

Schisandrin B alleviates LPS induced mitochondrial damage in C28I2 cells

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Abstract

Osteoarthritis is a common joint disease characterized by damage to the joint cartilage that occurs throughout the entire joint tissue. This damage primarily manifests as pain in the affected area. In clinical practice, medication is commonly used to relieve pain, but the treatment's effectiveness is poor and recurrent attacks are likely. Schisandrin B is the most abundant biphenylcyclohexene lignan found in the traditional Chinese medicine *Schisandra chinensis*, and it possesses various pharmacological effects. This study aims to investigate the protective effect of Schisandrin B on mitochondrial damage in osteoarthritis (C28I2 cells) under an inflammatory environment induced by LPS. Cell proliferation and activity, scratch tests, and LDH release tests are utilized to assess cell growth and migration ability. The immunofluorescence assay was used to detect the expression levels of proliferation and apoptosis proteins. The Western Blot assay was used to detect the expression levels of mitochondrial fusion and division proteins. The JC-1 assay was used to detect changes in mitochondrial membrane potential. The mitochondrial fluorescence probe assay was used to detect mitochondrial activity. Through research, it was found that Schisandrin B promotes the proliferation, growth, and migration of C28I2 cells, reduces apoptosis of C28I2 cells, balances mitochondrial fusion and division, stabilizes mitochondrial membrane potential, and promotes mitochondrial activity in an LPS-induced inflammatory environment.

Introduction

Osteoarthritis (OA) is the most prevalent degenerative musculoskeletal disease, primarily characterized by the deterioration of articular cartilage. It progressively affects the entire articular tissue, leading to mutations, fibrosis, fractures, and defects in the articular cartilage (Abramoff and Caldera. 2020; Molnar et al. 2021). It occurs in middle-aged and elderly individuals, with a higher prevalence in women than in men. The primary clinical manifestations include joint pain, stiffness, hypertrophy, limited activity, and gradually developing walking difficulties. These symptoms also contribute significantly to disability among the elderly (Nedunchezhiyan et al. 2022; Sacitharan 2019). The risk factors for osteoarthritis (OA) include obesity, lack of exercise, fatigue, and emotional factors. As the prevalence of these risk factors increases, the prevalence of OA also increases each year (Jiang 2022; Colletti and Cicero. 2021). Currently, certain clinical non-surgical interventions are utilized to provide temporary pain relief and enhance the functionality of osteoarthritis joints (Hawker and King. 2022; Cho et al. 2021). Nevertheless, thus far, no treatment has been able to restore damaged osteoarthritis tissue, leading patients with advanced osteoarthritis to inevitably undergo total joint replacement surgery. Osteoarthritis significantly impacts the quality of life for older individuals, and the long-term medical expenses also impose a substantial financial burden on families. Consequently, discovering an effective medication for treating osteoarthritis is of utmost importance.

Mitochondria are dynamic organelles that constantly mix with surrounding mitochondria and divide into daughter mitochondria. They serve as energy factories for cell life activities (Annesley and Fisher. 2019; Krako et al. 2021; Al Amir and Thierry. 2023). The main functions of mitochondria include producing ATP, coordinating the production of reactive oxygen species (ROS), regulating cell metabolism, proliferation,

aging, and apoptosis (Andrieux et al. 2021; Bader and Winklhofer 2020; Averbek and Rodriguez 2021). Mitochondrial dysfunction is a hallmark of joint aging and osteoarthritis (Liu et al. 2022). Recent studies have shown that the occurrence and development of osteoarthritis are related to an imbalance of mitochondrial homeostasis, including increased oxidative stress, excessive ROS production, dysmetabolism of cartilage matrix, increased inflammatory response, and increased chondrocyte death (Chan 2020; Luo et al. 2020; Adebayo et al. 2021). The metabolic pathology associated with mitochondrial dysfunction is also a significant factor in osteoarthritis. Regulating mitochondrial function offers a promising diagnostic and therapeutic strategy for OA.

Schisandrin B (Sch B, 1) is a plentiful bioactive dibenzocyclooctadiene derivative found in *Schisandrin fruit* (Yang et al. 2022). Research has demonstrated that Sch B plays a role in various biological activities in different disease models, such as liver protection, anti-oxidation, anti-aging, anti-tumor, and anti-anxiety (Nasser et al. 2020; Li et al. 2020; Wu et al. 2019). Furthermore, previous studies have confirmed the association between Sch B and mitochondrial function. Sch B can significantly enhance mitochondrial antioxidant capacity and respiratory function, maintain mitochondrial structural integrity, and reduce ROS production in the brain of experimental animals (Shi et al. 2021; Shi et al. 2022). The enhancement of mitochondrial function by Sch B has been demonstrated to stimulate the production of mitochondrial ATP in the brains of aging mice. This activation can trigger anti-apoptotic, anti-inflammatory, and anti-stress responses, while also regulating the aggregation of proteins linked to neurodegenerative diseases. Consequently, it can prevent or delay the onset of nerve damage (Hu et al. 2019; Qi et al. 2020).

Currently, there is limited literature on the effectiveness of Sch B in treating osteoarthritis (OA). This study focuses on C28I2 cells as the subject of research and uses lipopolysaccharide (LPS) stimulation to induce inflammation, simulating an OA disease model. The study aims to investigate the therapeutic impact of Sch B on C28I2 cells in an inflammatory environment and its relationship with mitochondrial function. The findings of this study offer theoretical support for the treatment of osteoarthritis and establish a basis for the clinical application of Sch B.

Materials and methods

Cell culture and treatment

C28I2 purchased from ATCC were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc), 100U/ml penicillin and 100 µg/ml Streptomycin at 37°C in a humidified cell incubator containing 5% CO₂. Sch B(1) powder (purity > 98%, SS8230) was purchased from Solarbio and dissolved in DMSO, LPS (HY-D1056, MCE) dissolved in DMSO.

Cell Proliferation Assay

In order to study the stimulation concentrations of LPS and Sch B, the kit 8 counting method (Beyotime, C0038) was used. Transfer C28I2 cells (1×10^4 cells/well, 100µl) were transferred to a 96 well plate, and

after overnight adhesion, 100µl of different concentrations of LPS and Sch B solution were added to each well to stimulate for 24 h. After the cultivation time is over, add 10µl to each well. After incubation with CCK8 solution for 1–4 h, measure the absorbance at 450 nm using an enzyme-linked immunosorbent assay.

Scratch experiment

Plant C28I2 cells in 6 wells, inoculate 5×10^5 cells per well, and incubate overnight in a 37 °C incubator. The next day, use 10µl gun head draws a line along the ruler. The gun head should be vertical and not tilted. Then wash the cells three times with PBS, remove the scraped cells, and add LPS and Sch B prepared in serum free culture medium. Continue cultivation in the incubator and take photos. All experiments have been conducted at least 3 times.

Cytotoxicity detection experiment of lactate dehydrogenase

To measure the protective effect of Sch B on C28I2 cells, we used the LDH assay kit (Beyotime, C0016) to detect the cytotoxicity of C28I2 cells. In short, add 60µl LDH detection working solution to each hole separately. Incubate at room temperature in dark for 30 min, and then measure the absorbance at 490 nm using an enzyme-linked immunosorbent assay. Cytotoxicity or mortality rate (%) = $(\text{absorbance of treated sample} - \text{absorbance of sample control well}) / (\text{absorbance of maximum enzyme activity of cells} - \text{absorbance of sample control well}) \times 100\%$

Immunofluorescence staining

Inoculate C28I2 cells onto 12 well plate slides and treat with LPS and Sch B for 24 h. Place the climbing slide on a glass slide, fix it with 4% paraformaldehyde for 15 min, and wash it in PBS three times, each time for 3 min; Then add 0.5% Triton X-100 (prepared with PBS) and let it penetrate at room temperature for 20 min. Soak the slide in PBS three times for 3 min each time. Suck off excess PBS, add goat serum dropwise, and seal at room temperature for 30 min; After the closure time, add the first antibody and incubate at 4 °C overnight. On the second day, add abundant secondary antibodies and incubate at room temperature for 1 h. Soak the slide in PBST three times, each time for 3 min. Use absorbent paper to absorb excess liquid, add DAPI dropwise, stain for 5 min, and observe under a confocal microscope.

Western Blot Analysis

For protein extraction, the cell homogenate is lysed in RIPA lysate containing a mixture of protease inhibitors. Collect the supernatant and centrifuge at 12000 rpm for 10 min at 4 °C. Quantitative analysis of protein extracts using the BCA assay kit (Beyotime, China). Protein samples were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes (Bio Rad, Hercules, CA, USA). Seal the membrane with 5% skim milk at room temperature for 1 h, incubate the first antibody overnight at 4 °C, wash with PBST three times for 5 min each time, and then incubate the second antibody at room temperature for 1 h. The gel imaging and analysis system (Tanon science and Technology Co., Ltd.) was used to detect protein bands. primary antibodies: β -actin (1:1000, 4ab020185, 4A Biotech), MFN1 (1:1000,

66776-1-Ig, proteintech), MFN2 (1:1000, 67487-1-Ig, proteintech), DRP1 (1:1000, 12957-1-AP, proteintech), OPA1 (1:1000, 66776-1-Ig, 27733-1-AP).

Measurement of mitochondrial membrane potential (MMP)

To measure mitochondrial MMP, we used the JC-1 kit (Beyotime, C2006) for detection. Cells incubate in JC-1 working solution (5 μ M) for 0.5 h. After complete washing with PBS, replace with fresh culture medium and use a fluorescence detection microscope (Leica DMIL LED, Germany).

Mitochondrial Green Fluorescence Probe Experiment

Follow the instructions for using the kit (Beyotime, C1048), add Mito-Tracker Green working solution to each well and incubate with cells at 37 °C for 45 min. Remove Mito-Tracker Green staining solution and add fresh cell culture medium incubated at 37 °C. Subsequently, observation was conducted using a laser confocal microscope.

Statistical analysis

SPSS 19.0 was used for data analysis, and GraphPad primer 8 was used for image generation. Data that conforms to a normal distribution is represented by an average $\pm$ standard value. Two sets of data were compared using T-test, and the above two sets of data were compared using one-way ANOVA. $P < 0.05$ indicates a statistically significant difference.

Results and Discussion

Determine the stimulation concentration of Sch B and LPS

Firstly, the CCK8 experiment selected the concentration of Sch B to stimulate C28I2 cells. The results are shown in Fig. 1A. When the concentration of Sch B is 20ng/ml, the protective effect on C28I2 cells is strongest. When the concentration of Sch B is 40 μ g/ml, it slightly inhibits cell activity, with a cell activity of about 70%. Similarly, the CCK8 experiment showed that the activity of C28I2 cells was 50–60% when the LPS concentration was 10 μ g/ml. Therefore, in the subsequent experiment, the stimulation concentration of Sch B was selected as 20ng/ml, and the LPS concentration was 10 μ g/ml.

Sch B can enhance the activity and migration ability of C28I2 cells

Next, we will explore the effect of Sch B on the activity of C28I2 cells. The CCK8 experiment showed (Fig. 2A) that the LPS stimulation group reduced the activity of C28I2 cells, and Sch B stimulation can alleviate cell damage and protect cells. The LDH experiment showed the same experimental results (Fig. 2B). After LPS stimulation, LDH release increased, while Sch B reduced LDH release. Further testing of the migration ability of C28I2 cells was conducted, and the results were shown in Fig. 2C. Sch B improved the inhibitory effect of LPS on the migration ability of C28I2 cells. Statistical analysis showed significance (Fig. 2D).

Sch B can improve LPS induced apoptosis in C28I2 cells

Further explore the effect of Sch B on apoptosis of C28I2 cells through immunofluorescence experiments. Firstly, the fluorescence expression level of apoptotic protein Caspase3 was detected. The results showed that (Fig. 3A) compared to the ctrl group, the green fluorescence of Caspase 3 was enhanced after LPS stimulation, while it was weakened after Sch B stimulation. Figure 3B shows the fluorescence expression level of proliferative protein PCNA. Compared with the ctrl group, the green fluorescence of PCNA in the LPS group decreased, while the green fluorescence of PCNA increased after stimulation with Sch B. This indicates that Sch B can improve LPS induced apoptosis in C28I2 cells.

Sch B can improve LPS induced mitochondrial dynamic imbalance in C28I2 cells

Further exploring the impact of Sch B on mitochondrial function, Fig. 4A detected the expression levels of mitochondrial outer membrane fusion proteins MFN1, MFN2, inner membrane fusion protein OPA1, and mitochondrial mitotic protein DRP1 through WB experiments. The results showed that the expression of mitochondrial fusion proteins MFN1, MFN2, and OPA1 decreased in the LPS group, while the expression of mitochondrial mitotic protein DRP1 increased. After stimulation with Sch B, the expression of mitochondrial fusion protein increased and the expression of mitotic protein decreased. It is suggested that Sch B can improve the inhibition of LPS induced mitochondrial functional damage.

Sch B improves LPS induced mitochondrial activity and membrane potential damage in C28I2 cells

Finally, mitochondrial activity and membrane potential changes were detected. Firstly, JC-1 experiment was used to detect changes in mitochondrial membrane potential. The results are shown in Fig. 5A. Compared with the control group, the LPS group showed a decrease in mitochondrial Aggregates red fluorescence and an increase in Monomers green fluorescence, while Sch B improved this phenomenon by enhancing mitochondrial red fluorescence and decreasing green fluorescence. It is suggested that Sch B can alleviate LPS induced mitochondrial membrane damage. Figure 5B detected mitochondrial activity through the mitochondrial green fluorescence probe experiment, and the results showed that compared with the control group, the LPS group showed a significant decrease in mitochondrial green fluorescence. After the action of Sch B, mitochondrial green fluorescence was enhanced. This indicates that Sch B has a protective effect on mitochondria.

OA is a chronic disease and the most common musculoskeletal disease. It can lead to cartilage degeneration, synovial inflammation, cartilage remodeling, and osteophyte formation (Hunter DJ and Bierma S. 2019; Rice et al. 2020). It is the most common cause of chronic joint pain in the elderly population and the leading cause of disability. Although OA is primarily known as a joint disease with cartilage damage, its symptoms are diverse and its pathogenesis is complex, affecting all tissues within the joint and other organs throughout the body (Li et al. 2021; Abdel-Aziz et al.2021). Currently, there is no

effective method to treat OA, and joint replacement is the only option for patients with total joint dysfunction

Research has shown that defective organelles affect the survival and metabolism of chondrocytes. Additionally, dysfunctional mitochondria lead to excessive production of reactive oxygen species, which induces damage to protein and DNA stability. This damage hinders the synthesis and metabolism of cell matrix in chondrocytes (Blanco and Fernández. 2020; Zhong et al.2022). The pathways involved in mitochondrial dysfunction-mediated osteoarthritis (OA) process include oxidative stress, chondrocyte apoptosis, cartilage matrix calcification, autophagy, impaired chondrocyte synthesis, and cytokine-induced inflammation (Coryell et al. 2021). Furthermore, the mitochondrial activity of chondrocytes in patients with osteoarthritis decreases compared to healthy individuals of the same age.

Sch B is the most important component in *Schisandra chinensis*, and studies have confirmed that it has various effects such as anticancer, anti-inflammatory, liver protection, and antibacterial properties (Ding et al.2018). Previous studies have also shown that Sch B can block the activation of NLRP3 and IL-1 β , thereby inhibiting inflammation (Chen et al.2021). However, there is limited literature on the effects of Sch B on mitochondrial function. In this study, C28I2 cells were chosen as the research subject, and LPS stimulation was used to induce an inflammatory environment in order to observe the protective effect of Sch B on C28I2 cells in such an environment. Firstly, the concentrations of Sch B and LPS for stimulation were determined through the CCK8 experiment, which confirmed that Sch B can enhance the activity of C28I2 cells in inflammatory environments and reduce LDH release. Next, it was discovered that Sch B can decrease apoptosis induced by LPS in C28I2 cells. Further investigation into its mechanism revealed that Sch B can regulate the equilibrium between mitochondrial fusion and division, enhance mitochondrial activity, and decrease mitochondrial membrane damage. This study determined that Sch B can enhance the mitochondrial function of C28I2 cells in an inflammatory environment, reduce apoptosis in C28I2 cells, and provide protection for these cells.

In summary, this study found that under inflammatory conditions, Sch B can enhance the survival and activity of C28I2 cells at the cellular level by improving mitochondrial function and reducing cell apoptosis. This provides a theoretical foundation for further research on the treatment of OA using Sch B. Additionally, as a widely used traditional Chinese medicine with various functions, Sch B offers an experimental basis for its functional research.

Declarations

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Author Contributions

FH and WJH conducted experiments; FH wrote the manuscript; HMX designed the study and revised the manuscript. All authors have read and approved the final version of the manuscript.

Data Availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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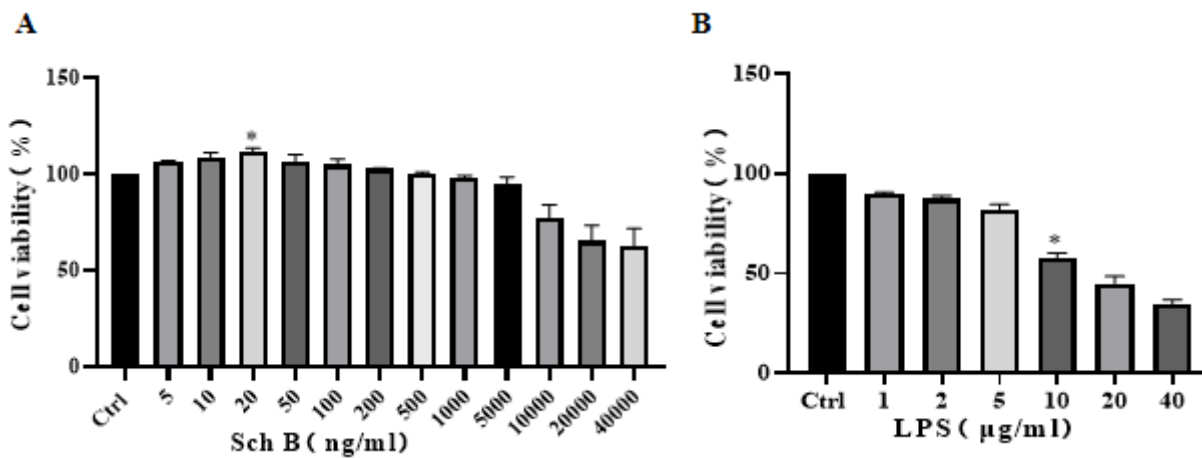


Figure 1

Determine the stimulation concentration of Sch B(1) and LPS in C2812 cells. (A) Select the concentration of Sch B for the CCK-8 assay; (B) Select the concentration of LPS for the CCK-8 assay. Data are presented as mean±SD of three independent experiments.

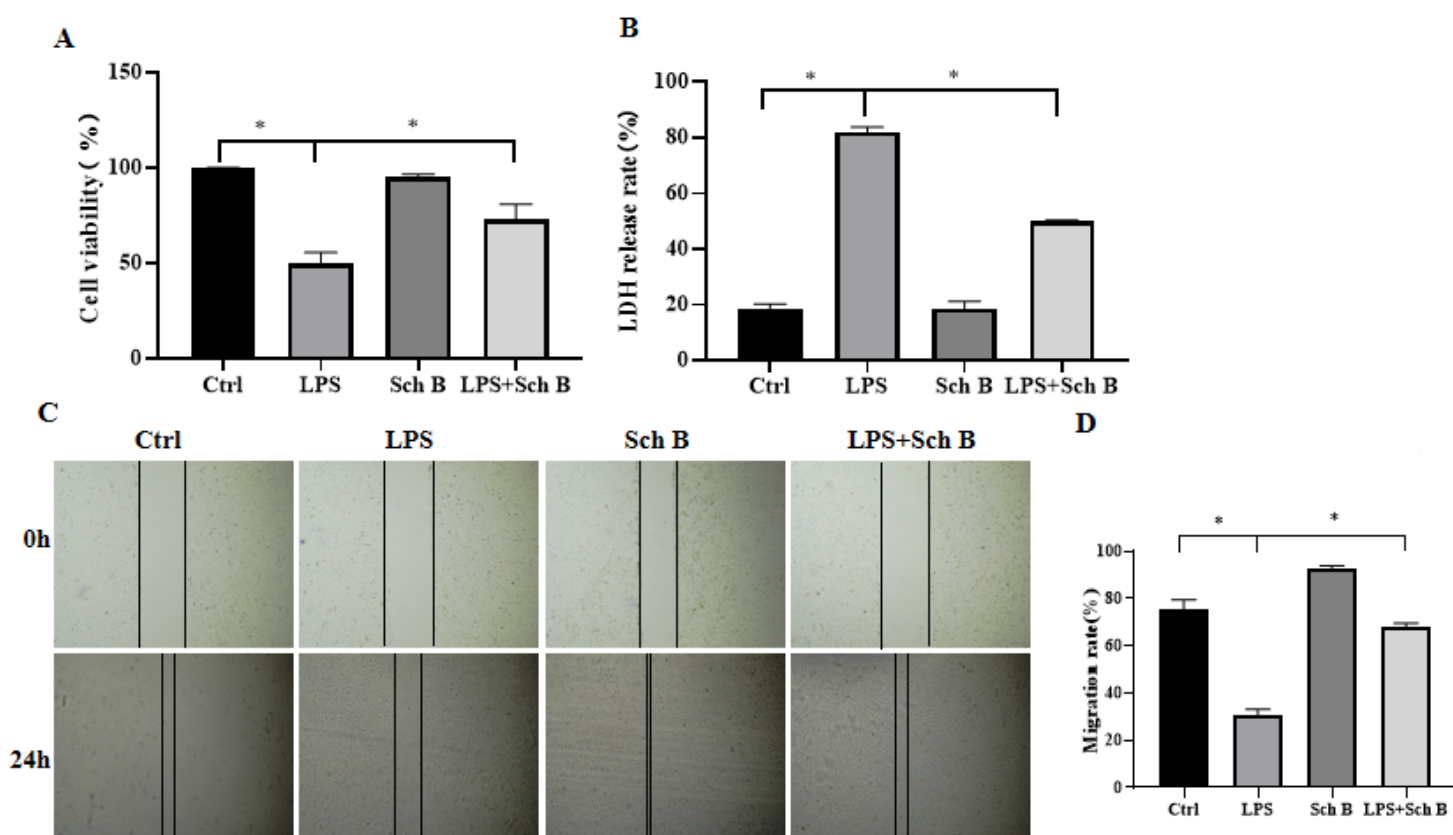


Figure 2

Sch B can enhance the activity and migration ability of C2812 cells. (A) Cells were incubated with sch B and LPS for 24 h, and cell viability was determined by the CCK-8 assay. (B) Incubate cells with Sch B and LPS for 24 h, and perform an LDH release experiment to determine LDH release. (C) C2812 cells were

stimulated with Sch B and LPS for 24 h, and the migration ability was detected through a scratch test. (D) Statistical analysis. Data are presented as mean±SD of three independent experiments.

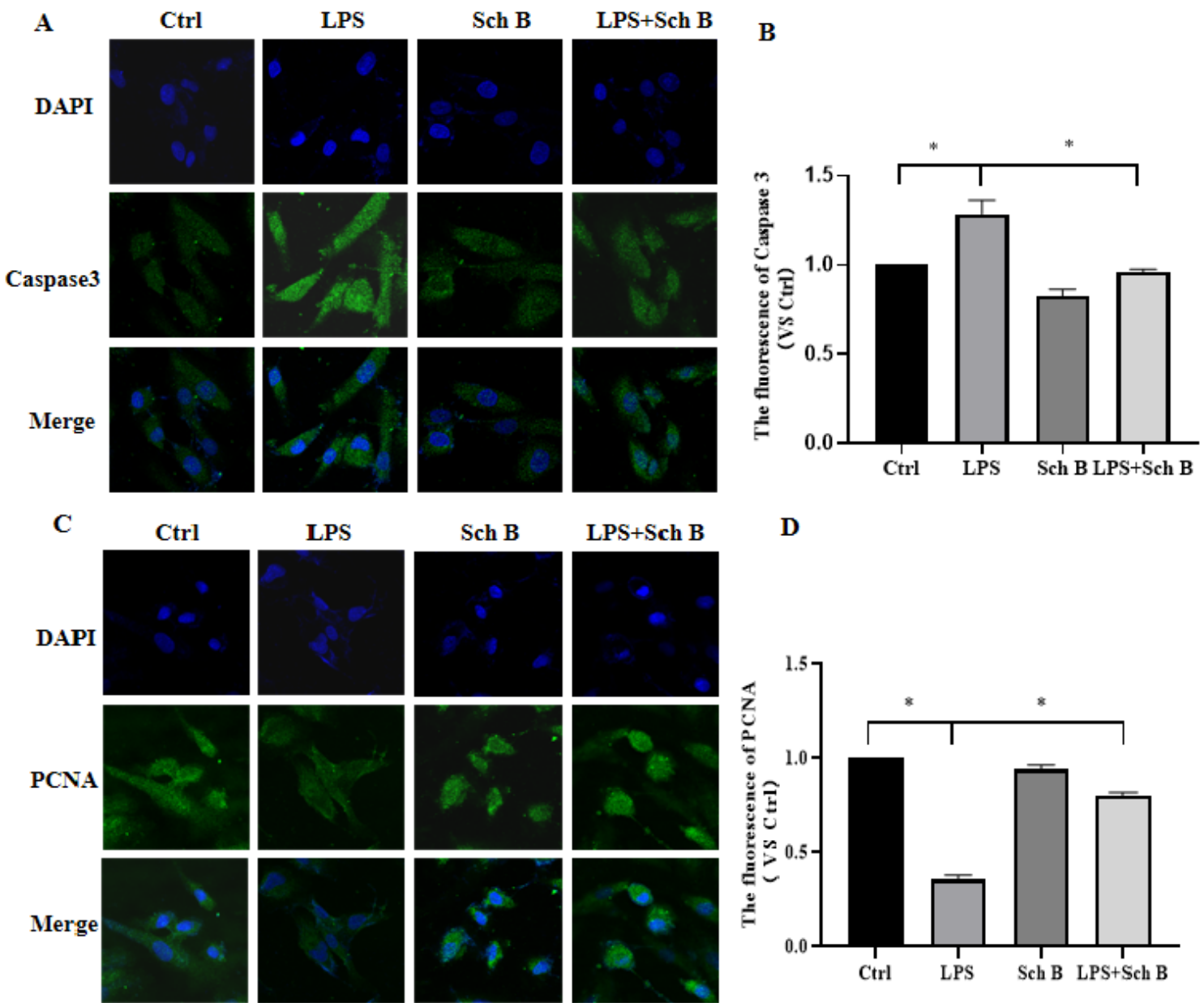


Figure 3

Sch B can enhance the activity and migration ability of C28I2 cells. (A, C) Incubate cells with Sch B and LPS for 24 h, and perform immunofluorescence detection of the expression levels of Caspase3 and PCNA. (B, D) Statistical analysis. Diferent data are expressed as the mean±SD of three independent experiments.

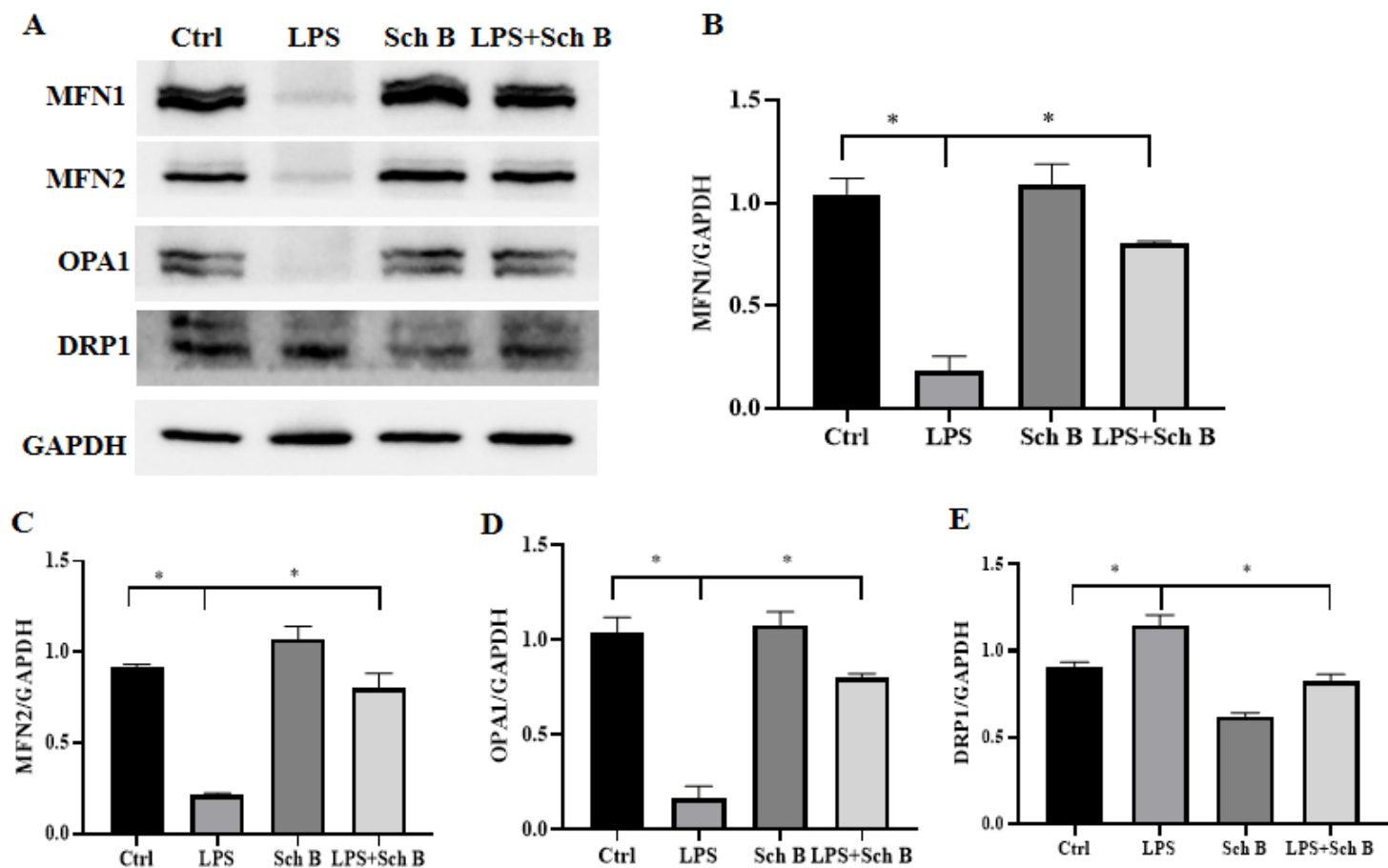


Figure 4

Sch B can improve LPS induced mitochondrial dynamic imbalance in C2812 cells (A) Incubate cells with Sch B and LPS for 24 h, WB detection of mitochondrial fusion and division protein expression levels.(B-E) Statistical analysis.Diferent data are expressed as the mean±SD of three independent experiments.

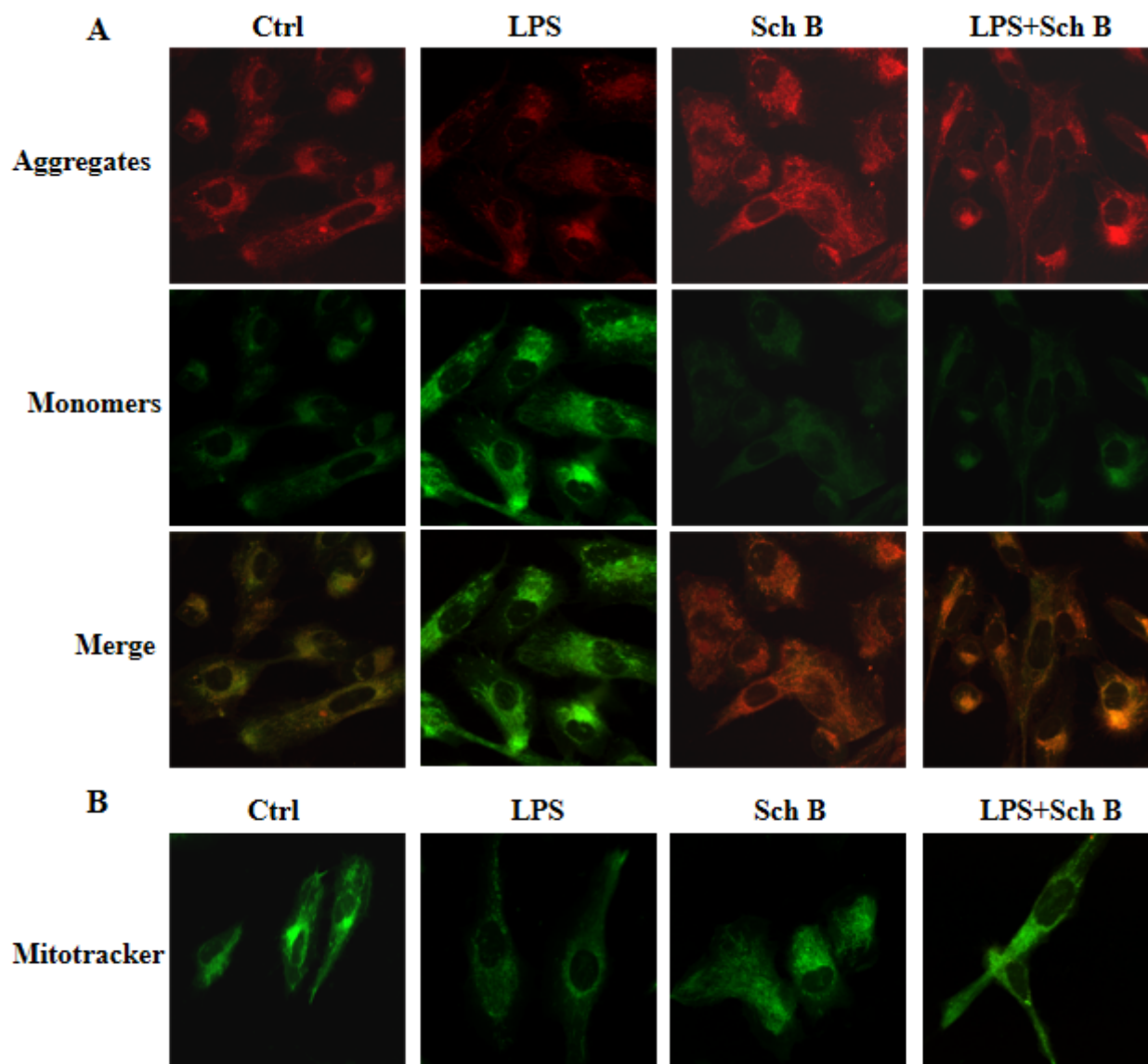


Figure 5

Sch B improves LPS induced mitochondrial activity and membrane potential damage in C28I2 cells. (A) Incubate cells with Sch B and LPS for 24 h, JC-1 reagent kit for detecting changes in mitochondrial membrane potential. (B) Incubate cells with Sch B and LPS for 24 h, mitochondrial fluorescence probe assay for detecting mitochondrial activity.

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