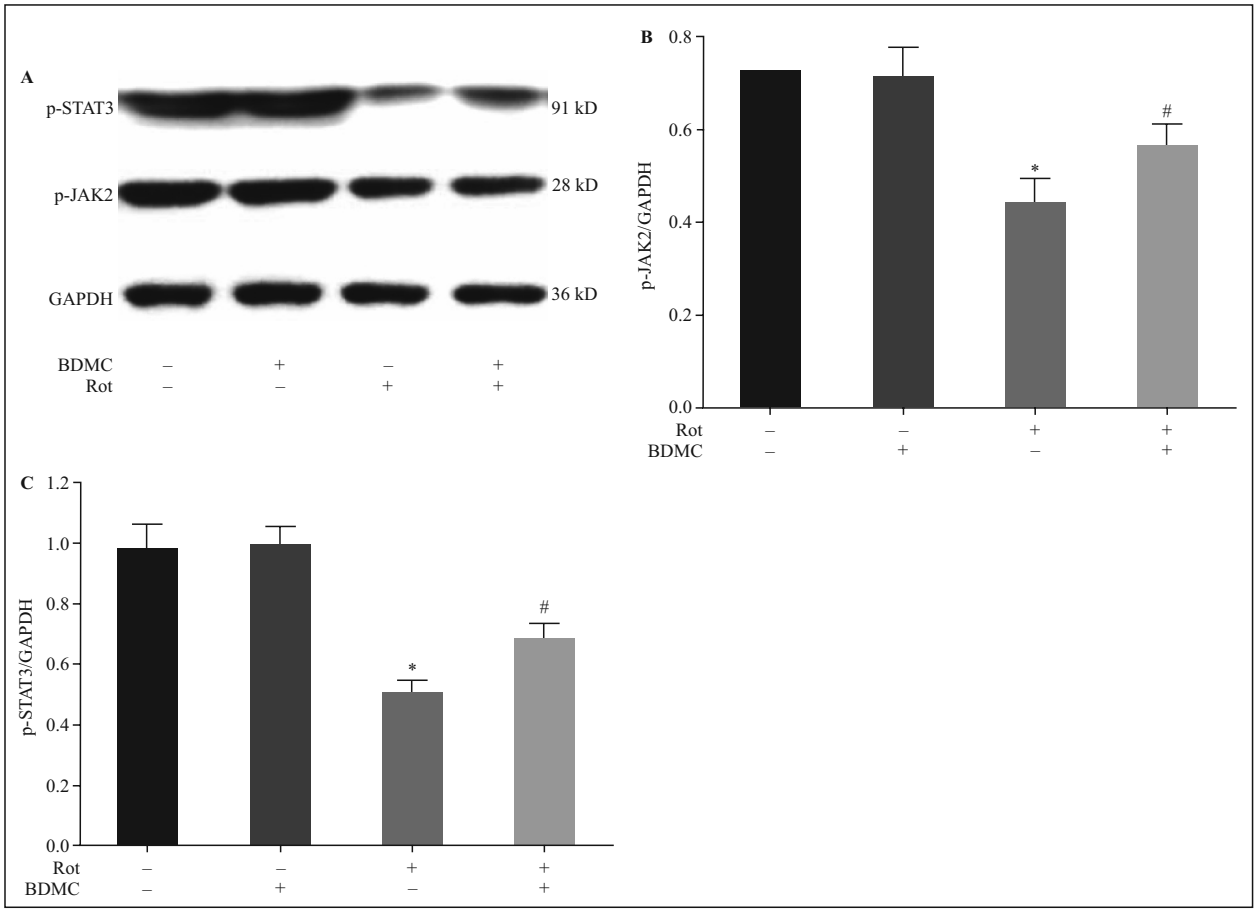

Figure 2. Determination of the effects of BDMC treatment on the JAK/STAT3 signaling pathway in SH-SY5Y cells treated with rotenone. (**A**) A total of 1×10^4 SH-SY5Y cells were pretreated with 15 μM BDMC for 30 min prior to a 24-h co-culture with 100 nM rotenone, and western blotting was performed. Representative western blots are shown. The phosphorylation levels of (**B**) JAK2 and (**C**) STAT3 were upregulated following the treatment with BDMC. $^*P < 0.05$ *vs*. control group; $^{\#}P < 0.05$ *vs*. rotenone-induced group from 5 independent experiments.

PD is a slow, progressive neurodegenerative disease that is associated with the degeneration of dopaminergic neurons [28]. The loss of dopamine accounts for numerous of the symptoms that accompany the disease, including motor dysfunction, mood alterations and cognitive impairment [29]. Due to difficulties in studying the progression of PD in humans, the treatment of the catecholaminergic neuroblastoma cell line SH-SY5Y with various neurotoxins has been widely used to construct a model closely mimicking PD *in vitro* [30–32]. In the present study, BDMC treatment was discovered to decrease the cell death of SH-SY5Y cells induced by rotenone.

Oxidative stress has been identified to account for the progressive death of the dopaminergic neurons [33]. The apoptotic death of nigrostriatal dopaminergic neurons is initiated by oxidative stress [34]. SOD and GSH are important antioxidants that scavenge oxygen free radicals, thus protecting against oxidative stress. In PD model rats, the levels of GSH, SOD and other free radical scavenging enzymes in the brain of the model rats were significantly reduced, further indicating that the rat model of PD demonstrated a reduced ability to scavenge oxygen free radicals; this led to the accumulation of ROS in the tissue cells, which in turn induced the continuous progression of PD [35]. In the present study, BDMC was discovered to protect against oxidative stress.

The activation of the JAK2/STAT3 signaling pathway serves as a protective mechanism against oxidative stress [36]. In the present study, the phosphorylation levels of the JAK2/STAT3 signaling pathway were identified to be upregulated in response to BDMC treatment, whereas following the inhibition of this pathway, BDMC was unable to exert its functional roles.

In conclusion, the findings of the present study suggested that BDMC treatment may alleviate the

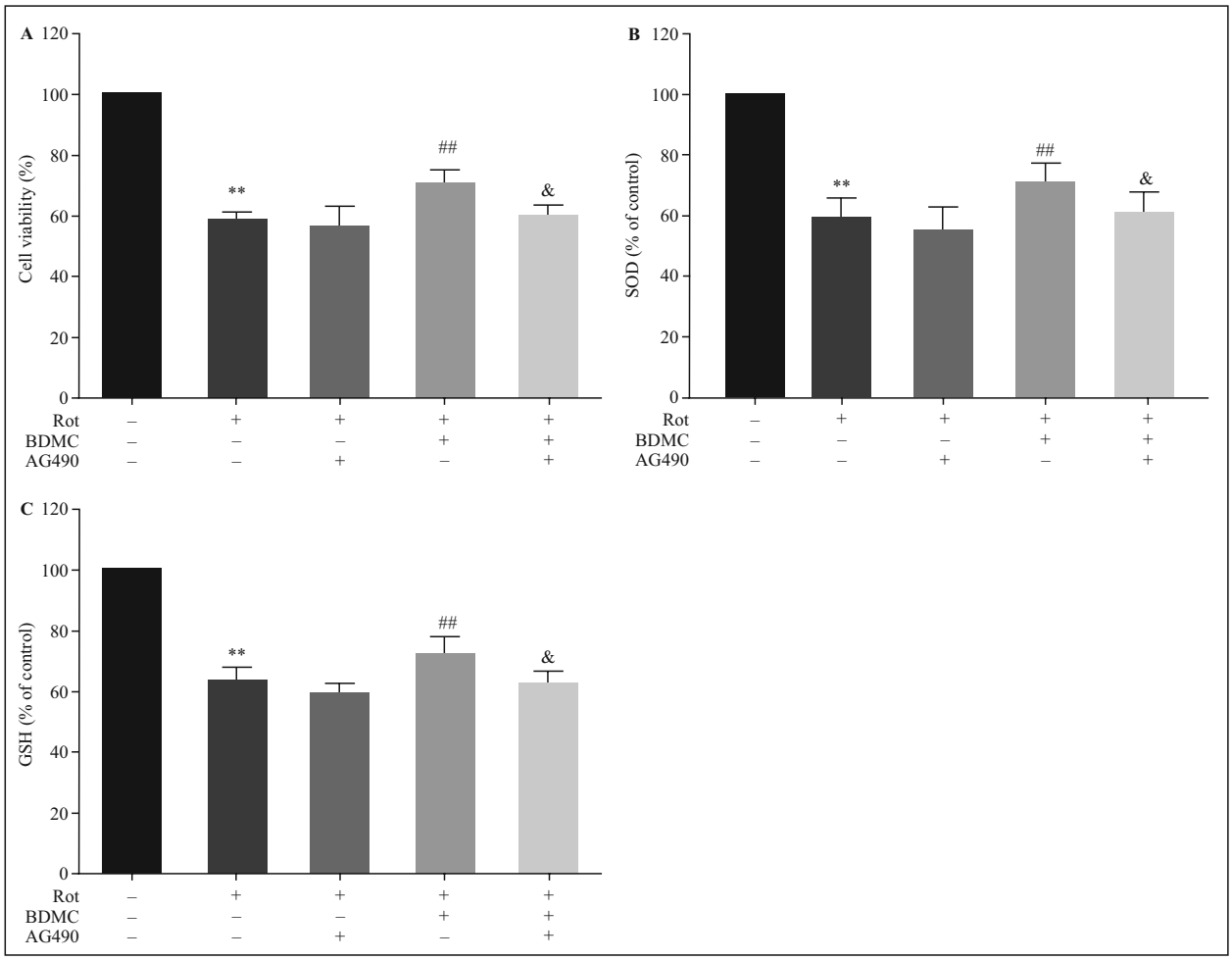

Figure 3. Determination of the effects of BDMC treatment on the cell survival and oxidative stress in SH-SY5Y cells treated with rotenone following the inhibition of the JAK/STAT3 signaling pathway. A total of 1×10^4 SH-SY5Y cells were co-pretreated with 10 μM AG490 (an inhibitor of the JAK/STAT3 signaling pathway) and 15 μM BDMC for 30 min prior to a 24-h co-culture with 100 nM rotenone. The (**A**) cell survival rate, (**B**) SOD activity and (**C**) GSH activity were not increased in response to the treatment with BDMC following the inhibition of the JAK/STAT3 signaling pathway. $^{**}P < 0.01$ *vs*. control group; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ *vs*. rotenone-induced group; $^{\&}P < 0.05$ *vs*. BDMC + rotenone-induced group from 5 independent experiments.

neurotoxicity of rotenone in SH-SY5Y cells by improving cell survival and exerting antioxidative stress properties. These effects were indicated to occur through the inhibition of the JAK/STAT3 signaling pathway, which may provide a foundation for BDMC treatment to be considered as a novel therapeutic strategy for the treatment of PD.

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Declaration of competing interest

All authors declare no competing interests.

Disclosure of conflict of interest

None.

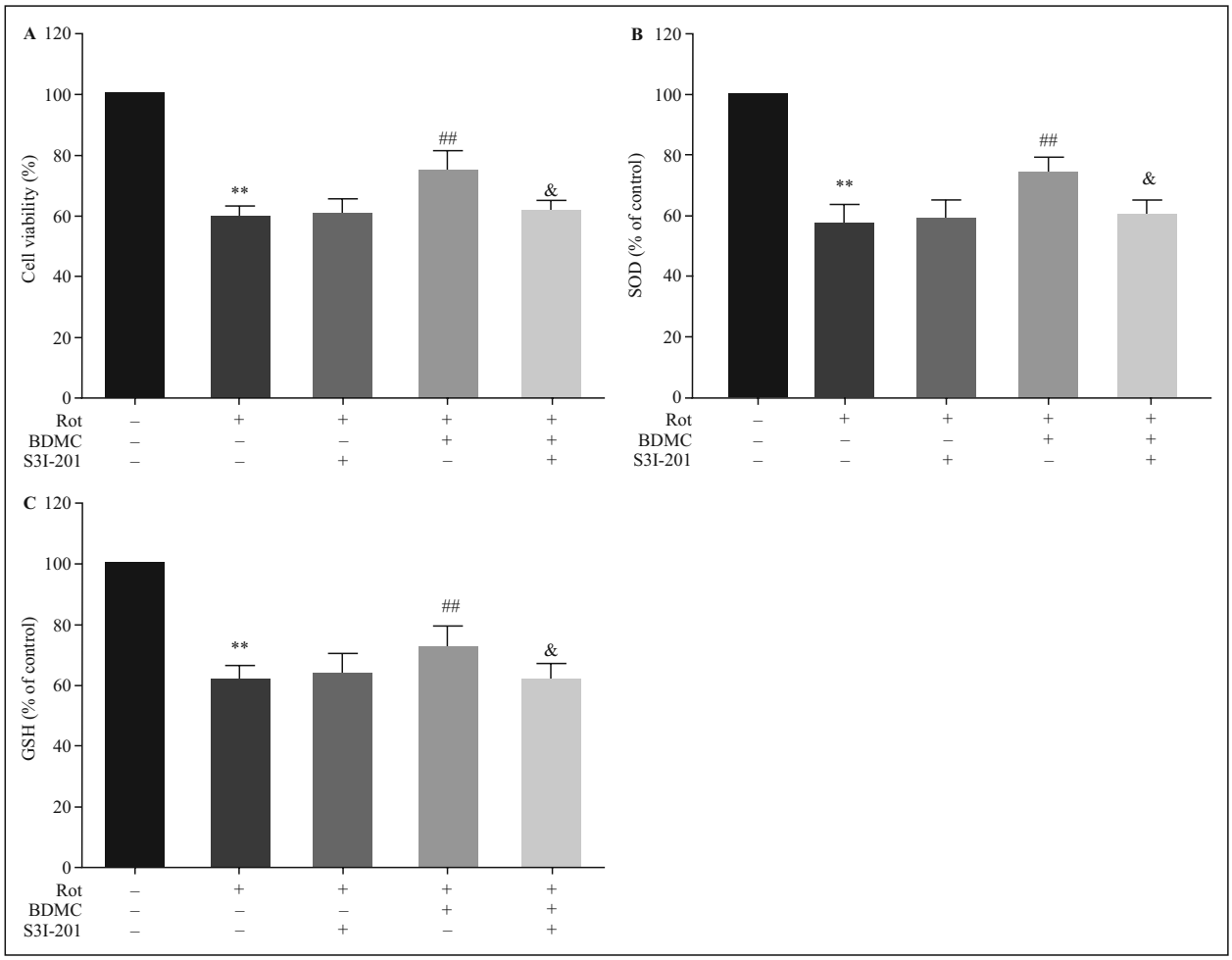

Figure 4. Determination of the effects of BDMC treatment on the cell survival and oxidative stress in SH-SY5Y cells treated with rotenone following the inhibition of the STAT3 signaling pathway. A total of 1×10^4 SH-SY5Y cells were co-pretreated with 100 nM S3I-201 (an inhibitor of the STAT3 signaling pathway) and 15 μM BDMC for 30 min prior to a 24-h co-culture with 100 nM rotenone. The (**A**) cell survival rate, (**B**) SOD activity and (**C**) GSH activity were not increased in response to the treatment with BDMC upon the inhibition of the STAT3 signaling pathway. $^{**}P < 0.01$ *vs*. control group; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ *vs*. rotenone-induced group; $^{\&}P < 0.05$ *vs*. BDMC + rotenone-induced group from 5 independent experiments.

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