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Research Article

Neuroprotection of TSG Against Mechanical Trauma Injury Through an Anti-inflammatory Mechanism in Human Neuroblastoma SH-SY5Y Cells

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Abstract

Background and Objective: Mechanical trauma injury is caused by some external force which does harm to the vasculature, tissues and neighboring neuronal cells. This injury is a serious insult to neuronal cells which may release lactate dehydrogenase as the characteristics of cell damage. The release of inflammatory cytokines in injury cells is a normal immune response but the over expression of some pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor- α are detrimental to wound recovery. Suppression of pro-inflammatory cytokines is beneficial to alleviate mechanical trauma injury-induced cell damage. The present study aims to establish the mechanical trauma injury model *in vitro* and investigate the protective effect of 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside on this model and its mechanism.

Materials and Methods: The SH-SY5Y cells were used to establish the mechanical trauma injury model *in vitro* by scratching out the monolayer and generating an area devoid of cells. Then, the extent of cell damage of the model was measured by lactate dehydrogenase content determination and 12 h was confirmed as the key time point to explore 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside concentration given. The cell viability was measured by cell counting kit-8 to determine the optimal concentration of drug administration. The extent of cell damage was detected by immunofluorescence analysis to observe whether 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside can protect the integrity of the cell structure. TUNEL staining was used to detect whether it can decrease cell apoptosis. Finally, Tested the inflammatory cytokine levels (interleukin-6, interleukin-10 and tumor necrosis factor- α) by enzyme-linked immunosorbent assays, reverse transcription-polymerase chain reaction and western blotting to clarify the mechanism of cytoprotection. Data were assessed by the SPSS version 13.0. **Results:** The 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside increased viability of SH-SY5Y cells and the migrative ability, protected the integrity of cell structure, reduced apoptosis, decreased pro-inflammatory cytokine levels (interleukin-6 and tumor necrosis factor- α) and increased anti-inflammatory cytokine level (interleukin-10) in mechanical trauma injury-induced SH-SY5Y cell model. **Conclusion:** These studies demonstrate that 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside relieves the mechanical trauma injury-induced damage in SH-SY5Y cells by attenuating the levels of inflammatory responses. This might help us to further understand the pharmacological role of 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside in anti-inflammation and neuroprotection in the neural cells.

Key words: 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside, pro-inflammatory cytokines, anti-inflammatory cytokines, neuroprotection, inflammation, interleukin-6, interleukin-10, tumor necrosis factor- α , SH-SY5Y cells, mechanical trauma injury

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The 2,3,5,4'-tetrahydroxystilbene-2-O-glucoside (TSG) is one of the major active components of rhizome extract of *Polygonum multiflorum* thunb¹ which has been widely used as a traditional Chinese medicinal herb. The TSG has a similar polyphenolic structure to resveratrol and also has been reported to possess anti-inflammatory effects²⁻⁴. It may act on experimental colitis by inhibiting production of inflammatory mediators and attenuating the inflammatory responses⁵⁻⁸. It is shown that TSG protect against cerebral ischemia/reperfusion injury². Besides, TSG was demonstrated to attenuate neuroinflammation through the inhibition of microglial activation. These findings give further support to the neuroprotective role of TSG in inhibiting apoptosis⁹.

Mechanical Trauma Injury (MTI) is caused by some external force which does harm to the vasculature, tissues and neighboring neuronal cells. The MTI in cell lines is an *in vitro* insult mimicking traumatic brain injury¹⁰. For injured cells, the release of inflammatory cytokines is a normal immune response. The over expression of some pro-inflammatory cytokines such as interleukin (IL-6) and tumor necrosis factor (TNF- α) can induce cell apoptosis and death¹¹. While, some other anti-inflammatory cytokines such as IL-10 are beneficial to wound healing¹¹. Inflammation and apoptosis are closely related, inflammation can cause apoptosis^{12,13} while, apoptosis may develop to cell death.

Neuroblastoma (NB) as originally described¹⁴ by Virchow in 1863 is an embryonal tumor of the autonomic nervous system and originating from neural-crest tissues^{15,16}. It is generally known that neuroblastoma cells represent an early stage in neuronal development where the cells are pluripotent and retain the capabilities for expressing multiple neural crest-derived phenotypes^{17,18}. The purpose of this study is to establish the mechanical trauma injury model for neurons then to study the influence of the release of inflammatory cytokines induced by injury on damage and non-damage cells. However, some inflammatory cells such as microglia^{19,20} and astrocytes^{21,22} are unable to replace neurons and they can secrete inflammatory cytokines which may exist disturbance on the release of inflammatory cytokines after injury in this study. So, didn't choose these cells for study. Moreover, SH-SY5Y cells have a structure similar to neurons such as the axons, dendrites and perikaryon²². Also, SH-SY5Y cells had been used as an inflammatory cell model in other studies²³⁻²⁶ just like Song *et al.*¹³ used MTI-induced inflammatory SH-SY5Y cell model to evaluate the anti-inflammation of arctigenin.

In another study, Zhang *et al.*²⁷ used H89-induced SH-SY5Y cell model to replace the neurons. In this study, choose SH-SY5Y as the cell model replacing neurons to study the neuroprotection of TSG against MTI-induced cell damage²⁸.

The mortality of MTI is particularly high. The recovery of nerve function is so unsatisfactory that it brings unfavourable impact on patients' lives after injury without death²⁹. Nerve cells are non-renewable so that nerve function can not recover^{30,31}. Then, the scientists pay attention to cell transplantation and to promote endogenous neuronal renewal study but the application of cell transplantation is limited because of ethics and low survival rate after cell transplantation and the poor effect of endogenous neuronal renewal promotion. The secondary injury is the main factor that affects the repair after injury. At present, there is no effective treatment for MTI. The purpose of this study is to protect the injury and normal cells via reducing the secondary injury inflammatory responses thus, reducing the neuron death.

The TSG-mediated neuroprotective effects have been well demonstrated. The TSG exhibits a significant neuroprotection against ischemic brain injury *in vitro* and *in vivo*². However, the neuroprotective actions of TSG on MTI cells are unknown³². Therefore, this study investigated whether²⁵ TSG had the anti-inflammatory and neuroprotective effects on MTI-induced inflammation in the neuronal cell model, SH-SY5Y³³ by measuring the ratio of dead cells, production of pro-inflammatory cytokines (TNF- α and IL-6) and anti-inflammatory cytokines (IL-10)³⁴.

MATERIALS AND METHODS

Preparation of TSG: The TSG (C₂₀H₂₂O₉, 406.39 Da, structure shown in Fig. 1a, 98% purity) was purchased from the National Pufuide for the Control of Pharmaceutical and Biological Products (141101; Chengdu, China).

Cell culture: The SH-SY5Y cells human neuroblastoma-derived cell line, possessing neuron-like characteristic³⁵ was obtained from Capital Medical University (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, v/v) supplemented with 10% Fetal Bovine Serum (FBS) and 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin³³ (1% P/S; all from Gibco Invitrogen corporation, New York, NY, USA) at 37°C in humidified 5% CO₂ to 95% air³⁵. The cells were passaged every 3 days until 75% confluence was achieved³⁶ (Fig. 1b).

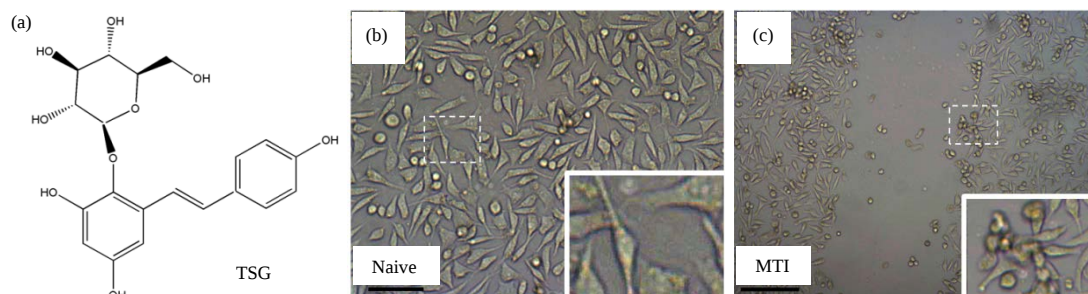


Fig. 1(a-c): Chemical structure of TSG and MTI model (a) Chemical structure of TSG, (b) SH-SY5Y cells and (c) MTI model *in vitro*, the portion enclosed by the dotted line was enlarged in the lower right corner of the image, scale bar: (b) 50 μm and (c) 100 μm

Preparation of *in vitro* MTI cell model: The SH-SY5Y cells have a structure similar to neurons such as the axons, dendrites and perikaryon²³. After MTI, the structure of SH-SY5Y cells will be destroyed just like the loss of axons and dendrites and the breakage of perikaryon. So, chose SH-SY5Y cells as the cell model replacing neurons to perform this study. The SH-SY5Y cells (5×10^5 cells mL^{-1}) were seeded in 12-well plates. After 24 h, the cells were scratched with a 10 μL pipette tip (axygen) according to a preset grid³⁷ (Fig. 1c) by scratching out the monolayer and generating an area devoid of cells. In this study, did not change the culture medium after wounding to keep the medium conditioned with cell debris and factors released from the detached cells¹³. The MTI cells were put into the incubator and draw 100 μL supernatant every time at 0, 0.5, 1, 3, 6, 12, 24, 36 and 48 h, respectively without changing the medium. The supernatant was collected for the measurement of lactate dehydrogenase (LDH) levels which is an indicator of plasma membrane integrity.

LDH assay: The LDH is a stable cytoplasmic enzyme present in all cells and it is rapidly released into the cell culture supernatant upon damage of the plasma membrane¹⁸ after scratch injury³⁸. Thus, assess the MTI model by detecting LDH concentration in the supernatant, using LDH Cytotoxicity assay kit according to the supplier's recommendation (Bi Yuntian Biological Technology Institution, Shanghai, China)³⁹.

Cell viability assay: In order to find the optimal concentration of TSG, SH-SY5Y cells were seeded into 96-well plates at 37°C in a humidified air incubator with 5% CO_2 . Then, the cells were separated into three main groups, naive, MTI and TSG+scratch groups, respectively. For TSG+scratch group, the cells were pretreated with different concentrations of TSG for 12 h then scratched immediately as described above and were

incubated for another 12 h after scratched. For MTI group, the cells were scratched as the same time as TSG+scratch group. For the naive group, the cells were incubated with culture medium for 24 h. The cell viability was assessed by Cell counting kit-8 (CCK-8) (Dojindo, Japan) assay. Approximately, 10 μL , CCK-8 was added into each well and incubated²⁵ at 37°C for 4 h. Then, OD value was read at 450 nm using a Bio-Rad enzyme-linked immunosorbent assays (ELISA) microplate reader (Bio-Rad laboratories, CA, USA)⁴⁰.

Setting of TSG concentrations: 0.1 $\mu\text{mol L}^{-1}$ (0.04 $\mu\text{g mL}^{-1}$), 1 $\mu\text{mol L}^{-1}$ (0.4 $\mu\text{g mL}^{-1}$), 10 $\mu\text{mol L}^{-1}$ (4 $\mu\text{g mL}^{-1}$), 50 $\mu\text{mol L}^{-1}$ (20 $\mu\text{g mL}^{-1}$), 100 $\mu\text{mol L}^{-1}$ (40 $\mu\text{g mL}^{-1}$) and 150 $\mu\text{mol L}^{-1}$ (60 $\mu\text{g mL}^{-1}$).

Immunofluorescent staining: The SH-SY5Y cells in 96-well plates were fixed in 4% paraformaldehyde for 30 min followed by washing 2 times with PBS and blocked in PBS containing 0.1% (W/V) triton X-100 for 30 min at room temperature⁴¹. Cells were incubated at 4°C overnight with antibodies against the following proteins: Doublecortin (DCX), neuronal nuclei (NEUN) (All at 1:150; Stem Cell Technologies, Vancouver, Canada) and incubated at 4°C overnight. After washing off the primary antibody, cells were incubated with CyTM3-conjugated goat anti-rabbit IgG (1:200) (all secondary antibodies from Jackson Immuno Research Lab, West Grove, PA, USA) and incubated at room temperature for 1 h and counter stained with 4'6-diamidino-2-phenylindole (DAPI) followed by 3 times PBS washes. Cells were mounted with anti-fade aqueous mounting medium (Vector Laboratories, Burlingame, CA, USA) and viewed using the Nikon Eclipse E800 microscope. Image J (NIH; Bethesda; MD USA) was used for quantitative analysis³⁶.

TUNEL staining: Cell death was measured by TUNEL staining as previously described⁴². The SH-SY5Y cells cultured on cover slips were fixed with fresh 4% paraformaldehyde in 0.1 M of PBS for 15 min at room temperature then, permeabilized with

0.1% of triton X-100 and 0.1% sodium citrate in PBS for 5 min on ice. The TUNEL staining was performed using the Fluorescence *in situ* Cell Death Detection kit (Roche, Chicago, IL, USA) according to the manufacturer's instructions. The cover slips were then mounted on glass slides to be observed under a fluorescence microscope. The percentage of cell death was determined by the ratio of the number of TUNEL-positive cells over the total of 100 cells in one count⁹.

IL-6, IL-10 and TNF- α assay: The SH-SY5Y cells (2×10^5 cells mL⁻¹) were seeded into 4 of 12-well plates after digested at 37°C in a humidified air incubator with 5% CO₂. Given TSG of the selected concentration in previous studies after 12 h, incubated for additional 12 h then scratched as mentioned above and the supernatant was collected for the measurement of IL-6, TNF- α and IL-10 levels²⁸ by ELISA⁴³ according to the instruction for the kit (R and D Systems, Minneapolis, MN, USA) supplied by the manufacturer.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

analysis: The SH-SY5Y cells were treated as mentioned above. Then, cells were harvested and the total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. Reverse transcribed to cDNA was carried out using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania)⁴¹. The PCR reaction (35 cycles) was carried out using a Dream Taq Green PCR master mix kit (Thermo Scientific). Quantitative RT-PCR was performed using the following forward and reverse primer sets designed using Premier Biosoft 5 (Palo Alto, CA, USA): TNF- α , 5'-ATA AGA GCA AGG CAG TGG AG-3' and 5'-TCC AGC AGA CTC AAT ACA CA-3'; IL-6, 5'-AGC CAG AGT CCT TCA GAG AG-3' and 5'-TCC TTA GCC ACT CCT TCT GT-3'; IL-10, 5'-TTC TCA TTC CTG CTT GTG GC-3' and 5'-ATC TGA GTG TGA GGG TCT GG-3' and β -actin (Control), 5'-GGG AAA TCG TGC GTG ACA T-3' and 5'-TCA GGA GGA GCA ATG ATC TTG-3'. Products were resolved by 1.5% agarose gel electrophoresis with ethidium bromide staining. The mRNA levels of TNF- α , IL-6 and IL-10 were detected at 3 dpi. Quantitative analysis was performed using a Tanon 4100 Gel Imaging System (Tanon Science and Technology Co., Shanghai, China).

Western Blotting (WB) analysis: Proteins were extracted from SH-SY5Y cells after 12 h of TSG treatment with a Ready Prep Protein Extraction kit according to the manufacturer's instructions (R and D, CA, USA). Equal amounts of protein (50 μ g) were loaded onto 10% SDS-PAGE gels and transferred onto polyvinylidenedifluoride membranes. The membranes

were blocked for 1 h with a blocking buffer containing 5% BSA in tris-buffered saline solution and tween 20 (10 mM tris-HCl, 150 mM NaCl and 0.05% tween 20 and TBS-T). The membranes were incubated overnight at 4°C with different primary antibodies diluted in the same blocking buffer. Next, the membranes were incubated with the HRP-conjugated secondary antibody for 1 h at room temperature and visualized by quantitative chemiluminescence using ECL western blotting detection reagents (Millipore, USA)⁴⁴. The signal intensity was quantified using Image J. The following antibodies were used: Primary rabbit anti-IL-6, TNF- α and IL-10 antibodies (1:1500, Abcam, Cambridge, MA, USA) and the secondary HRP-conjugated goat anti-rabbit IgG antibody (1:2000, Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL, USA). To control for loading, the blots were stripped and reprobed with rabbit anti- β -actin (1:2000; Abcam, Cambridge, MA, USA). Quantitative analysis was performed using a Tanon-4200SF Gel Imaging System (Tanon Science and Technology Co., Shanghai, China).

Statistical analysis: Data were expressed as Mean $\pm$ Standard Deviation (SD). Significance was assessed by one-way analysis of variance with the SPSS version 13.0 (SPSS, Chicago, IL, USA) and *post hoc* multiple comparisons were carried out with the Student-Newman-Keuls test. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

LDH leakage presented time-dependent tendency in MTI

cell model: The LDH as an intracellular marker enzyme has a very stable biological and chemical property. When the cell membrane permeability changed, LDH release will sharply increase. So, the amount of LDH in the cytosol is always taken as an indicator for cell damage and cell membrane permeability. Therefore, LDH leakage rate reveal the extent of cell damage³⁸. The MTI can destroy cell membrane thus, this study observed LDH concentration increased with time and reached the peak value at 12 h (Fig. 2). So, this study took 12 h as the key time point to explore TSG concentration given.

TSG increased SH-SY5Y cell viability of TSG treatment

group: According to previous test and studies, choose 12 h after scratched as the best time point to detect the optimal concentrations of TSG administrated. Figure 3 shows that the highest viability point of different concentrations of TSG treatment groups was 10 μ mol L⁻¹. So, select 10 μ mol L⁻¹ as the final concentration to evaluate the cell viability.

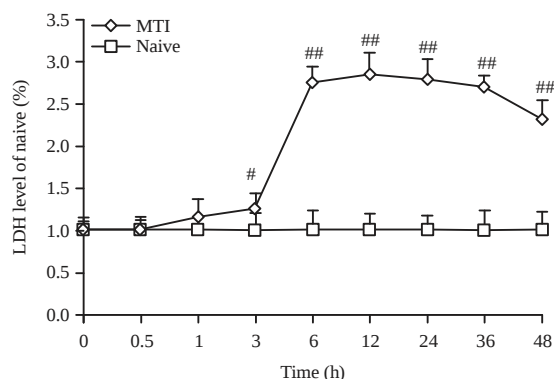


Fig. 2: LDH ratio of scratch group, the horizontal axis represents time point, the vertical coordinate represents the LDH ratio of scratch group vs naive group, data are presented as Mean $\pm$ SD, n = 6 per group and ##p<0.01 vs naive

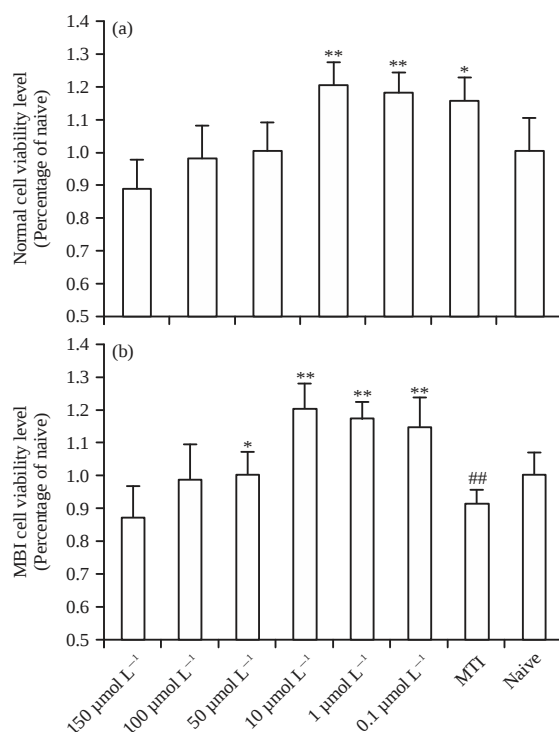


Fig. 3(a-b): Cell viability of various concentrations of TSG to normal and scratched cells, the horizontal axis represents different concentrations, the vertical coordinate represents the cell viability ratio of TSG treatment group vs naive group, (a) Viability of various concentrations of ARC to normal cells by CCK-8 and (b) Viability of various concentrations of TSG to scratch injury cells by CCK-8, data are presented as Mean $\pm$ SD, n = 6 per group, ##p<0.01 vs naive and *p<0.05 and **p<0.01 vs MTI

TSG treatment reduced cell damage and enhanced the migrative ability (Wound healing):

Since SH-SY5Y cells possess the neuron-like characteristic²³, detect the DCX and NEUN expression of SH-SY5Y cells to observe the neuroprotection of TSG using immuno cytochemistry. As shown in Fig. 4a and b, DCX- and NEUN-positive cells in MTI group around the scratched trace were less than those in TSG treatment group. The higher magnifications in the insets showed that the cell structure of TSG treatment group was more complete than that of MTI group. To quantify the results, measured the pixel intensity of DCX and NEUN immunoreactivity using the Image J software (DCX: $38.45 \pm 4.12\%$ vs $66.89 \pm 8.11\%$, **p<0.01, NEUN: $35.59 \pm 8.63\%$ vs $54.36 \pm 7.16\%$ and **p<0.01) (Fig. 4c, d). Moreover, TSG treatment group exhibited an enhanced migrative ability and the scratch width in MTI group was larger than in TSG treatment group (Fig. 4a, b; green). The results showed that the pixel intensity of TSG treatment group was higher than the pixel intensity in MTI group. The results above indicated that TSG can protect the integrity of SH-SY5Y cell structure against MTI to some extent.

TSG treatment reduced the cell apoptosis induced by MTI

in vitro: As shown in Fig. 5, the TUNEL-positive cells in TSG treatment group were less than those in MTI group (TUNEL: $11.35 \pm 4.3\%$ vs $16.23 \pm 3.62\%$ and *p<0.05). Nuclei along the scratch section in MTI group were less than those in TSG treatment group. The scratch width in MTI group was larger than that in TSG treatment group. All of which demonstrated that TSG probably had protective function against MTI-induced apoptosis and cell death.

TSG treatment significantly reduced the release of TNF- α and IL-6 and increased the release of IL-10 induced by ELISA:

The TNF- α and IL-6 are pro-inflammatory cytokines that are detrimental to wound healing. In contrast, some anti-inflammatory cytokines such as IL-10 are beneficial to injury recovery¹¹. It was shown in Fig. 2 that the LDH level dramatically increased and reached the peak value at 12 h after scratched. So, chose 12 h after scratched as the final time point to do TNF- α , IL-6 and IL-10 assays by ELISA. From Fig. 6, the TNF- α and IL-6 levels of MTI group increased significantly compared with that of naive group (TNF- α : 4.87 ± 0.216 vs 0.96 ± 0.063 , ##p<0.01 and IL-6: 1.08 ± 0.093 vs 0.065 ± 0.0070 , ##p<0.01) (Fig. 6b) that of the TSG treatment group decreased obviously (TNF- α : 0.13 ± 0.009 vs 4.87 ± 0.216 , **p<0.01 and IL-6: 0.0057 ± 0.0022 vs 1.08 ± 0.093 , **p<0.01) (Fig. 6a) especially for IL-6. The IL-10 level of MTI group increased little vs naive group (IL-10: 0.17 ± 0.030 vs 0.06 ± 0.039 , ##p<0.01)

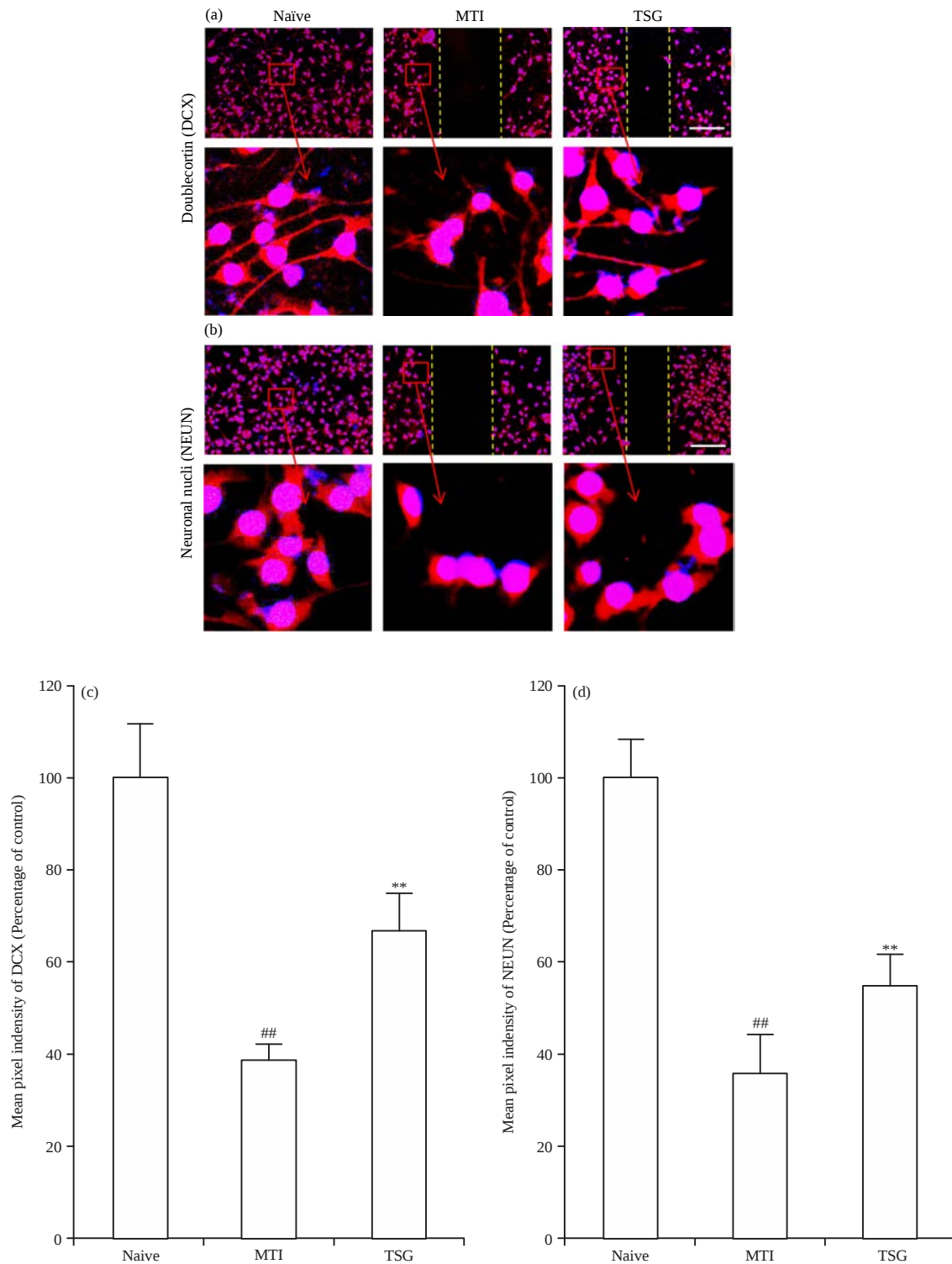


Fig. 4(a-d): TSG treatment reduced cell damage, (a-b) SH-SY5Y cells were detected by DCX and NEUN immunostaining (red) and boxed areas appear at higher magnifications in the insets and (c-d) Staining cells were quantified by measurement of pixel intensity of DCX and NEUN immunore activity using Image J software, the horizontal axis represents groups, the vertical coordinate represents the pixel intensity ratio of TSG treatment and untreatment group vs naive group, scale bar: 50 μ m, nuclei were stained with DAPI (blue), data are presented as Mean $\pm$ SD, n = 6 per group, ^{##}p<0.01 vs naive and ^{**}p<0.01 vs MTI

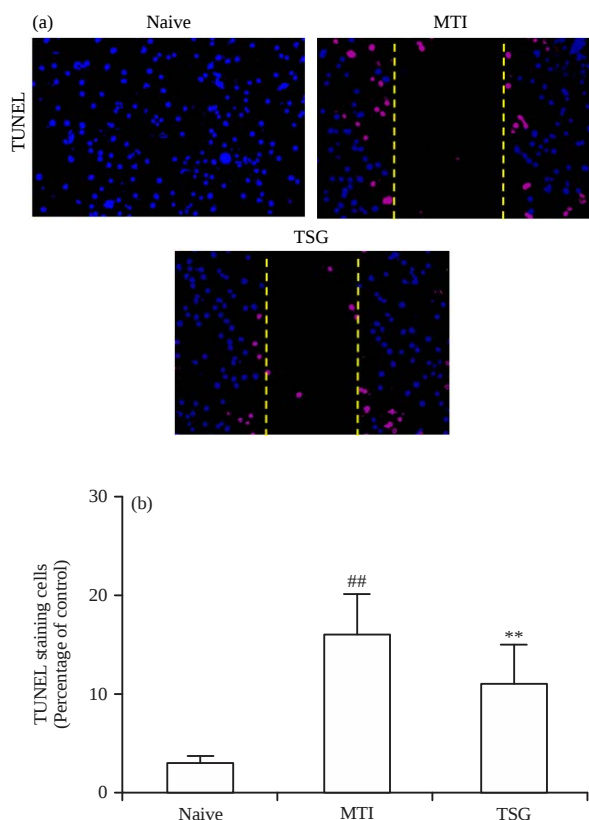


Fig. 5(a-b): TSG treatment reduced apoptosis, (a) SH-SY5Y cells were analyzed by TUNEL staining (red) and (b) Apoptotic cells were quantified by measurement of the number of TUNEL-positive cells using the Image J software, the horizontal axis represents the groups, the vertical coordinates represent the percentage of TUNEL stained cells, scale bar: 50 μ m, nuclei were stained with DAPI (blue), data are presented as the Mean $\pm$ SD, n = 6 per group, ^{##}p<0.01 vs naive and ^{*}p<0.05 vs MTI

(Fig. 6c) while that of TSG treatment group increased to a large extent (IL-10: 1.67 ± 0.240 vs 0.17 ± 0.030 and ^{**}p<0.01) (Fig. 6c). These results illustrated that TSG possessed the anti-inflammatory effects in MTI cell model.

TSG treatment reduced the expression of TNF- α and IL-6 and increased the expression of IL-10 induced by MTI at gene level *in vitro*: As shown in Fig. 7a, the mRNA expression levels of TNF- α and IL-6 in TSG treatment group obviously increased while, IL-10 expression decreased compared to MTI group (TNF- α : 0.57 ± 0.084 vs 0.99 ± 0.088 , ^{**}p<0.01, IL-6: 0.42 ± 0.050 vs 0.54 ± 0.059 , ^{**}p<0.01 and IL-10: 1.02 ± 0.124 vs 0.89 ± 0.115 , ^{**}p<0.01) (Fig. 7b-d). This result was consistent

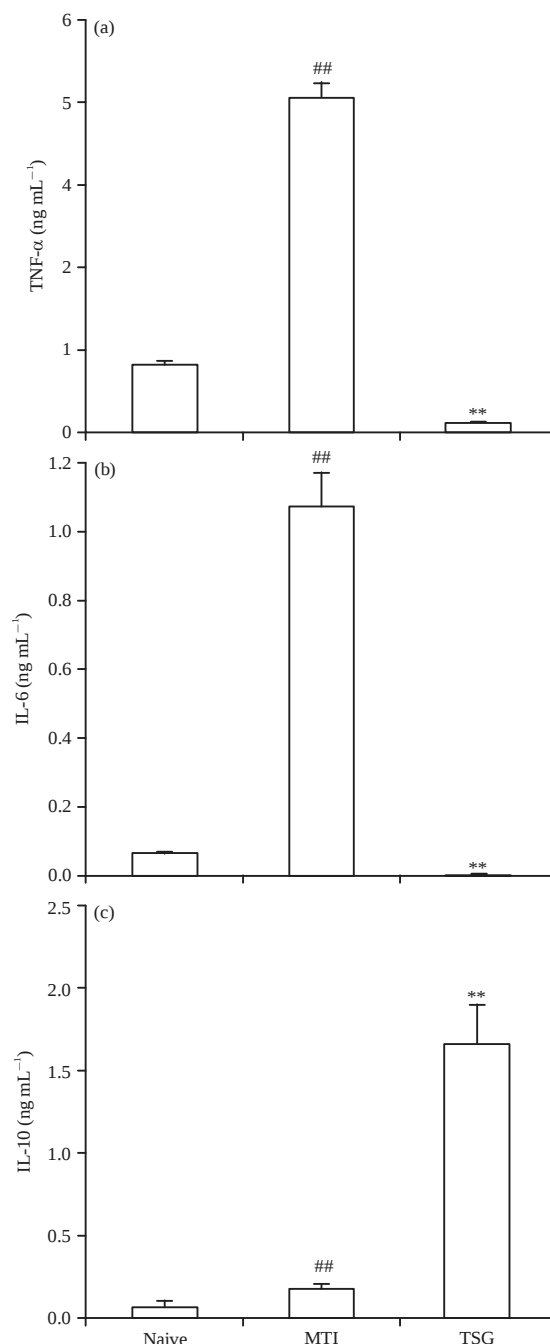


Fig. 6(a-c): Inflammatory cytokine levels in SH-SY5Y cells by ELISA, the horizontal axis represents groups, the vertical coordinate represents the concentrations of the inflammatory cytokines, (a) Concentrations of the TNF- α , (b) Concentrations of the IL-6 and (c) Concentrations of the IL-10, data are presented as Mean $\pm$ SD, n = 6 per group, ^{##}p<0.01 vs naive and ^{**}p<0.01 vs MTI

with that of ELISA detection which confirmed that TSG had the anti-inflammatory effect on MTI-induced SH-SY5Y cells.

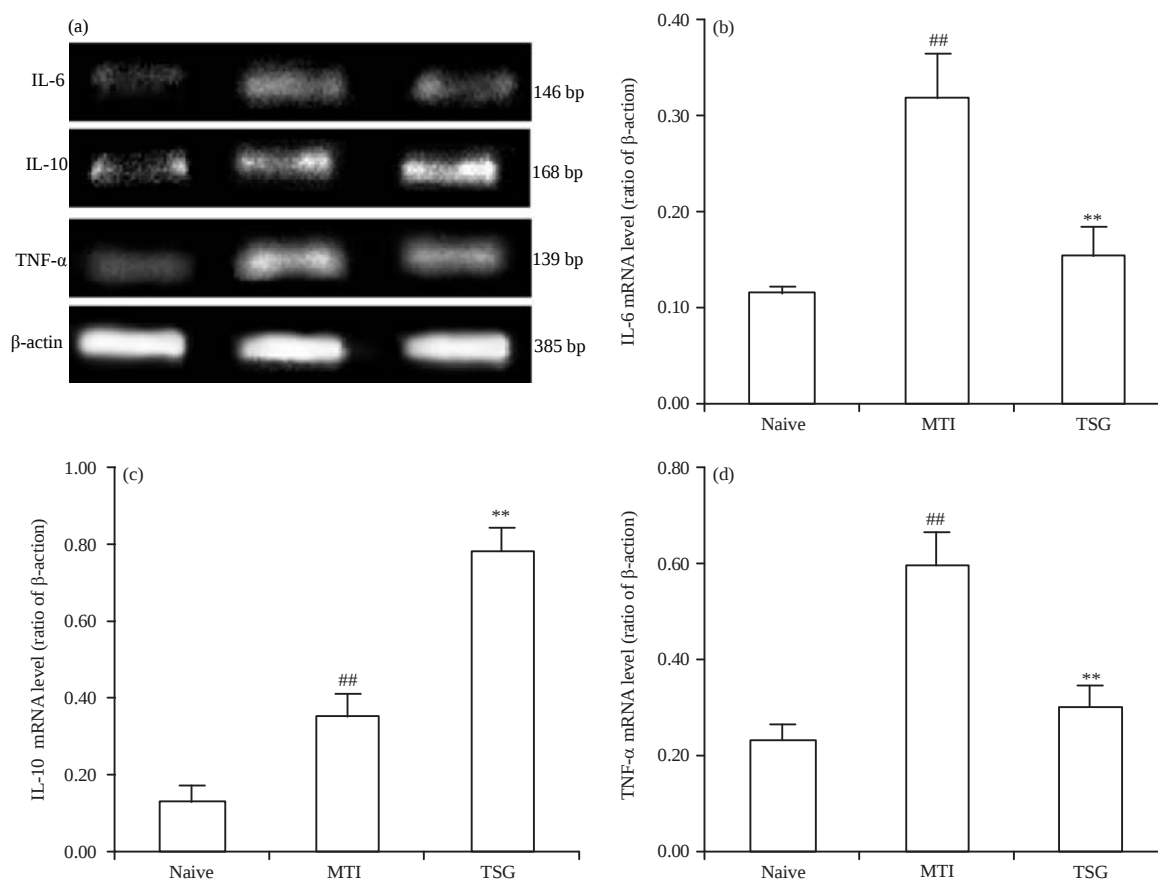


Fig. 7(a-d): Changes of gene expression in injured cells after TSG administration, (a) mRNA of IL-6, IL-10 and TNF- α expression revealed by RT-PCR and (b-d) Quantitative analysis of mRNA Levels of IL-6, IL-10 and TNF- α by Image J, the TSG administration up-regulated mRNA expression of IL-10 and down-regulated mRNA expression of TNF- α and IL-6 significantly as compared with the naive group, data are presented as Mean $\pm$ SD, n = 6 per group, ^{##}p<0.01 vs naive and ^{**}p<0.01 vs MTI

TSG treatment suppressed the inflammatory response at protein level *in vitro*: As shown in Fig. 8a, the protein expression levels of the pro-inflammatory cytokines TNF- α and IL-6 obviously decreased in TSG treatment group whereas, IL-10 expression increased compared to MTI group (TNF- α : 0.48 ± 0.066 vs 0.95 ± 0.104 , ^{**}p<0.01, IL-6: 0.57 ± 0.114 vs 0.93 ± 0.160 , ^{**}p<0.01 and IL-10: 1.11 ± 0.155 vs 0.66 ± 0.111 , ^{**}p<0.01) (Fig. 8b-d). The above results were consistent with the ELISA and gene level results and further confirmed that TSG had an anti-inflammatory effect on MTI cell model.

DISCUSSION

After MTI, the cells will be damaged by the scratch. The MTI leads to immediate primary and subacute secondary effects. Primary effects on the cells after MTI include breakage of the scratched cells. Secondary effects on the cells after MTI include inflammatory responses, cellular stress and apoptotic

cascades⁴⁵. Secondary effects caused by primary effects affect the recovery from damage in later stages. In this study, use the scratch method to mimic the MTI cell model which was explored in other investigations for anti-inflammation study¹⁰.

In this study, TSG treatment reduced LDH leakage with time-dependent tendency in MTI cell model, protected SH-SY5Y cells against MTI-induced cell death, reduced the cell damage and apoptosis and enhanced the migrative ability. The TSG reduced the expression of TNF- α and IL-6 and increased the expression of IL-10 while, suppressed the inflammatory response at gene and protein levels induced by MTI *in vitro*.

The SH-SY5Y cells possess neuron-like characteristic²³ with a structure that is similar to neurons and possess the axons, dendrites and perikaryon. The structures (perikaryon, axon and dendrites) of SH-SY5Y cells were destroyed after MTI and the damage could lead to secondary injury. The DCX is a microtubule-associated protein that is widely distributed in

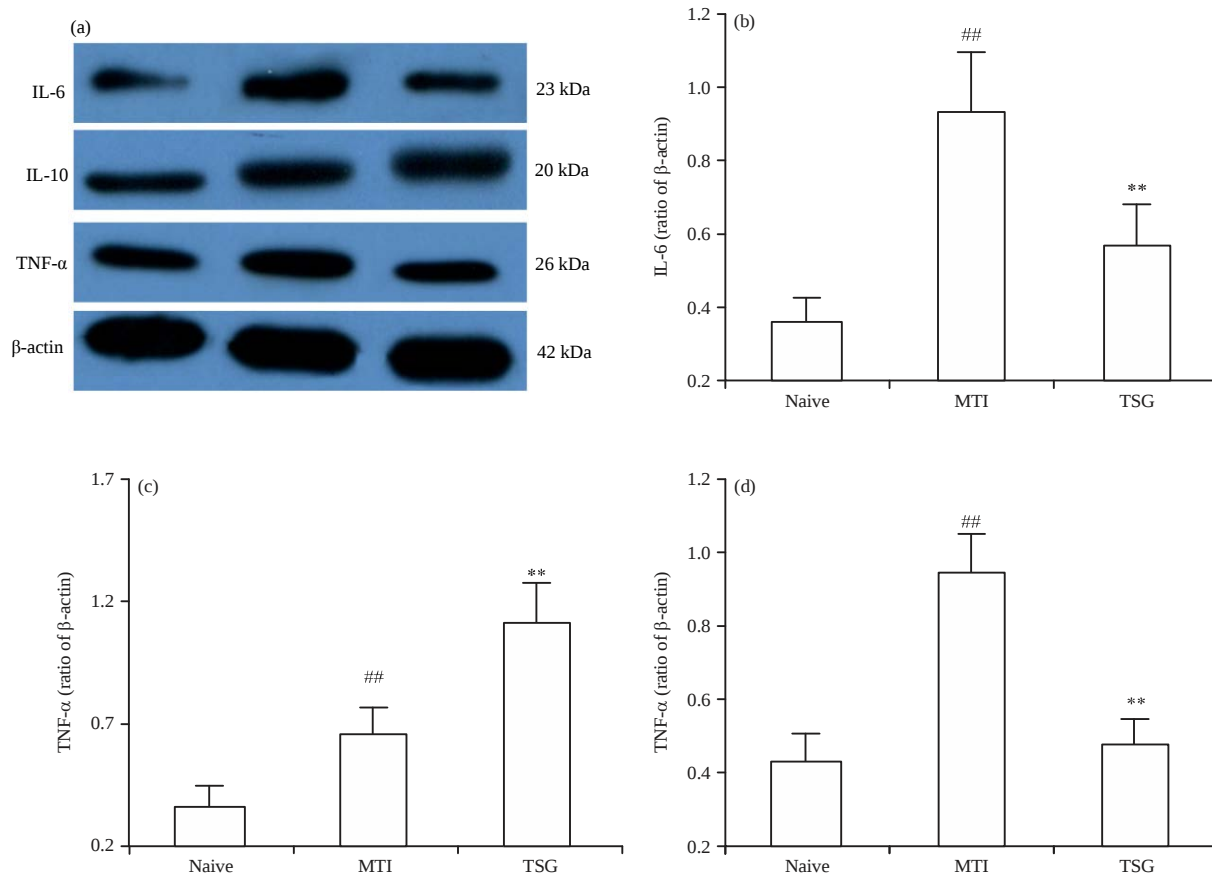


Fig. 8(a-d): Changes of protein expression in injured cells after TSG administration, (a) Protein expression of IL-6, IL-10 and TNF-α revealed by RT-PCR and (b-d) Quantitative analysis of protein levels of IL-6, IL-10 and TNF-α by Image J, the TSG administration up-regulated protein expression of IL-10 and down-regulated protein expression of TNF-α and IL-6 significantly as compared with the naive group, data are presented as Mean ± SD, n = 6 per group, ^{##}p<0.01 vs naive and ^{**}p<0.01 vs MTI

the axon and dendrites^{46,47} and NEUN is a soluble nuclear protein that is distributed in the perikaryon⁴⁸. This study measured DCX and NEUN expression in the SH-SY5Y cells to observe neuroprotection of TSG using immunofluorescence. The DCX and NEUN expression decreased in the injured cells and indicating the loss of axon, dendrites and perikaryon. Figure 4a and b showed that TSG treatment increased DCX and NEUN expression and the cells in TSG treatment group had a more complete cell structure compared to the MTI cells, indicating that TSG possessed neuronal protection via maintaining the cell structure integrity. Moreover, the scratch trace became narrower that demonstrated TSG treatment probably increased the cell migratory ability and reduced cell apoptosis (Fig. 5a, b). Secondary pathophysiological insults result in delayed cell apoptosis and death in surrounding or distant regions⁴⁹. As shown in Fig. 5, the number of TUNEL-positive cells significantly decreased in TSG treatment group compared to MTI group. The apoptotic cells were

concentrated along both sides of the scratch trace which indicated that the direct MTI acquired and the little distance to injury cells resulted in the severity of cell damage along the scratch trace. Moreover, the scratch width in TSG treatment group was narrower than that in MTI group, as a result of the reduced cell apoptosis and the increased cell migrative ability which were consistent with the results of the immunofluorescence (DCX and NEUN). These results demonstrated that TSG was neuronal protective and apoptosis inhibitive.

The IL-6 is a pleiotropic cytokine with a central role in immune regulation and inflammatory response⁵. The TNF-α is a major pro-inflammatory mediator and one of the primary stimuli to induce apoptosis²⁴. The most important function of IL-10 is to limit and eventually terminate the inflammatory response^{50,51}. The release of inflammatory cytokines in injured cells is a normal immune response but the over expression of some pro-inflammatory cytokines such as IL-6 and TNF-α

are detrimental to wound recovery. Suppression of inflammatory cytokines is beneficial to alleviate MTI-induced cell damage. Inflammation is involved in neuronal death and a wide range of pro-inflammatory cytokines such as TNF- α and IL-6 might be toxic to neurons^{24,52,53}. Both TNF- α and IL-6 increase the permeability of the blood-brain barrier⁵⁴. The IL-10 can decrease the expression of IL-6 and TNF- α ^{55,56}. The TNF- α can increase IL-6 expression^{57,51}. The TSG exhibits its function through anti-inflammation²³, anti-apoptosis² and anti-oxidation^{40,58}. The TSG lowered the protein over expression of the pro-inflammatory mediators TNF- α , IL-6 and enhanced the IL-10 levels⁵. Increasing evidence indicates that IL-10 has the ability to improve neurological outcome after central nervous system injury and this ability relies on its anti-inflammatory effects⁵⁹⁻⁶¹. But, TSG has not been used for anti-inflammation and neuroprotection in MTI-induced SH-SY5Y cells before. This model is used to observe the anti-inflammation and neuroprotection of TSG in this study. As shown in the results of ELISA (Fig. 6) TSG can significantly decrease the expression of the pro-inflammatory cytokines such as TNF- α and IL-6 while, increase the IL-10 expression dramatically and indicating that TSG has obviously anti-inflammatory effect. From the results of PCR (Fig. 7) and WB (Fig. 8) TSG decrease TNF- α and IL-6 expression and increase IL-10 expression at gene and protein levels which demonstrates that TSG possesses anti-inflammatory effect on MTI cell model. The neuroprotective effect of TSG is probably mediated by decreasing the expression of inflammation-associated and apoptosis-associated factors induced by MTI and increasing the anti-inflammatory cytokine IL-10 production³⁴.

CONCLUSION

The TSG treatment protects against cell damage and secondary injury via anti-inflammatory mechanisms in an MTI cell model. The potential mechanisms underlying the anti-inflammatory functions of TSG include two aspects in this study: The first one is that TSG positively regulates the release of anti-inflammatory cytokine IL-10 and the second one is that TSG negatively regulates the release of pro-inflammatory cytokine IL-6 and TNF- α . This study indicated that TSG might become a promising candidate for the treatment of MTI-induced neuro inflammation.

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