ORIGINAL ARTICLES

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Neuroprotective and neurotrophic effects of ginkgetin and bilobalide on MPTP-induced mice with Parkinson' disease

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Objectives: The neuroprotective and neurotrophic effects of natural products ginkgetin and bilobalide were investigated to explore their underlying mechanisms in Parkinson's disease (PD). *Methods*: Mice were treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (25 mg/kg) and probenecid (250 mg/kg) for five consecutive days to induce PD. Ginkgetin (5, 10, 20 mg/kg), bilobalide (10, 20 mg/kg) and bromocriptine (10 mg/kg) were administered orally for 26 days including five days of pretreatment in Parkinson mice respectively. Subsequently, behavior analysis and oxidative stress were detected. Tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP) and brain-derived neurotrophic factor (BDNF) in the substantia nigra (SN) were tested by immunostaining method. ELISA was used to detect tumor necrosis factor -α (TNF-α). *Results*: In MPTP-induced PD mice, movements and muscle functions improved by ginkgetin and bilobalide. TH positive cells were reduced to 7% ($P < 0.001$) and then recovered to 69% and 63% after treatment, later with no degenerations ($P < 0.001$). The GFAP levels decreased, while the BDNF levels increased significantly after treatment of ginkgetin and bilobalide. *Conclusions*: Ginkgetin and bilobalide showed effective dopaminergic neurons protection by reducing oxidative damage, activating microglial, and enhancing the potential of neurotrophic, which demonstrated that the two natural products were prospective candidates for the treatment of PD.

1. Introduction

Parkinson's disease (PD) is a common and slowly developing neurodegenerative disease which causes the degeneration of dopaminergic neurons in the substantia nigra (a region to control movements in the brain)(De Virgilio et al. 2016). The initial symptoms of PD include resting tremor, muscle stiffness, slow motion, and instability, which could be treated by dopamine (DA) replacement therapy (Garcia Ruiz et al. 2011). Nevertheless, continuous use of DA receptor agonist or levodopa will lead to adverse reactions, such as a severe motoric and non-motoric effects (Perez-Lloret and Rascol 2010), therefore, drugs with nerve protection and/or disease control effects would be a dramatic breakthrough in PD treatment options.

Oxidative stress has been reported to play a key role in the neurodegeneration associated with PD (Zhang et al. 2000). It was discovered that this negative effect decreases the levels of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) from dissection's results that the striatum and the substantia nigra pars (SNpc) in PD patients exposed (Jenner and Olanow 1996). Moreover, NF-κB was reported to be triggered by oxidative stress and accelerate the processes of inflammation and apoptosis (Jenner 2003; Tóbon-Velasco et al. 2014). Therefore, drugs with expected neuroprotection and anti-inflammatory effects may contribute to a delay in neural degeneration (Mohanakumar et al. 2000; Muralikrishnan et al. 2003). Additionally, drugs with potential neurotrophic effects may be help to promote the survival of neurons (Bender et al. 2015; Migliore et al. 2014).

Ginkgetin and bilobalide were selected based on the literature surveys and experimental screenings to evaluate their neuroprotective and neurotrophic effects (Defeudis 2002; Li et al. 2019; Wang et al. 2015; Zheng et al. 2000). It is well known that natural products from many plants are profitable in some physiological processes, especially in free radical scavenging (Abushouk et al. 2017). Ginkgetin is a flavonoid compound isolated from *Ginkgo biloba*, which can enhance the activities of SOD, even improve the abilities of learning and memory to various degrees in mice (Zuo et al. 2017). Hence, ginkgetin shows potential neuroprotective effects. Bilobalide is a terpenoid compound extracted from *Ginkgo biloba*, which could ameliorate learning and memory impairment caused by Aβ, regulate-apoptosis related genes, scavenge free radicals and protect brains (Defeudis 2002). Bilobalide is also capable of protecting dopaminergic neurons from inflammation-mediated injury by inhibiting the activations of microglia (Feng et al. 2019). Ginkgetin and bilobalide were demonstrated to suppress inflammatory mediators in vitro microglia studies, indicating that they were likely to have a neuroprotective function in neurodegenerative diseases (Abushouk et al. 2017). Therefore, the main purpose of this study was to appraise the neuroprotective and neurotrophic potentials and mechanism of ginkgetin and bilobalide in PD mice.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a well-known drug to induce PD in mice (Gubellini and Kachidian 2015; Zeng et al. 2018). The rotation was implemented along with open field tests for behavioral analysis. Degeneration degrees of dopaminergic neurons were assessed by tyrosine hydroxylase (TH) positive cells in the SNpc using immunohistochemistry. Besides, the levels of oxidative stress in midbrain were estimated by the SOD and CAT activities, as well as the amount of GSH reduction and lipid peroxidation (LPO) levels. Inflammatory mediators such as TNF-α and glial fibrillary acidic protein (GFAP) were detected to ascertain whether ginkgetin and bilobalide would diminish inflammatory reaction induced by MPTP. Brain-derived neurotrophic factor (BDNF) levels had been chosen to represent the neurotrophic activity further.

2. Investigations and results

2.1. Ginkgetin and bilobalide administrations improve movements in MPTP-induced mice

The rotarod and the open field test are classical methods to judge whether drugs improve dyskinesia in Parkinson mice (Kraeuter et al. 2019; Shiotsuki et al. 2010). Consequently, the two tests were performed. As can be seen in Fig. 1A, ginkgetin 5 mg/kg (P<0.01), 10, 20 mg/kg (P<0.01) led to a dose-dependent improvement of the rotational activities in MPTP induced PD mice. Similarly, bilobalide 10 mg/kg (P<0.01), 20 mg/kg (P<0.001) significantly increased the movements of MPTP-induced PD mice. The rotational activity of mice in the bromocriptine (10 mg/kg) group at 4th and 20th days was significantly better than that in mice treated with MPTP alone (P<0.001).

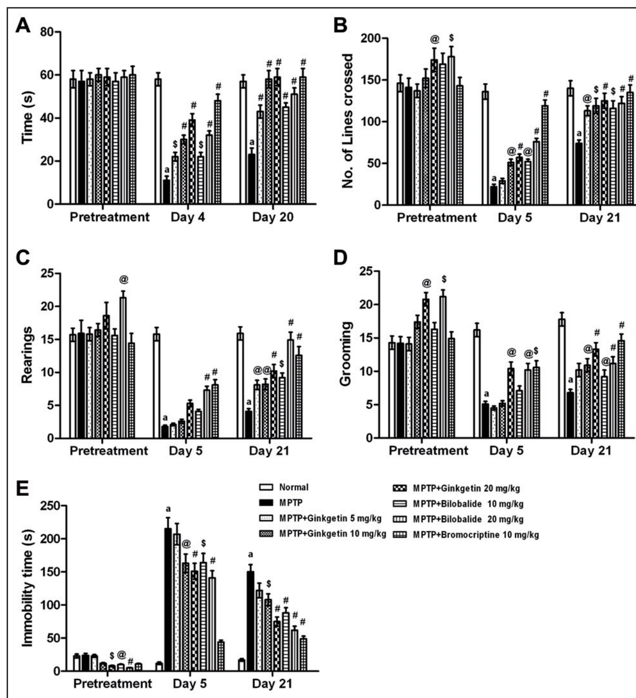

Fig. 1: Rotarod and open field performance in different experimental groups. A: Time;B: Lines crossings; C: Rearing; D: Grooming; E: Immobility time. Data expressed as mean±SEM. N-10; df (between columns) = 7; F values for time: Pretreatment: F = 0.85; for Day 4: F = 26; for Day 20: F = 16; F values for Lines crossings: Pretreatment F = 4.7; Day 5 F = 26; Day 21 F = 7.1. Rearing: Pretreatment F = 3.1; Day 5 F = 23; Day 21 F = 12. Grooming: Pretreatment F = 4.3; Day 5 F = 11; Day 21 F = 12. Immobility time: Pretreatment F = 5.4; Day 5 F = 53; Day 21 F = 26. a P < 0.001 compared with normal group and @ P < 0.05, $ P < 0.01, #P < 0.001 compared with MPTP group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

On the 5th day after MPTP administration, the movements of mice treated with ginkgetin 10 mg/kg (P<0.05), ginkgetin 20 mg/kg (P<0.001), bilobalide 10 mg/kg (P<0.05) and bilobalide 20 mg/kg (P<0.001) increased dramatically compared with those of mice in the MPTP only group (Fig. 1B). On the 21st day, the movements of mice in all the drug treated groups increased significantly. Inevitably, the movements of MPTP induced PD mice in the bromocriptine (10 mg/kg) treated group (P<0.001) increased on the 5th and 21st days.

After the administration of MPTP, the feeding quantity of MPTP mice was remarkably lower (P<0.001) than that of the normal mice (Fig. 1C). On the 5th day, the feeding behaviors of mice treated with ginkgetin 20 mg/kg (P<0.01) and bilobalide 20 mg/kg (P<0.001) were significantly improved, though mice in all drugs administrated groups on the 21st days improved in comparison with those in the MPTP only treated group. As we predicted earlier, the group of MPTP induced PD mice treated with bromocriptine also exhibited the same results on the 5th and 21st day (P<0.001). Similarly, the grooming behaviors of mice treated with ginkgetin 20 mg/kg (P<0.05) and bilobalide 20 mg/kg (P<0.05) were strikingly increased on the 5th day. Additionally there was a significant difference between the drug treated groups of mice and the MPTP only group, where the former had been greatly improved on the 21st day. (Fig. 1D). Before MPTP injections, all mice had explored the open fields and displayed the lowest quiescent times (Fig. 1E). The movements of mice in the pre-treatments with ginkgetin 20 mg/kg, bilobalide 10 and 20 mg/kg group were strengthened compared with those in MPTP only treated group (P < 0.01, P < 0.05, P < 0.01). MPTP administration resulted in mice movements lower than normal. On the 5th day, ginkgetin 10, 20 mg/kg (P < 0.05, P < 0.01) and bilobalide 10, 20 mg/kg (P < 0.01, P < 0.001) to the treatment group of the immobility time and MPTP control group were significantly decreased in comparison, surprisingly the same results were obtained in the test time. Ginkgetin (5 mg/kg) lowered the silence of MPTP induced PD mice, although it was not obvious. The movements of mice in all the other groups were significantly enhanced.

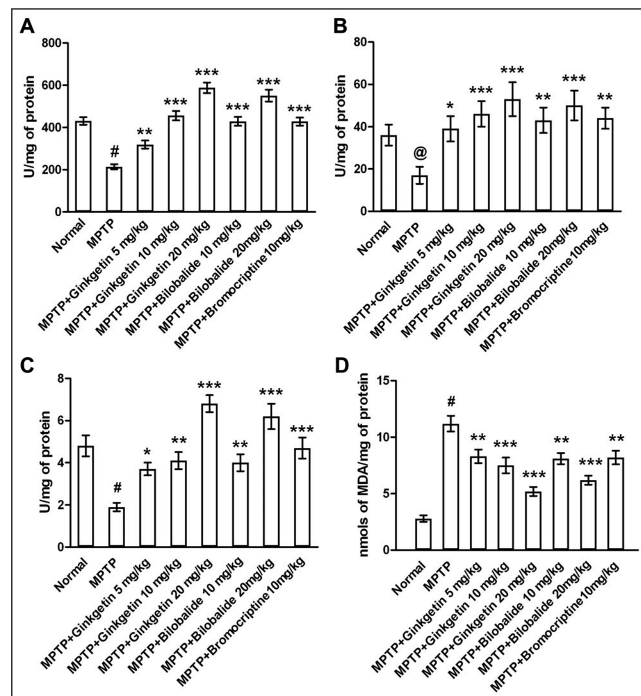

Fig. 2: Effect on SOD, CAT, GSH and LPO in midbrain. A: SOD; B:CAT; C: GSH; D: LPO. Data expressed as mean±SEM. N-6; df (between columns) = 7; F value for SOD = 37, CAT = 6.6, GSH = 16 and F = 22. P < 0.05, #P < 0.001 compared with normal group and *P < 0.05, **P < 0.01, ***P < 0.001 compared with MPTP group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

2.2. Ginkgetin and bilobalide administrations relieve oxidative stress in MPTP-induced mice

In order to identify the precise cellular damage caused by oxidative stress, we measured the lipid peroxidation level in the midbrain. Oxidative stress assessment included the analysis of SOD, CAT and GSH, which were the most critical substances in the natural antioxidant defense system (Fig. 2). The SOD (P<0.001), CAT (P<0.05) activities and GSH (P<0.001) levels were significantly reduced in mice compared to normal, demonstrating that MPTP administration could lead to the production of oxidative stress.

The SOD activity was increased dose-dependently in the 5, 10 and 20 mg/kg ginkgetin administration groups (P<0.01, P<0.001, P<0.001, respectively) (Fig. 2A). Similarly, the SOD activity in 10 and 20 mg/kg bilobalide administration groups was higher than that in the MPTP group. Attractively, we found that the

increases of SOD activities in ginkgetin and bilobalide groups were larger than that in normal mice. Although bromocriptine was found to protect the body from oxidative stress by increasing the activity of SOD (P < 0.001) compared with MPTP group, the effect was weaker than that in the ginkgetin and bilobalide groups.

The activity of CAT was significantly improved after administration of ginkgetin 5, 10, 20 mg/kg (P<0.05, P<0.001, P<0.001) and bilobalide 10, 20 mg/kg (P<0.01, P<0.001) (Fig. 2B). The levels of reduced protective GSH in ginkgetin 5, 10, 20 mg/kg (P<0.05, P<0.01, P<0.001) group and bilobalide 10, 20 mg/kg (P<0.01, P<0.001) group increased compared with the MPTP group (Fig. 2C). Interestingly, the GSH values in high-dose ginkgetin and bilobalide (20 mg/kg) groups recovered more than lower doses groups (5, 10 mg/kg), even much better than the control group. The antioxidant defense system (SOD, CAT and GSH) in these cells was better than in the normal control group after administrations of ginkgetin and bilobalide. The capacities to enhance the antioxidant defense system of ginkgetin and bilobalide were more significant than that of bromocriptine.

In the MPTP control group, it was higher than that in the normal control group (P<0.001) (Fig. 2D). The lipid peroxidation levels in the ginkgetin 5, 10, 20 mg/kg (P<0.01, P<0.001, P<0.001) groups was evidently lowered compared with that in the MPTP control group, while that in bilobalide 10, 20 mg/kg (P<0.01, P<0.001) and bromocriptine group was also decreased similarly. High doses of ginkgetin and bilobalide (20 mg/kg) demonstrated stronger protections of organisms from oxidative damage in MPTP induced damage than bromocriptine.

2.3. *Ginkgetin and bilobalide administrations enhance neuroprotection and neurotrophic effects in MPTP-induced mice*

The combined administrations of MPTP and probenecid resulted in significant dopaminergic neuron toxicities. Generally, the levels of TH-positive cells, GFAP and BDNF were used to characterize the effects of drugs. Only 7% of the TH-positive cells (P < 0.001) were found in the SNpc of MPTP group compared with that in the normal group (Fig.3 and Fig.4). Comparatively, the TH-positive cells in ginkgetin 10 and 20 mg/kg group were 54% and 69% separately, while in the bilobalide10 and 20 mg/kg groups, they were 46% and 63% respectively. Nonetheless, only 25% TH-positive cells exited in the bromocriptine group, much lower than those in ginkgetin and bilobalide (10 and 20 mg/kg).

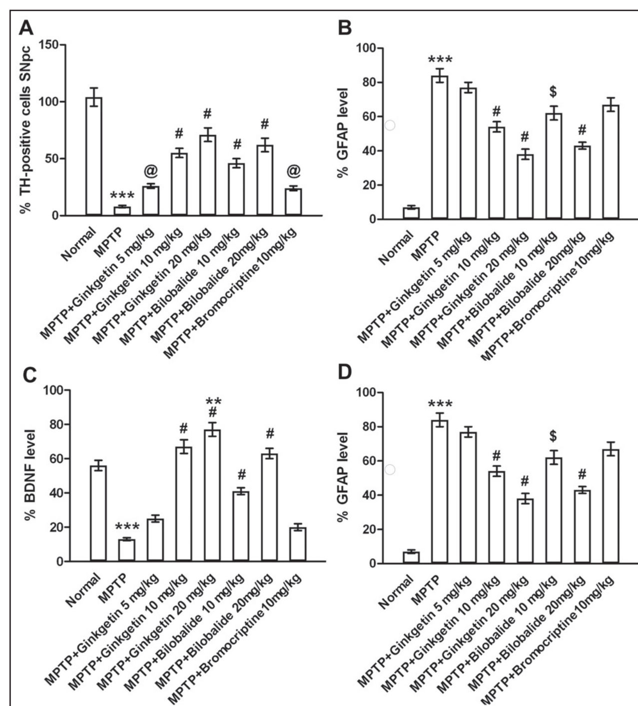

Fig. 3: Immunostaining of tyrosine hydroxylase TH-positive neurons, level of GFAP, BDNF and TNF-α in the SNpc of midbrain. A: TH-positive neurons; B: GFAP; C: BDNF; D: TNF-α. Data expressed as mean±SEM. N-6; df (between columns) = 7; for TH-positive neurons: F = 67.75.for GFAP: F=47. for BNDF: F = 40.for TNF-α F = 12. ***P < 0.001 compared with normal group and P < 0.05, $ P < 0.01, #P < 0.001 compared with MPTP group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

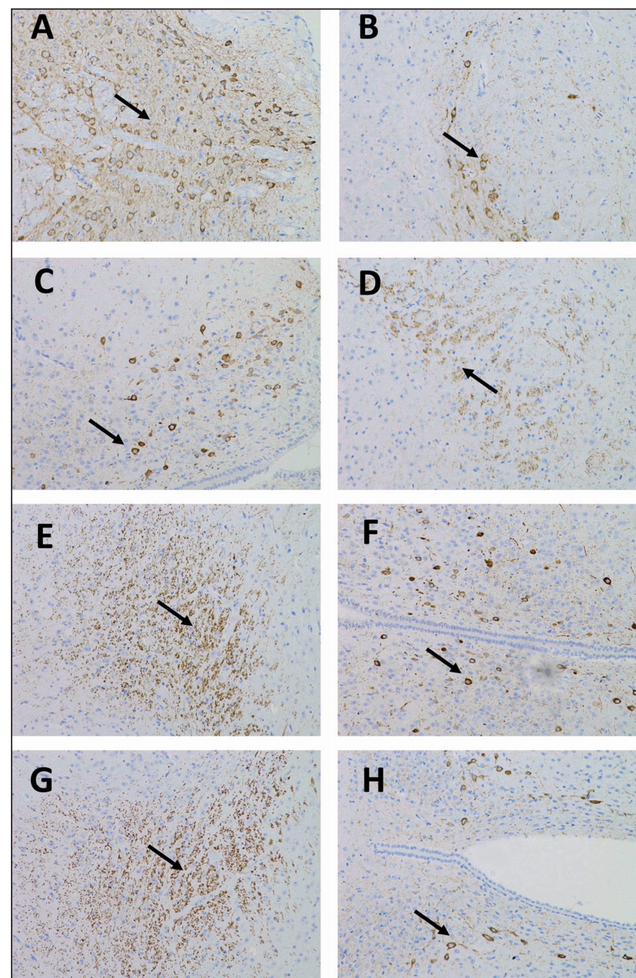

Fig. 4: TH immunohistochemistry N-4. Representative photomicrographs of TH-immunoreactive neurons in the SNpc. A-Normal, B-MPTP, C-MPTP + ginkgetin 5 mg/kg, D-MPTP + ginkgetin 10 mg/kg, E-MPTP + ginkgetin 20 mg/kg, F-MPTP + bilobalide 10 mg/kg, G-MPTP + bilobalide 20 mg/kg, H-MPTP + bromocriptine 10 mg/kg.

What is shown in Fig. 5 is the histochemical staining of GFAP, especially in the SNpc, and brown staining was on behalf of active GFAP positive astrocytes. The GFAP level in the MPTP group increased by 85% (P<0.001) than that in the control group (Fig. 3B). After administration of ginkgetin 10, 20 mg/kg (P<0.001) and bilobalide 10, 20 mg/kg (P<0.01, P<0.001), the GFAP levels decreased significantly in MPTP mice. High doses of ginkgetin and bilobalide (20 mg/kg) were found to improve GFAP levels better than bromocriptine.

The level of BDNF in the SNpc was about 60% in normal control mice, while that was significantly lowered (14%, P<0.001) in MPTP group (Fig. 6 and Fig. 3C), on the contrary, increased statistically in ginkgetin 10, 20 mg/kg group(P<0.001). Interestingly, the BDNF level in ginkgetin 20 mg/kg was 77% higher than that in the normal control group. Similarly, the BDNF positive neurons in bilobalide 10, 20mg/kg groups were 46% and 63%, respectively, which were only higher than the MPTP control group (P<0.001).

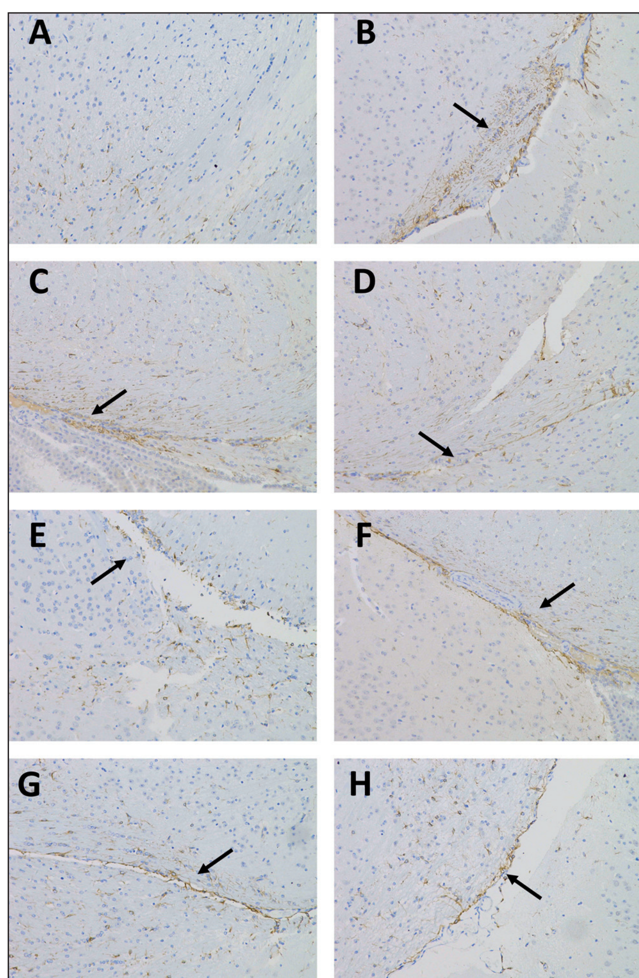

Fig. 5: GFAP immunohistochemistry N-4. Representative photomicrographs of GFAP-immunoreactive astroglial cells in the SNpc. A-Normal, B-MPTP, C-MPTP + ginkgetin 5 mg/kg, D-MPTP + ginkgetin 10 mg/kg, E-MPTP + ginkgetin 20 mg/kg, F-MPTP + bilobalide 10 mg/kg, G-MPTP + bilobalide 20 mg/kg, H-MPTP + bromocriptine 10 mg/kg.

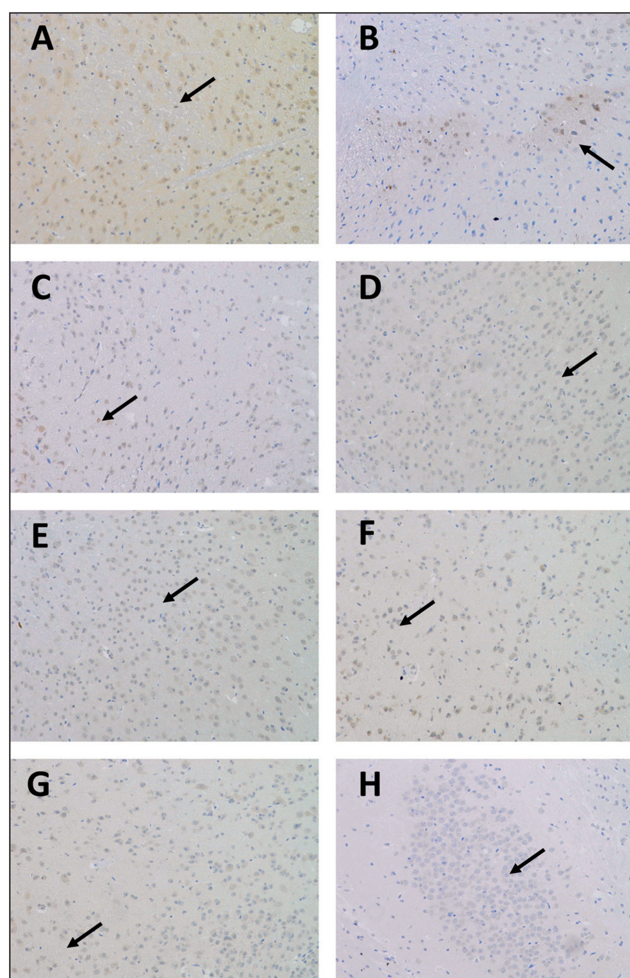

Fig. 6: BDNF immunohistochemistry N-4. Representative photomicrographs of BDNF immunoreactivity in the SNpc. A-Normal, B-MPTP, C-MPTP + Ginkgetin 5 mg/kg, D-MPTP + ginkgetin 10 mg/kg, E-MPTP + ginkgetin 20 mg/kg, F-MPTP + bilobalide 10 mg/kg, G-MPTP + bilobalide 20 mg/kg, H-MPTP + bromocriptine 10 mg/kg.

2.4. Ginkgetin and bilobalide administrations eliminate neuroinflammation in MPTP-induced mice

Compared with normal mice, the level of TNF-α was increased in the midbrain of MPTP-induced neurotoxic mice significantly (P<0.001) (Fig. 3D). After administration of ginkgetin 5 mg/kg, the TNF-α level did not change, but higher doses of ginkgetin (10, 20 mg/kg) could significantly decreased the TNF-α level in MPTP mice. Bilobalide 10, 20 mg/kg could also reduce TNF-α level in MPTP mice (P<0.001). Bromocriptine was found to show no effects on TNF-α level in MPTP mice.

3. Discussion

It may be promising that neural protection and nutrition strategies are applied to neurodegenerative diseases, for the reason that the technology not only slows the progression of neurodegenerative diseases, but also prompts the body to recover from disease conditions. Our study, firstly demonstrated the neuroprotective effects of ginkgetin and bilobalide in MPTP induced Parkinson's mice. The treatments of ginkgetin and bilobalide for 4 weeks exhibited many effects, such as protecting tyrosine hydroxylase (TH), reducing oxidative stresses and inflammations, enhancing neurotrophic activities, and ultimately improving Parkinson's disease mice motor coordination and motor behaviors. These are the possible mechanisms by which ginkgetin and bilobalide are effective against PD. Bromocriptine is commonly used to treat PD, and was therefore regarded as a reference drug in our study (Seeman 2015). Bromocriptine has a role in reducing oxidative stress and may have a neuroprotective potential besides the dopaminergic agonist activities (Le and Jankovic 2001).

We took into consideration that there were some limitations in the traditional MPTP mice model, thus another mice model was established in the present study. In this model, MPTP and probenecid were combined to contribute a decrease in cell apoptosis and striatal dopaminergic neurons (Jackson-Lewis and Przedborski, 2007; Shi et al. 2016). Probenecid can reduce the excretion of MPTP, and thus resulting in serious neurotoxicity. After MPTP administration, it was metabolized into MPP+, an active toxic metabolite, by monoamine oxidase B (MAO-B) in the brain (Smeyne and Jackson-Lewis 2005; Zeng et al. 2018). MPP+ had a high degree of affinity with dopamine transporter (DAT), and it entered into the dopaminergic neurons through DAT to lead to the degeneration of neurons (Ito et al. 2013). Ginkgetin and bilobalide were found to have no effect on MAO-B activities in 1~100 mM concentration in selective MAO-B inhibition experiments, suggesting that the two compounds were not involved in MPTP induced neurotoxicity.

Oxidative stress plays a more and more important role in the process of aging, so it is considered the most important risk factor of Parkinson (Jiang et al. 2016). MPTP administration led to serious free radical formation and oxidative stress in the substantia nigra, ultimately resulted in the degeneration of dopaminergic neurons (Smeyne and Jackson-Lewis 2005). In this study, the activities of SOD, CAT and the levels of GSH, LOP were determined as biomarkers of oxidative stress in the midbrain.

There are two critical enzymes, called SOD and CAT, hunting reactive oxygen species (Ighodaro and Akinloye 2018). Therefore,

the increase of the SOD and CAT after ginkgetin and bilobalide administrations indicated that the two compounds had a good protective effect against oxidative stress induced by MPTP. The reason why the cells were susceptible to be damaged is that glutathione is exhausted during the oxidative stress (Smeyne and Smeyne 2013). Ginkgetin and bilobalide showed strong abilities to scavenge reactive oxygen species and increased the glutathione activity, which might be due to the flavonoid and terpene compounds resulted in an overall reduction in oxidative stress. Bromocriptine also reduced oxidative stress activities, supporting the results for ginkgetin and bilobalide (Kline et al. 2004).

The free radicals induced by MPTP reacted with free oxygen molecules to generate the free radical, which caused the lipid membrane damage and then led to lipid peroxidation (Peña-Bautista et al. 2019). MDA is a diagnostic marker of lipid peroxidation, which was used as oxidative stress markers caused by PD (Tsikas, 2017). In the current study, the level of MDA in the substantia nigra striatum region of MPTP mice, representing the level of lipid peroxidation, was significantly elevated. The treatments of ginkgetin and bilobalide decreased the levels of MDA, and prevented the lipid peroxidation and degeneration of cells further. The reduction of LOP was related with the promising effects of ginkgetin and bilobalide to scavenge free radical.

We also found that, in our study, the immune reactivity of GFAP increased after MPTP administration, which was consistent with the previous studies (Zhang et al. 2019). Up to regulation of GFAP after the injury and astrocyte aggregation was Parkinson's long-term pathology (Brenner 2014). The treatments of ginkgetin and bilobalide lowered the activation of astrocytes, even reduced GFAP immunoreactivity. As a pro inflammatory mediator, TNF-α plays an important role in the initiation and regulation of cytokine cascades of the inflammatory response (Reale et al. 2009). Several genetic and pharmacological studies indicated that TNF-α inhibitors could improve disease status (Reale et al. 2009; Tweedie et al. 2007). Our results confirmed that TNF-α level was increased after the administration of MPTP. Ginkgetin and bilobalide decreased the level of TNF-α, which is in accordance with previous studies, thereby down-regulating the inflammatory cascade (Wang et al. 2015; Zhou et al. 2016). Importantly, the effects of ginkgetin and bilobalide to reduce TNF-α were more significant than that of bromocriptine.

The immune response of TH was determined, due to the fact that TH is the rate limiting enzyme that converts L-DOPA to dopamine, to evaluate the function of dopamine neurons and fibers in the substantia nigra (Rangasamy et al. 2019). This study produced results that MPTP administration significantly reduced the immunoreactivity of TH. The main mechanism for the loss of dopaminergic neurons in these animals, by all accounts, was the oxidative stress response induced by MPTP (Smeyne and Jackson-Lewis 2005). Ginkgetin and bilobalide (10, 20 mg/kg) showed stronger protections on dopaminergic neurons compared with the only MPTP treated group due to the antioxidant and anti-inflammatory activities. It was also shown that bromocriptine exhibited protective effects on nigral dopamine neurons, however, this effect was weaker than that of ginkgetin and bilobalide.

As is known to all, neuronal development and survival require neurotrophic support. BDNF and GDNF are the most promising neurotrophic factors in the treatment of neurodegenerative diseases (Sampaio et al. 2017). Hence, inducing endogenous neurotrophic factors expressing or enhancing their signal transmission has attracted more and more attentions currently. We found that ginkgetin and bilobalide increased the levels of BDNF in Parkinson mice in this study. Attractively, the BDNF level in Parkinson's mice treated with ginkgetin and bilobalide at a high dose (20 mg/kg) was higher than normal levels, while that in bromocriptine treated Parkinson mice was not increased. Ginkgetin and bilobalide had the potential of neurotrophic and may be helpful in dopamine cell survival.

Motor dysfunction is a clinical symptom of PD (Garcia Ruiz et al. 2011). In the behavioral analysis, Parkinson mice treated with ginkgetin and bilobalide showed better grip strength and muscle coordination compared with only MPTP treated mice in the rotation test, and they exhibited stronger movement functions in the open field test. Ginkgetin and bilobalide protected mice substantia nigra dopamine cell to be invariance, therefore, enhancing coordination of movements and the overall movement capacity. Bromocriptine improved the movement capacities of Parkinson mice more significantly than ginkgetin and bilobalide. Interestingly, in the preprocessing stage, high doses of ginkgetin and bilobalide showed the abilities to strengthen the movements in the open field test, which may be associated with its anxiolytic potential (Ma et al. 2012; Wang et al. 2015).

In conclusion, ginkgetin and bilobalide showed neuro-protection on Parkinson mice induced by MPTP. Although, bromocriptine, as a dopamine receptor agonist, showed better enhancement activities/symptom relief in the early stages of the disease compared with ginkgetin and bilobalide. However, ginkgetin and bilobalide exhibited superior reversal effects on biochemical markers than bromocriptine, which might be attributed to their additional neuroprotective (antioxidant and anti-nerve inflammation) and neural nutrition potential. This neuroprotective effect may be due to its potent antioxidant potential and the inhibitory effects on nerve inflammation in different important processes. Ginkgetin and bilobalide increased the BDNF level in the SNpc, indicating that they have the neurotrophic potential. In view of this, the combined treatment of ginkgetin and bilobalide could relieve symptoms and delay the disease process, which may be a reasonable method for the treatment of PD and other neurodegenerative disorders.

4. Experimental

4.1. Chemicals and reagents

Ginkgetin (98%), bilobalide (96%) and 1-mehtyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP-HCl) were purchased from Sigma-Aldrich (USA). Primary monoclonal mouse antibody to tyrosine hydroxylase, glial fibrillary acidic protein and brain derived neurotrophic factor were purchased from Abcam (USA). ELISA kit for the estimation of TNF-a was procured from eBioscience (USA). All other chemicals and reagents used in the experiments were of analytical grade.

4.2. Animals

Adult male Swiss-albino mice (22-25 g) were procured from Envigo (Indianapolis, IN, USA). Animals were kept on a 12 h light/dark cycle in the controlled temperature (25±2 °C) and relative humidity (50-70%) for a minimum of 3 days before experiments. All experiments were approved by the animal ethics committee at China Pharmaceutical University. Mice were randomly divided into eight groups containing 10 animals in each group. Group I: Normal control, Group II: MPTP 25 mg/kg, Group III: MPTP 25 mg/kg + ginkgetin 5 mg/kg, Group IV: MPTP 25 mg/kg + ginkgetin 10 mg/kg, Group V: MPTP 25 mg/kg + ginkgetin 20 mg/kg, Group VI: MPTP 25 mg/kg + bilobalide 10 mg/ kg, Group VII: MPTP 25 mg/kg + bilobalide 20 mg/kg, Group VIII: MPTP 25 mg/ kg + bromocriptine 10 mg/kg.

4.3. MPTP and drug treatment

This experiment was inclusive of 5 days of pretreatment (P1-P5) and 21 days of treatment (Day 1–Day 21). Except for the normal control group, all animals were treated intraperitoneally with MPTP (25 mg/kg) along with probenecid (250 mg/kg) once a day for five consecutive days (Day 1–Day 5) to induce parkinsonian symptoms(Jackson-Lewis and Przedborski 2007; Shi et al. 2016). Probenecid was administered 30 min before MPTP as it decreased the clearance of MPTP and intensified neurotoxicity (Shao et al. 2019). A volume of oral administration was calculated based on 1 ml/100 g, using an oral gavage needle. Both ginkgetin and bilobalide were suspended in the distilled water using Tween 80. On the last day, animals were sacrificed and their brains were isolated for further biochemical parameters and immunohistochemistry studies.

4.4. Behavioral studies

4.4.1. Rotarod test

Rotarod equipment, which has been previously described, was exploited to estimate motor performance (Hamm et al. 1994; Shiotsuki et al. 2010). The premise that all mice had stable behavior was that they were pre-trained on the carousel, consisting of three sessions per day for five days consecutively (P1-P5). Their motor activity was evaluated on a rotarod at a fixed speed of 30 rpm. The time they spend was recorded meanwhile determined the average of the retention time. This test was implemented on P4, Day4 and Day20.

4.4.2. Open field test

The open field was a square arena surrounded by walls (42 cm×42 cm ×42 cm), where it was divided into nine sub-squares. The test was initiated by placing the mice in the center of the arena, lasting 5 min. The number of line crossings (crossing the square's boundaries with both forepaws), rearing (standing on its hind legs), grooming

(rubbing the body with paws or mouth and rubbing the head with paws) and duration of immobility were measured. At the end of each test, the apparatus was thoroughly cleaned with a cotton pad wetted with 70% ethanol. Video recording and scoring was done by blind observers (Kraeuter et al. 2019; Nogueira Neto et al. 2013). This test was implemented on P5, Day5 and Day21.

4.5. Perfusion and tissue processing

For immunohistochemistry analysis, 4 mice were selected to perfuse with 4% paraformaldehyde while 6 mice were perfused with normal saline solution (37 °C) for biochemical markers determination (Paletzki and Gerfen 2019; Torres et al. 2006). Brains were rinsed in ice-cold isotonic saline for further biochemical evaluations at -4 °C. Then the midbrain was segregated, some of which were homogenized with 1 ml of ice-cold 0.1 M phosphate buffer saline (pH 7.4). The homogenate was centrifuged at -4 °C (10,000 rpm; Centrifuge 5804/ 5804 R, Eppendorf, Germany) for 15 min, so biochemical parameters were estimated using aliquots of homogenates.

4.6. Assessment of SOD, CAT and GSH

The ability that SOD in the brain homogenate (as described in 4.5) scavenged superoxide radicals generated by automatic oxidation of pyrogallol in the alkaline medium was monitored to characterize its activity. Traditionally, the method for determining SOD drew lessons from the proposed point previously with minor modifications (Nozik-Grayck et al. 2005; Ramasarma et al. 2015; Weydert and Cullen 2010). Total volume of the reaction mixture was 3 ml, containing 2.8 mL of potassium phosphate buffer (0.1 M, pH 7.4), 0.1 ml tissue homogenate and 0.1 ml pyrogallol solution (2.6 mM in 10 mM HCl). The rate of increase in absorbance at 325 nm was recorded at 30 s intervals for a period of 5 min. The amounts of enzymes required to engender 50% inhibition of pyrogallol auto-oxidation per 3 ml of the test mixture represented one unit of SOD.
CAT activity was assessed using similar procedures with some adjustment (Hadwan and Ali 2018; Mueller et al. 1997). A volume of 0.1 ml tissue homogenate (as described in 4.5) was incubated with 2.9 ml 10 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7) for 2 min. The rate of decrease in absorbance at 240 nm was documented for 3 min. The results were expressed as units of CAT activity/mg protein.
Reduced GSH was estimated according to the earlier theories with some changes (Cribb et al. 1989; Smith et al. 1988). A volume of 0.1 ml of tissue homogenate (as described in 4.5) was gently mixed with 2.9 ml of 5,5-dithiobis (2-nitrobenzoic acid) dissolved in potassium phosphate buffer (0.1 M, pH 7.4). Soon afterward the mixture was incubated at 37 °C for 15 min and GSH/mg protein was used to present what obtained in absorbance at 415 nm.

4.7. Determination of LPO

A classic spectrophotometric method was used to determine LPO content in the brain homogenate (as described in 4.5) on the basis of recommendations proposed by Reilly and Aust (2001); Schmedes and Hølmer (1989). Firstly, 0.2 ml tissue homogenate was added into the mixture containing 8.1% SDS (0.2 ml), 20% acetic acid solution adjusted to pH 3.5 with NaOH (1.5 ml), and 0.8% aqueous solution of TBA (1.5 ml). Secondly, distilled water was added to adjust the final volume to 4 ml. It was then heated at 95 °C for 60 min in a water bath. Thirdly, 1 ml of distilled water and 5 ml of the mixture of N-butanol and pyridine (15:1, v/v) were added and shaken vigorously after the above reaction mixture cooled. Finally, all samples were centrifuged at 4000 rpm for 10 min, and the absorbance of the organic layer was measured at 532 nm. The expression of LPO is in terms of nmol of MDA/mg protein.

4.8. Estimation of protein concentration

One of the most commonly used approaches was the dye-binding method given by Bradford, where bovine serum albumin (BSA) was used as a standard, consequently, it was chosen to measured protein content in the brain homogenate (as described in 4.5). Specifically, 5 ml of tissue homogenate was added into 200 ml of Bradford reagent and then incubated together at 37 °C for 15 min. What to do next was to measure the absorbance at 596 nm with the help of a microplate spectrophotometer (Epoch, Biotek, USA) (Data not showed).

4.9. Immunohistochemistry analysis

Frozen brain slices, with a thickness of 3 mm, which was fixed by 4% paraformaldehyde were to reflect immunohistochemistry. Normally, these sections were the regions that pass through the SNpc, containing 14~15 sections. Sections were fixed on poly-L-lysine-coated slides, transferred by three xylene changes for 30 min, and then rehydrated with 95%, 70%, and 50% anhydrous ethanol. In order to block the activity of peroxidase, an experiment was extremely essential, which was incubated with 3% hydrogen peroxide in methanol for 5 min. Primary anti-TH, GFAP and BDNF monoclonal antibodies were incubated for 30 min at room temperature and then washed with Tris buffer solution (pH 7.4) for 10 min, the same went for sections, except for incubating with poly-horseradish peroxide (Poly-HRP). Further the two above were incubated with the substrate and examined whether the color changed to brown within 5~10 min. The amounts of immune-positive cells in the stained sections was assessed through a light microscope which magnified 200 times. The procedures were performed without the examiner knowing the experimental protocol (Cregger et al. 2006; Ramos-Vara, 2017; Ward and Rehg 2014).

4.10. Determination of TNF-a by ELISA

According to the method provided by the kit, TNF-α which existed in brain homogenate prepared as stated in 2.5 was estimated by using an ELISA kit.

4.11. Statistical analysis

All the data were presented as mean ± SEM. Statistical analysis was performed with the help of analysis of variance (ANOVA) followed by Dunnett's test. For behavioral test and immunohistochemistry quantification $P < 0.05$, \$ $P < 0.01$, #$P < 0.001$ and for oxidative stress biomarkers *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ were considered to be statistically significant when compared with MPTP group.

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