POTENTIAL NEUROPROTECTIVE EFFECT OF TETRAMETHYLPYRAZINE IN AN EXPERIMENTAL ANIMAL MODEL OF PARKINSON’S DISEASE

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ABSTRACT

Parkinson's disease (Powers et al.) is a slowly progressive neurodegenerative movement disorder and it is the second most common neurodegenerative disorder after Alzheimer's disease. Several hypotheses have been proposed to explain the pathogenesis of PD, of which apoptotic cell death, neuroinflammation and oxidative stress are the most prevalent. Tetramethylpyrazine (TMP) is the major bioactive component of Ligusticum wallichii Franchat (ChuanXiong), Family Apiaceae, which exhibits anti-apoptotic, anti-inflammatory and antioxidant roles. In the present study, the possible neuroprotective effect of TMP against rotenone-induced model of PD in rats was investigated and the possible mechanisms were elucidated. Results showed that systemic rotenone administration significantly impaired rats’ movement and induced postural instability, compared to the control group. Co-administration of TMP significantly improved rats movement and attenuated postural instability in rotenone-treated rats. Moreover, rotenone-treated rats exhibited altered midbrain histology, evidenced by severe hemorrhage, hyalinosis and blood vessels’ congestion in addition to a significant loss of tissue histo-architecture, where the neurons appeared hyperchromatic, lost the round form of normal ones and phantom nuclei appeared scattered in the field. Co-treatment with TMP showed restoration of normal histological structure in the midbrain and the global appearance of the tissue in sections co-treated with TMP was closer to normal with apparent increased number of normal neurons as compared to rotenone-treated rats. Immunohistochemical staining of the midbrain sections showed that rotenone increased caspase-3 expression compared to the control, while co-treatment with TMP reduced caspase-3 expression compared to rotenone treated rats. In conclusion, the present study demonstrated that TMP has neuroprotective effects in rotenone-induced PD. Therefore, TMP can be a promising candidate for further investigations in other neurodegenerative disorders.

Introduction

Parkinson's disease –PD– is the second most common neurodegenerative disorder after Alzheimer's disease. Nowadays, this disorder is recognized as being clinically characterized by four cardinal symptoms; resting tremor, rigidity,
bradykinesia (slowness of movement) and postural instability (Jankovic and Stacy, 2007; Smeyne and Jackson-Lewis, 2005). The incidence of PD increases with age (Tan and Jankovic, 2006) where it affects about 1% of those over the age of 60 rising to over 4% by age 85 (de Lau and Breteler, 2006). The development of these classical motor symptoms can be attributed to the selective loss of dopaminergic (DA) neurons, primarily in the substantia nigra pars compacta (SNpc). This hallmark loss of neurons results in striatal dopamine depletion and a resultant dysfunction of the basal ganglia, a cluster of nuclei involved in the initiation and execution of movement (Rodriguez-Oroz et al., 2009). Pathologically, the disease is also characterized by proteinaceous inclusions known as Lewy bodies and Lewy neurites (Agid, 1991) that can be found localized to the soma and processes of neurons, respectively, in many areas of the PD brain. Lewy bodies and Lewy neurites are composed of several proteins including α-synuclein and ubiquitin, as well as lipids (Spillantini et al., 1997).

Rotenone is an insecticide and pesticide extracted from Leguminosae plants (Hisata, 2002) that has been used extensively as a prototypic mitochondrial toxin in cell cultures; exposure to it has been linked to a higher risk of PD (Tanner et al., 2011). Rotenone is highly lipophilic and is able to cross the blood–brain barrier (Talpade et al., 2000). The highlight of the use of chronic rotenone treatment of rats to model PD has been the generation of proteinaceous inclusions in some of the surviving dopaminergic neurons that cannot be found in the standard 6-hydroxydopamine and 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine models. These inclusions were first described in osmotic pump administration experiments as Lewy body-like inclusions, positive for ubiquitin and α-synuclein, and composed of a dense core with fibrillar peripheral elements (Betarbet et al., 2000; Sherer et al., 2003).

TMP, also known as ligustrazine, is a purified chemical that has been identified as a component of *Ligusticum wallichii* Franchat (ChuanXiong), a Chinese herb that is largely used in the treatment of neurovascular and cardiovascular diseases (Chun-Sheng et al., 1978; Guo et al., 1983; Jiao et al., 2004). TMP exhibited potent antidepressant-like effects in the forced swimming test and tail suspension test in mice and this was mediated by promoting BDNF signaling pathway (Jiang et al., 2015). Moreover, TMP partly alleviated kainate-induced status epilepticus (Li et al., 2010) and reversed scopolamine-induced memory deficits in rats (Wu et al., 2013). Thus, the current study aimed at investigating the potential neuroprotective effect of TMP in rotenone-induced model of PD in rats.

**Methodology**

**Animals**

Twenty four male albino rats weighing 200–250 g were purchased from the Nile company, El Amyria, Cairo, Egypt. They were housed in plastic cages at constant temperature (21 ± 2°C), with alternating 12 h light/dark cycle where animal chow and water were provided *ad libitum*. Few days before the experiment, animals were acclimatized to laboratory conditions. All animal treatments adhered strictly to institutional and international ethical guidelines of the care and use of laboratory animals. The experimental protocol was approved by Ain Shams University Faculty of Pharmacy Review Committee for the use of animal subjects.
Drugs and chemicals

Rotenone, TMP and all chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), rabbit polyclonal caspase-3 antibody was purchased from Thermo Fisher Scientific, (UK).

Rotenone was suspended in sunflower oil and administered subcutaneously at a dose of 2 mg/kg for 4 weeks (Huang et al., 2011). TMP was dissolved in saline and administered intraperitoneally at 20 mg/kg 1 h before rotenone injection for 4 weeks (Kao et al., 2006; Kao et al., 2013).

Experimental groups

Male albino rats were divided into four groups, six animals each. The first group, serving as a control group, received intraperitoneal saline (Tetramethylpyrazine – TMP– vehicle) injection and sunflower oil (rotenone vehicle) subcutaneously for 4 weeks. The second group received TMP vehicle (i.p.) and rotenone (2 mg/kg/d, s.c.) for 4 weeks. The third group was intraperitoneally injected with the selected TMP dose in addition to rotenone (2 mg/kg/d, s.c.) for 4 weeks. The fourth group received TMP (20 mg/kg, i.p.) and rotenone vehicle (s.c.) for 4 weeks.

Rats were tested for general movement analysis using Ludolph scale and postural instability 24 h after the last injection, then they were decapitated. Whole brains were excised and fixed in 10% formalin saline (pH 7.4) for the preparation of paraffin blocks.

General movement analysis

Animals were monitored for induction of general motor anomalies, which were analyzed over a scoring system adapted from (Ludolph et al., 1991). Data were obtained 24 h after the last dose. The scoring system used in the analysis was – 0: normal behavior, 1: general slowness of displacement resulting from mild hind limb impairment, 2: incoordination and marked gait abnormalities, 3: hind limb paralysis, 4: incapacity to move resulting from forelimb and hind limb paralysis, and 5: recumbency

Postural instability test

The animal was held vertically, while one forelimb was allowed to contact the table surface, which was lined with medium-grit sand paper. The animal's center of gravity was then advanced until the animal initiated a “catch-up” step. The displacement distance required for the animal to regain the center of gravity was recorded. At each time-point 3 trials for each forelimb were recorded and the average reported (Cannon et al., 2009).

Histological examination

Autopsy samples were taken from the brains and fixed in 10% formalin saline for 24 h. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene then embedded in paraffin at 56°C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 lm thickness by slide microtome. The obtained tissue
sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin, and examined using a light microscope (Hopwood et al., 1996)

**Toluidine-blue staining and evaluation of the neurodegeneration**

Paraffin sections of 5 μm thickness were mounted on slides, washed three times for 5 min with PBS-Triton X-100 (0.4%) and subsequently stained with toluidine blue 0.1% for approximately 2 min. Finally, dehydration of the sections was done and mounted with Canada balsam and the slides were visualized under a light microscope (Wilson and Gamble, 2002). Degenerated midbrain neurons were counted by capturing six non-overlapping fields from the levels of midbrain using a magnification of 200X in six sections per group (n = 6 rats/group). The number of midbrain degenerated neurons was then calculated and expressed as a percentage of the total number of neurons [% degenerated neurons = number of degenerated neurons × 100 / total number of neurons]. Neurons with rounded nuclei and visible nucleoli were considered undamaged, while deeply stained shrunken neurons were considered damaged neurons (Vafaee et al., 2012).

**Immunohistochemical estimation of caspase-3**

According to the techniques described by (Johansson et al., 2002; Yuan et al., 2006), after deparaffinization of brain paraffin sections (4-μm-thick), endogenous peroxide was quenched with 3% hydrogen peroxide in deionized water for 10 min. Nonspecific binding sites were blocked by incubating the sections in 10% goat serum for 10 min. Sections were then incubated with polyclonal rabbit anti-caspase-3 overnight at 4°C, followed by incubation with biotinylated goat-anti-rabbit IgG at room temperature for 10 min. After 3x3 min phosphate buffer saline (PBS) rinses, sections were incubated for 10 min with streptavidin horseradish peroxidase. The antibody binding sites were visualized by incubation with diaminobenzidine-H$_2$O$_2$ solution. Sections incubated with PBS instead of the primary antibody were used as negative controls. Yellowish brown granules in cytoplasm or nuclei were recognized as positive staining for caspase-3.

**Statistical analyses**

Statistical analysis was achieved using a software program (GraphPad Instat, version 2.0, Philadelphia, 1993). Comparisons between means of data were analyzed by one-way analysis of variance (ANOVA). If the overall F-value was found to be statistically significant, post hoc comparisons among groups were made according to Tukey test.

**Results**

**Effect of rotenone and/or TMP on rat movement**

Systemic administration of rotenone (2 mg/kg) significantly impaired rats movement compared to the control group. However, co-administration of TMP (20 mg/kg) significantly attenuated this effect as shown in table 1.

Table 1: Effect of rotenone and/or TMP on rat movement

<table>
<thead>
<tr>
<th>Group</th>
<th>Ludolph scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1 ± 0.001</td>
</tr>
<tr>
<td>Rotenone (2 mg/kg)$^1$</td>
<td>3.8 ± 0.5$^a$</td>
</tr>
<tr>
<td>Rotenone + TMP (20 mg/kg)$^2$</td>
<td>2.2 ± 0.3$^a$</td>
</tr>
<tr>
<td>TMP (20 mg/kg)</td>
<td>0.2 ± 0.001</td>
</tr>
</tbody>
</table>

- $^1$Rotenone (2 mg/kg, s.c.) was administered for 4 weeks.
TMP (20 mg/kg, i.p.) was co-administered with rotenone or vehicle for 4 weeks.
- Data are presented as means ± SEM (n=6).
- *, # statistically significant compared to control and rotenone-treated groups, respectively at p<0.001 using one way ANOVA followed by Tukey’s test for multiple comparisons between groups.

**Effect of rotenone and/or TMP on postural instability**

Rotenone-treated animals exhibited significant postural instability (Fig. 1) compared to the control group. Concomitant administration of TMP (20 mg/kg) significantly attenuated rotenone-induced postural instability by 28.6%.

![Fig.1: Effect of rotenone and/or TMP on postural instability in rats](image)

Histological examination

Histological examination of the midbrains of the control (Fig. 2A) and TMP-alone treated groups (Fig. 2D) showed normal histological structure. Sections from rats treated with rotenone (2 mg/kg, s.c.) for 4 weeks showed severe hemorrhage, hyalinosis and blood vessels’ congestion in the midbrain (Fig. 2B). Co-treatment with TMP (20 mg/kg, i.p.) showed restoration of normal histological structure in the midbrain (Fig. 2C).
Fig. 3 shows the neuronal population in the midbrains of the different experimental groups stained with toluidine blue. Midbrain neurons of the control (Fig. 3A) and TMP-alone treated (Fig. 3D) groups showed a conservation of the general tissue histo-architecture, with neurons having intact body, clear cytoplasm and defined nucleus. In rotenone-treated rats (Fig. 3B), there is a significant loss of tissue histo-architecture and neurons appear hyperchromatic and have lost the round form of normal ones and phantom nuclei appear scattered in the field. However, the global appearance of the tissue in sections co-treated with TMP (20 mg/kg, i.p.) was closer to normal with apparent increased number of normal neurons as compared to rotenone-treated rats (Fig. 3C).

Quantitation of the percentage of degenerated neurons (Fig. 4) showed a significant increase in the number of degenerated neurons in the midbrain by 417% in the rotenone-treated group as compared to the control. However, co-treatment with
TMP (20 mg/kg, i.p.) significantly reduced damaged neurons in the midbrain by 60% as compared to the rotenone-treated group.

Fig. 3: Representative photomicrographs of toluidine-blue stained rat midbrain sections

The figure shows a normal distribution of healthy neurons with euchromatic nucleus and a conserved cytoplasm of the control (A) and TMP-alone treated groups (D). Sections from rats treated with rotenone (2 mg/kg, s.c.) show a predominance of degenerated neurons as shown by dark cytoplasm and pyknotic nuclei (B). However, TMP (20 mg/kg, i.p.) significantly decreased rotenone-induced neuronal death with the global appearance close to the control (C). 200X

Fig. 4: Quantitative analysis of midbrain and striatal neuronal degeneration calculated as the percentage of degenerated neurons in comparison to total neurons

- Data are presented as means ± SEM (n=6).
- *, # statistically significant compared to control and rotenone-treated groups, respectively at p<0.05 using one way ANOVA followed by Tukey’s test for multiple comparisons between groups.
Effect of rotenone and/or TMP on midbrain caspase-3 expression

Immunohistochemical staining of the midbrain sections showed that rotenone increased caspase-3 expression compared to the control and co-treatment with TMP reduced caspase-3 expression compared to rotenone treated rats as shown in fig (5).

**Fig. 5:** Immunohistochemical staining of midbrain caspase-3 positive cells immunized with goat-anti-rabbit antibodies of the control group (A), rotenone (2 mg/kg) treated group (B), rotenone + TMP (20 mg/kg) (C) and TMP (20 mg/kg)-alone treated group (D).
Discussion

PD is the most common neurodegenerative movement disorder. It is clinically characterized by bradykinesia, resting tremor, rigidity and postural instability (Moore et al., 2005; Soldner et al., 2009). Current medications for PD include Levodopa, dopamine agonists, monoamine oxidase inhibitors (MAOI), catechol-O-methyl transferase (COMT) inhibitors and anticholinergic drugs (Rascol et al., 2003). However, they only provide symptomatic treatment and none of them can delay the disease progression. In addition, the medication’s side effects in chronic condition, are difficult to deal with. Thus, new therapeutic agents and disease-modifying strategies are urgently needed in PD (Strecker and Schwarz, 2008).

Rotenone, a commonly used natural pesticide is a classical high affinity specific inhibitor of complex I. Owing to its lipophilicity, it crosses the blood–brain barrier rapidly and accumulates in subcellular organelles like mitochondria (Sherer et al., 2003). Chronic systemic exposure to rotenone is often used to model PD in rats since it induces dopaminergic neurodegeneration, Parkinson’s like behavior and occurrence of cytoplasmic inclusions similar to the Lewy bodies (Betarbet et al., 2000). In harmony, the present study showed rotenone-induced hypokinesia and postural instability. Interestingly, this effect was attenuated through TMP administration.

Histological examination of midbrains and whole striata revealed pathological changes following subcutaneous rotenone administration shown as neurodegeneration, hemorrhage, hyalinosis and congestion of blood vessels. These findings are in accordance with previous studies showing the deleterious effects of rotenone on different brain areas (Radad et al., 2013; Verma and Nehru, 2009). Histological examinations of rat brain sections co-treated with TMP (20 mg/kg, i.p.) revealed restoration of normal histological structure.

Apoptosis can result from the activation of two distinct molecular cascades, known as the extrinsic (or death receptor) and the intrinsic (or mitochondrial) pathways. The extrinsic pathway is recruited upon activation of cell-surface death receptors, such as Fas/CD95 and the tumor necrosis factor receptor 1 (TNFR1), whereas the intrinsic pathway is triggered by intracellular stimuli such as Ca$^{2+}$ overload or increased generation of reactive oxygen species (ROS). In both pathways, initiator caspases (caspase-8 and -9, respectively) are activated and catalyze the proteolytic maturation of executioner caspases, such as caspase-3, which are the final effectors of cell death. Numerous lines of evidence indicate that activation of apoptotic pathways may contribute to midbrain dopaminergic neurodegeneration in PD (Hartmann et al., 2001a; Hartmann et al., 2001b; Tatton, 2000). Also, increased activity of executioner caspase-3 has been found in SNpc dopaminergic neurons of PD patients (Hartmann et al., 2000; Tatton, 2000). In context, the present study showed increased caspase-3 immunoreactivity in rotenone-treated rats. Rotenone is known to produce such effect as previously reported (Angeline et al., 2012; Tapias et al., 2014). As expected, TMP co-administration decreased caspase-3 immunoreactivity in rotenone-treated rats.

Conflict of interest statement

The authors declare that there are no conflicts of interest.
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