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Original Research

Tormentic acid confers protection against oxidative stress injury in rats with Parkinson's disease by targeting the Wnt/β-catenin signaling pathway

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Abstract: Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (AD). Studies have shown that oxidative stress (OS) may contribute to the cascade of reactions leading to the degeneration of dopaminergic neurons in the brain. The present study investigated the protective effect of tormentic acid (TMA) on OS-induced injury in rat model of PD, and the underlying mechanism. Evaluation of learning and memorizing ability was done using Morris water maze (MWM) test. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), and level of malondialdehyde (MDA) in substantia nigra were determined using enzyme-linked immunosorbent assay (ELISA). The protein and mRNA expressions of β-catenin, GSK-3β, and GSK-3β-Ser9 were determined using real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting. The effect of TA on cell viability and proliferation was determined *in vitro* on rat adrenal pheochromocytoma (PC12) cell line using MTT assay. The results showed that the escape latency of rats in negative control group was significantly higher than that in normal control group ($p < 0.05$). However, treatment with TMA significantly and time-dependently reduced the escape latency of the rats ($p < 0.05$). The extent of apoptosis was significantly reduced after treatment with TMA ($p < 0.05$). Besides, treatment with TMA significantly increased the viability of brain cells ($p < 0.05$). The activities of SOD and GPx were significantly lower in negative control group than in normal control group, but were significantly increased after treatment with TMA ($p < 0.05$). The level of MDA was significantly higher in negative control group than in normal control group, but was significantly reduced after treatment with TMA ($p < 0.05$). The results of qRT-PCR and Western blotting showed that treatment with TMA significantly activated Wnt/β-catenin signaling pathway ($p < 0.05$). TMA treatment significantly reversed the effect of 6-hydroxydopamine on the expression levels of these proteins and their mRNAs ($p < 0.05$). These results suggest that TMA confers protection against OS-induced injury in rats with PD by targeting the Wnt/β-catenin signaling pathway.

Key words: Parkinson's disease; Tormentic acid; Oxidative stress; Superoxide dismutase; Expression.

Introduction

Parkinson's disease (PD) is a central nervous system (CNS) degenerative disease that is common among the elderly. The disease is characterized by loss of dopaminergic neurons in the substantia nigra and accumulation of misfolded α-synuclein in the form of eosinophilic protein deposits (1, 2). The symptoms of PD include extrapyramidal signs such as tremor and bradykinesia. In addition to motor symptoms, early non-motor symptoms such as cognitive, emotional, behavioral, and personality disorders may occur in PD (3, 4). Parkinson's disease (PD) is the second most common neurodegenerative disease after AD; it affects approximately 1 % of people over 65 years of age, and almost 3 % of people over 80 years of age, with age as its greatest risk factor (5, 6). Although several risk factors are involved in the development of PD, its pathogenesis is still not fully understood (7). Strategies currently employed in the management of PD serve as mere palliatives (8).

Potentilla is a genus containing over 300 species of annual, biennial and perennial herbaceous flowering plants in the rose family, *Rosaceae* . Tormentic acid (TMA) is a bioactive compound isolated from *Potentilla*. Studies have shown that TMA possesses pharmacological effects such as antioxidant (9), antihypertriacylglycerolemic, antihypercholesterolemic, hepatoprotective (10), antitumor (11), and hypoglycemic properties (12). It has also been reported that TMA confers significant protection against acute liver injury induced by lipopolysaccharide (LPS) and D-galactosamine (D-GalN) in mice. The underlying mechanism has been specu-

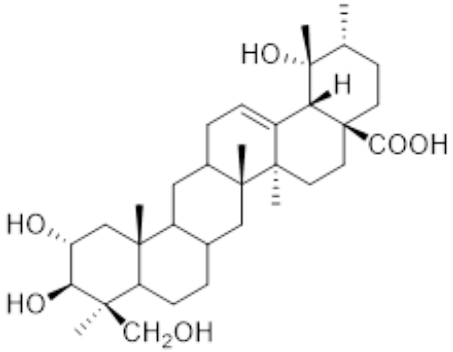

Figure 1. Chemical structure of TMA.

lated to involve anti-inflammatory response, inhibition of lipid peroxidation and apoptosis of hepatocytes (10). However, the protective mechanism of TMA on OS-induced injury in PD has not been reported. The present study investigated the protective effect of TMA on OS-induced injury in rat model of PD, and the underlying mechanism.

Materials and Methods

Materials and reagents

Rat adrenal pheochromocytoma (PC12) cell line was obtained from Shanghai Institute of Cellular Biosciences, Chinese Academy of Sciences (China). Tormentic acid (TMA) was provided by the Institute of Chinese Medicine, China Academy of Chinese Medical Sciences. Lithium chloride (LiCl, an activator of the Wnt/β-catenin signaling pathway), MTT solution, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA). Coomassie brilliant blue, SOD, MDA, and GP_X kits were products of Nanjing Jiancheng Institute of Biology (China). Octanoic acid was obtained from Thermo Fischer Scientific Co. Ltd (USA). Cyclin D1, β-catenin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were products of Cell Signaling Technology (USA), while GSK-3β and p-GSK- 3β-Ser9 primers were obtained from Abcam (UK).

Experimental rats and grouping

Adult Wistar rats (n = 40) of either sex weighing 180 – 220 g (mean weight = 200 ± 20 g) were obtained from Shanghai Jiake Biotechnology Co., Ltd. (China) and used for this study. Prior to commencement of the study, the rats were acclimatized to the laboratory environment for one week under controlled conditions at a temperature of 23 ± 2 °C, humidity of 55 – 65 %, and 12 h light/12h dark cycle. They were allowed access to standard rat feed and clean drinking water. The rats were randomly assigned to five groups of 8 rats each: normal control group, negative control group, TMA control group, LiCl control group, and treatment group. Parkinson disease (PD) was induced in all the rats through injection of 6-hydroxydopamine via intracerebral infusion, with the exception of normal control group. The rats were fasted 12 h before induction of PD. Rats in normal control group received normal saline at two points to their right striata. Rats in the TMA control group were intraperitoneally injected with TMA (250 mg/kg bwt), while the treatment group rats received intraperitoneal injection of TMA (250 mg/kg bwt) and LiCl (1 mEq/kg bwt). The LiCl control group rats were intraperitoneally injected with LiCl (1 mEq/kg bwt), while rats in the negative control group were not treated. Treatments were initiated 3 days prior to induction and continued till the 14th day post-induction (13).

Morris water maze (MWM) test

The MWM test was performed on the rats to test their spatial learning and memorizing ability (14). The Morris water labyrinth is a cylindrical pool made of stainless steel with a diameter of 200 cm and a height of 50 cm. Four points were marked on the wall of the Morris water maze as entry points into the water, and the pool was divided into four quadrants in the clockwise direction: A, B, C and D. The depth of water was approximately 30 cm and the temperature was maintained between 22 and 25 °C. A platform of 11 cm in diameter and 29 cm in height was fixed in one quadrant at a position 1 cm below the water mark. The monitoring camera was mounted above the water maze and connected to a computer for analysis of the swimming traces of the rats. The Morris water navigation test was performed twice daily, in the morning and afternoon, and lasted three days. The rats were placed in 4 quadrants separate from the influent site facing the pool wall, and the time taken to find the platform within 60 sec was recorded as the escape latency. Mice that could not find the platform within 60 sec were guided to it and kept there for 10 sec, and then put back into the cage. The time taken (latency time) by the rats to locate the platform was calculated using video tracking system (ANY-maze). After the completion of trial, the rats were warmed using infrared lamp in holding cages before being transferred to their cages.

Probe trials

On the fourth day of the location navigation test, memorising ability of the rats was determined using probe trials. The rats were put in opposite quadrant and the platform in the pool was removed. Then, the rats were given 60 sec to make attempts to locate the position of the submerged platform. Spatial learning/memory capabilities was assessed in terms of the time it took for rats to find and enter a given quadrant.

Cell culture

The PC12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing glucose and bovine serum (15 %) at 37 °C in a humidified atmosphere containing 5 % CO_2 and 95 % air. Cells in logarithmic growth phase were selected and used for this study. The medium was changed every 2 days, and the monolayer cells were concentrated to 80 % confluency. The cells were digested with 0.25 % trypsin to obtain a single cell suspension.

Tissue sample collection

Rats were euthanized with 10 % chloral hydrate intraperitoneally at a dose of 400 mg/kg bwt and then sacrificed according to the ethical laws approved by Sichuan Provincial People's Hospital Ethics Committee. Their brain tissues were excised and placed on an ice tray to separate the substantia nigra. The tissues were blotted dry, weighed and portions were used to prepare 10 % tissue homogenate. The homogenate was centrifuged at 3000 rpm for 15 min at 4 °C to obtain supernatant which was used for biochemical analysis.

MTT assay

The MTT assay was performed to determine the proliferative ability of the cells and their viability in the presence of TMA. After trypsinization with 0.25 % trypsin, the cells were seeded into 96-well plates (1×10^5 cells/well) and cultured in DMEM for 24 h. The TMA was added to the cells and incubated for 24 h at 37 °C. At the end of the second day, 20 µL of 5.0 mg/mL MTT solution was added to the wells, followed by incubation for

another 4 h. The medium was finally replaced with 150 µL of 0.1 % DMSO solution, agitated at 50 oscillations/min for 10 min. Absorbance was read in a microplate reader at 540 nm. The control cell cultures were incubated in medium containing DMSO alone. Cell viability was calculated thus:

Cell viability *(%) = (absorbance of the experimental group/absorbance of the control group) × 100 %.*

Determination of activities of SOD and GPx and level of MDA

The activities of SOD and GPx, and level of MDA in substantia nigra were determined using their respective ELISA kits.

qRT-PCR

The substantia nigra of rats was excised and blotted dry. The total mRNA was extracted using Trizol reagent. The concentration and purity of the extracted RNA was determined spectrophotometrically at 260/280 nm. The RNA was reverse-transcribed to cDNA using First Strand cDNA synthesis kit, and qRT-PCR was performed using QuantStudio™ 7 Flex Real-Time PCR System. The PCR conditions were: pre-denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 5 sec, and extension at 72 °C for 5 sec. Dissociation melt-curve analysis was performed to determine non-specific amplification. Each procedure was performed in triplicate, and the mean threshold value for each cycle was normalized to the expression of GAPDH which was used as an internal reference. The relative expression of RNA was calculated using $2^{-\Delta\Delta Ct}$ method.

Western blotting

Cell suspension resulting from trypsinization of substantia nigra tissues was washed twice with phosphate-buffered saline (PBS) and lysed with ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitors (1:5, v:v). The resultant lysate was centrifuged at 12, 000 rpm for 10 min at 4 °C, and the protein concentration of the supernatant was determined using bicinchoninic (BCA) assay kit. A portion of total cell protein (30 µg) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated for 2 hours to block non-specific binding of the blot. The blots were incubated overnight at 4 °C with primary antibodies of rabbit polyclonal anti-cyclin D1, β-catenin, GSK-3β, p-GSK-3β-Ser9 and GAPDH, each at a dilution of 1:500. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (diluted at 1:1000) for 2 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ECL. The various protein expression levels were normalized to that of GAPDH which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD, and the statistical analysis was performed using GraphPad Prism (7.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of TMA on 6-hydroxydopamine -induced changes in memory and behavior of rats

The escape latency of rats in negative control group was significantly higher than that of rats in the normal control group ($p < 0.05$). However, treatment with TMA +LiCl significantly and reduced the escape latency of the rats ($p < 0.05$; Figure 2A). TMA +LiCl co-adminstration significantly increased time spent for swimming compared to TMA or LiCl group ($p < 0.01$; Fig. 2B). Number of times rats crossed the removed hidden platform was significantly increased in TMA +LiCl group compared to TMA or LiCl group ($p < 0.001$; Fig. 2C).

Viability of PC12 cells in vitro

As shown in Figure 3, the extent of apoptosis was significantly higher in negative control group than in normal control group, but was significantly reduced after treatment with TMA ($p < 0.05$). There were only a few viable cells in negative control group, when compared with normal control group ($p < 0.05$). However, treatment with TMA significantly increased the viability of brain cells ($p < 0.05$). Besides, TMA significantly

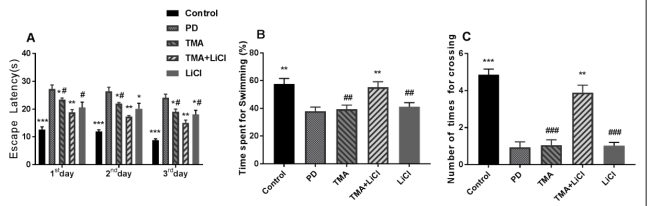

Figure 2. Measurement of escape latency in rats. (A): Escape latency determined using MWM test; (B): Time spent swimming in the training quadrant; and (C): Number of times rats crossed the removed hidden platform. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared with negative control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, when compared with the treatment group.

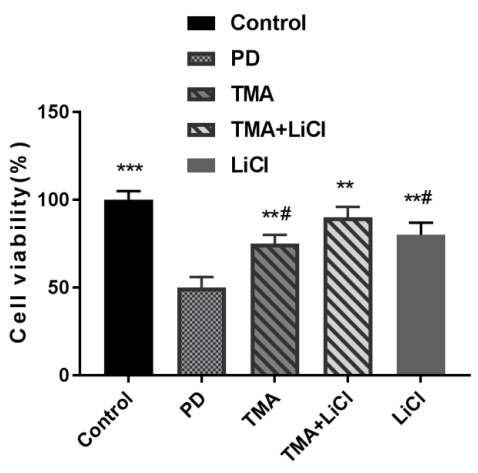

Figure 3. Effect of TMA on the viability of PC12 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ & $^{***}p < 0.001$, when compared with negative control group. $^{\#}p < 0.05$, when compared with treatment group.

increased the effect caused by LiCl ($p < 0.05$).

Effects of TMA on activities of SOD and GP_X, and level of MDA in subsTMAntia nigra of rats

The activities of SOD and GPx were significantly lower in negative control group than in normal control group, but were significantly increased after treatment with TMA ($p < 0.05$). The level of MDA was significantly higher in negative control group than in normal control group, but was significantly reduced after treatment with TMA . TMA enhanced the potency of LiCl in substantia nigra of rats. ($p < 0.05$; Figure 4)

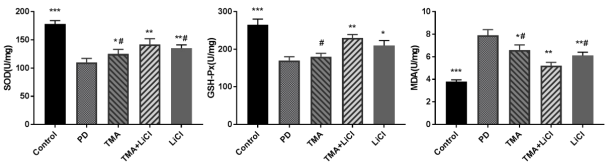

Figure 4. Effect of TMA on SOD and GPx activities and levels of MDA in substantia nigra of rats in the various groups. $^{*}p < 0.05$, $^{**}p < 0.01$ & $^{***}p < 0.001$, when compared with negative control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ & $^{\#\#\#}p < 0.001$, when compared with treatment group.

Levels of expressions of Wnt /β-catenin signaling pathway proteins

The results of qRT-PCR and Western blotting showed that treatment with TMA significantly activated Wnt/β-catenin signaling pathway ($p < 0.05$). 6-Hydroxydopamine significantly down-regulated the protein and mRNA expressions of β-catenin, and significantly upregulated the protein and mRNA expressions of GSK-3β ($p < 0.05$). However, treatment with TMA significantly reversed the effect of 6-hydroxydopamine on the expression levels of these proteins and their mRNAs ($p < 0.05$). TMA enhanced the potency of LiCl in Wnt/β-catenin pathway-related factors in substantia nigra of rats. These results are shown in Figure 5.

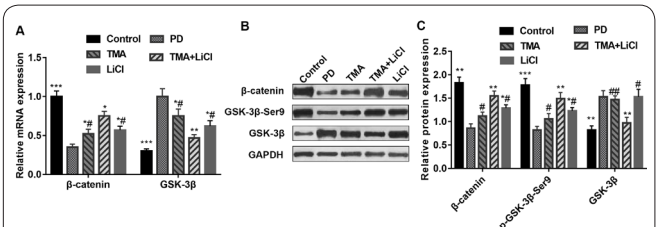

Figure 5. Effect of TMA on protein and mRNA expressions of Wnt/β-catenin pathway-related factors in substantia nigra of rats. (A): Expressions of β-catenin and GSK-3βmRNAs as determined using qRT-PCR; (B): Protein expressions of β-catenin, GSK-3β, and GSK-3β-Ser9 as determined using Western blotting; and (C): Density analysis of β-catenin, GSK-3β, and GSK-3β-Ser9 as determined using ImageJ. $^{*}p < 0.05$, $^{**}p < 0.01$ & $^{***}p < 0.001$, when compared with negative control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ & $^{\#\#\#}p < 0.001$, when compared with the treatment group.

Discussion

Parkinson's disease (PD) is usually accompanied by slow, progressive degeneration of dopaminergic neurons in substantia nigra of the brain (8). It is a disease of the elderly (15). Oxidative stress (OS) promotes CNS diseases, mitochondrial dysfunction, and neuroinflammation (16, 17). Studies have shown that in AD (a CNS degenerative disease), mutations in PSEN1 gene encoding presenilin-1 (PS1) leads to increased level of expression of GSK-3β, decreased level of expression of β-catenin, and inactivation of Wnt/β-catenin signaling pathway (18, 19). The Wnt/β-catenin pathway is the Wnt pathway that causes an accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional coactivator of the TCF/LEF family transcription factors (19). The Wnt co-receptor LRP6Δ3 inhibits Wnt/β-catenin signaling pathway and increases the risk of AD. On the other hand, overexpression of Wnt co-receptor (LRP5/LRP6) in SH-SY5Y cells results in abnormal activation of the Wnt signaling pathway. This protects neurons against OS-induced damage, and promotes their survival by inhibiting GSK-3β-mediated Tau hyperphosphorylation (20). However, reports on the protective effect of TMA on OS and its antiapoptotic effect on neurons of patients with PD are scanty.

In this study, treatment of PD rats with TA significantly reduced the escape latency of the rats, an indication that TMA may improve behavioral defects caused by PD. The results of MTT assay showed that TMA enhanced the protective effect of LiCl on PC12 cells. The activation of Wnt/β-catenin signaling pathway by TMA may ameliorate OS-induced damage in PD. Clinical studies have shown that the level of hydrogen peroxide in the substantia nigra of patients with PD is significantly higher than that in normal individuals, and the activity of GPx and level of reduced glutathione (GSH) are also significantly reduced in these patients (21, 22). Glutathione peroxidase (GPx) and SOD are important free radical scavenging enzymes which confer protection on cell membrane structure and function. Superoxide dismutase (Cu-Zn) also known as SOD1 is ubiquitously expressed in cells, and it is the main form of SOD. However, this protein has no specific receptors or channels, and cannot enter the cell by means of endocytosis or other similar mechanisms. Malondialdehyde (MDA) is a lipid-derived peroxide that reflects the degree of lipid peroxidation in a cell. The enzyme GPx blocks the secondary reaction of free radicals triggered by lipid peroxides, and protects the cell membrane from peroxidative damage (23). In this study, the activities of SOD and GPx were significantly lower in negative control group than in normal control group, but were significantly increased after treatment with TMA. The level of MDA was significantly higher in negative control group than in normal control group, but was significantly reduced after treatment with TMA. These results suggest that activation of the Wnt/β-catenin signaling pathway may improve antioxidant defense mechanism in rats with PD. It is also likely that TMA scavenges oxygen free radicals and reduces lipid peroxidation.

The results of qRT-PCR and Western blotting showed that treatment with TMA significantly activated Wnt/β-catenin signaling pathway. 6-Hydroxydopamine significantly down-regulated the protein and mRNA expressions of β-catenin and GSK-3β-Ser9, and significantly upregulated the protein and mRNA expressions of GSK-3β. However, treatment with TMA significantly reversed the effect of 6-hydroxydopamine on the expres-

sion levels of these proteins and their mRNAs. These results suggest that TMA may enhance the effect of LiCl on the expression of Wnt/β-catenin signaling pathway proteins. 6-Hydroxydopamine is a neurotoxin used for inducing PD in rats. It increases intracellular oxidants and cell damage which culminate in cell apoptosis.

The results obtained in this study suggest that TMA confers protection against OS-induced injury in rats with PD by targeting the Wnt/β-catenin signaling pathway.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xiaohua Jiang; Yamin Yue, Bai Qiao, Xiaohua Jiang collected and analysed the data; Yamin Yue wrote the text and all authors have read and approved the text prior to publication.

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