Andrographolide does not protect against neuroinflammation in a Parkinsonism model: Focus on tumor necrosis factor alpha

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Abstract. The incidence of Parkinson's disease has increased in recent years, with pathological theories newly emerging. One of the most acknowledged theories is the neuroinflammation theory, which involves microglial activation and the expression of proinflammatory cytokines. Previous studies have confirmed the effects of andrographolide on anti-inflammatory characteristics; however, the role of andrographolide as a neuroprotective agent in 1-methyl-4-phenyl-1,2,3,4-tetrahydropiridine (MPTP)-induced Parkinsonism remains unknown. In this study, we used several doses of andrographolide and investigated its potential as a neuroprotective agent in an MPTP-induced Parkinsonism model. Five types of treatment were used for five groups of C57BL/6 mice. These treatments included (1) normal control, (2) MPTP treated, (3) MPTP and selegiline treated, (4) andrographolide treated at 5 mg/kgbw, and (5) andrographolide treated at 50 mg/kgbw. Immunohistochemical analyses were performed to determine tumor necrosis factor-alpha (TNFα) levels. No significant differences were observed in TNFα levels between control groups and those treated with andrographolide. TNFα levels were determined to be 10.0 ± 3.5 for the normal group, 8.4 ± 2.9 for the selegiline-treated group, 12.8 ± 7.8 for the MPTP-treated group, 5.4 ± 2.4 for the andrographolide-treated group at 50 mg/kgbw, and 5.8 ± 1.6 for the andrographolide-treated group at 5 mg/kgbw. This study suggests that no significant reduction of TNFα levels occur after treatment with andrographolide at doses of 5 and 50 mg/kgbw in an MPTP-treated Parkinsonism model, indicating no neuroprotective effect of andrographolide.

1. Introduction
Parkinson's disease (PD) was first described in 1817 by the neurologist James Parkinson as what he called “Shaking Palsy,” which was characterized by a group of symptoms including involuntary movements with reduced muscular power when not in action, a tendency to bend the trunk forward, and cognitive damage [1]. Since then, studies investigating the pathology and potential therapies for the diseases have been under intense development. Some PD forms are thought to occur sporadically, without any clear pathological reason. However, theories of the underlying pathophysiology continue...
to be introduced. Unfortunately, although there have been pharmacological improvements to diminish PD symptoms, no cure of the disease itself has been found.

Along with the complexity of the disease, the number of PD cases appears to be increasing and has confounded researchers. Indeed, PD is currently the second most common neurodegenerative disease, just after Alzheimer's disease [2]. Moreover, underdeveloped countries are currently undergoing a rapid aging process, which is a concern for disorders of old age. A recent study reported that the number of PD cases will jump two-fold from 4.1–4.6 million in 2005 to 8.7–9.3 million by 2030 [3]. The densely populated country of Indonesia is estimated to have 6.17 million PD cases by the year 2030 [4].

Over the years, the pathophysiology of PD has been hotly debated and several predominant theories exist. One emerging theory in particular involves the neuroinflammation process. This theory started with a description of activated HLA-positive microglia in the substantia nigra pars compacta, along with increasing cytokine formation, increased production of reactive oxygen and nitrogen species, and decreasing secretion of trophic factors used for normal activities related to neuronal viability [5].

A recent study showed that andrographolide, a bioactive molecule taken from Andrographis paniculata, may potentially reduce neuroinflammation. Indeed, andrographolide has been shown to decrease the activation of nuclear factor-κB (NF-κB) p65, which is involved in inflammatory regulation and the inhibition of several cytokines, namely, tumor necrosis factor-alpha (TNFα), in an Alzheimer's disease model [6]. However, these results need further investigation to confirm whether andrographolide affects neuroinflammation and would be useful in neurodegenerative treatment strategies related to PD.

2. Methods

This study used a pre-clinical experimental study design with primary data and was conducted for 4 months starting from February 2016 to May 2016. Experimental laboratory work was performed at the Pharmacology and Therapeutic Department of the Faculty of Medicine, Universitas Indonesia in Jakarta, Indonesia. The study population was naïve C57BL/6 mice. Other samples included preserved mice brain tissue from mice subjected to past experimental research that fulfilled certain criteria.

The inclusion criteria in this study were male C57BL/6 mice, aged 8–12 weeks, weighing 25–30 g each and able to function normally. The exclusion criteria were female C57BL/6 mice and those not able to function normally. The dropout criteria were mice that died before the start of the procedure. The number of subjects used for the sample in this research was calculated using Federer's formula.

Prior to this research, preserved brain tissues were obtained from mice of past studies that had undergone several treatments during various experiment stages as follows. Each rat group provided brain tissue with the following treatment (see Table 1).
Table 1. Sample preparation.

| Rat Group | Name               | Day 0          | Day 1-14       |
|-----------|--------------------|----------------|----------------|
| 1         | Normal             | -              | -              |
| 2         | Negative control   | MPTP 20 mg/kgbw| 1 % DMSO in 0.1 mL normal saline |
| 3         | Positive control   | MPTP 20 mg/kgbw| Selegiline 10 mg/kgbw |
| 4         | Test group 1       | MPTP 20 mg/kgbw| Andrographolide 5 mg/kgbw |
| 5         | Test group 2       | MPTP 20 mg/kgbw| Andrographolide 50 mg/kgbw |

The samples received two stages of the experimental study:

2.1. Treatment of mice
Acclimatization of test animals was done in cages at Eijkman Molecular Biology Institute for 1 week. Test animals were then randomly placed into the five groups described above. Parkinson induction was performed using MPTP in normal saline through subcutaneous injection of 20 mg/kgbw four times with a period of 2 h after the previous injection. Starting the next day (at least 8 h after the last MPTP injection), andrographolide was injected intraperitoneally with a dosage of 5 and 50 mg/kgbw with a frequency of once every 14 days. The dose of andrographolide given was calculated to consider the increase in body weight every 3 days. During the research period, mice were treated according to the standard treatment for test animals and were observed for signs of general toxicity such as weight loss and death.

2.2. Behavioral observations
Behavioral observations of the mice were performed by a vertical grid test and a modified horizontal grid test for the evaluation of motoric dysfunction according to the method described by Kim et al.

After given anesthesia, five mice of each test group were perfused with normal saline, followed by paraformaldehyde (4%). The mouse brain was then taken out to be dehydrated, paraffinized, and cut using a microtome. The substantia nigra part of the brains was observed using an immunohistochemistry method with an antibody toward TNFα. The analysis was done by counting TNFα-positive cells as an indicator of the neuroinflammation process. The procedure continued by cutting and mounting sections on slides coated with a suitable tissue adhesive. Sections were then de-paraffinized in gradients of xylene (xylene 1, xylene 2, and xylene 3) for 5 min each; after which, sections were re-hydrated through a gradient of alcohols (100%, 95%, 80%, and 70%) for 5 min each. Slides were then washed in aquadest for 5 min, washed in phosphate-buffered saline (PBS) for 5 min, and neutralized for endogenous peroxidase using Peroxidase Block for 20 min. Slides were then incubated with Protein Block for 20 min, followed by incubation with 1:500 diluted primary antibody against TNFα overnight. Slides were then incubated with Post Primary for 30 min, followed by Novolink™ polymer for 30 min. Between steps, slides were washed twice in PBS for 5 min. Finally, slides were washed twice in PBS for 5 min to develop peroxidase activity and incubated with DAB working solution for a few seconds. Slides were rinsed in water for 10 min, followed by counterstaining with hematoxylin and a final rinse in water for 10 min. After that, slides were dehydrated through graded alcohols (70%, 80%, 95%, and 100%) for 5 min each, and sections were re-paraffinized in xylenes (xylene 1, xylene 2, and xylene 3) for 5 min each. Lastly, sections were mounted on slides with cover slips. The slides were then observed under a microscope and counted for TNFα expression.

3. Results
3.1. TNFα levels
After following the pre-determined steps of the laboratory experiments, the data obtained from the rat brain were as follows (Table 2).
Table 2. Mean TNFα levels and cell counts.

| Group                        | Mean | +/- Standard Deviation |
|------------------------------|------|------------------------|
| Normal                       | 10.0000 | 3.50999            |
| Positive control             | 8.3600 | 2.89275              |
| Negative control             | 12.8000 | 7.78203            |
| Andrographolide 50 mg/kgbw   | 5.4000 | 2.43311              |
| Andrographolide 5 mg/kgbw    | 5.8000 | 1.61864              |

The independent \(t\)-test revealed a significant difference between levels of TNFα in Group 1 (normal control) and the two test groups, Groups 4 and 5, which were the groups treated with andrographolide 50 mg/kgbw (\(p = 0.043\)) and 5 mg/kgbw (\(p = 0.041\)), respectively. However, the same test did not show a significant difference among the other groups (\(p > 0.05\)).

4. Discussion

Neuroinflammation is known to play a key role in the progression of dopaminergic neuron loss in neurodegenerative processes, including PD. In addition, postmortem studies have shown evidence of microglial activation within the substantia nigra [7] and cerebrospinal fluid of patients with PD [8]. However, contradictory results have also been demonstrated by several studies. In one study by Ferger et al. using a chronic MPTP mouse model, genetic ablation of TNFα was found to be related to decreased MPTP-induced striatal terminal damage, whereas the same did not happen to dopaminergic neurons in the substantia nigra. Another study that targeted the deletion of either Tnfsr1α or Tnfsr1b did not have the same effect [9], and neither did another study that observed single and double TNFα receptor knockout mice [10]. Sriram et al. investigated mice lacking TNF receptors 1 and 2 and reported that striatal damage was decreased after a single injection of low-dose MPTP. However, nigral dopaminergic cell loss was not observed, and the assessment was only short term. These findings suggest that these studies may differ because of the varying extents of MPTP-induced injury, as well as the survival time of the animal models [11,12]. Besides proinflammatory cytokines such as TNFα, neuroinflammation has also been suggested by the strong expression of RelA p65 in the substantia nigra pars compacta of the model, and NF-κB activation has been shown in an MPTP-induced mice PD model [13]. Therefore, a compound with a wide range of protective effects against NF-κB activation and TNFα expression may be beneficial to help save dopaminergic neuronal loss in PD.

On the basis of histopathological observation, in this particular study, the MPTP-induced Parkinsonism model showed a slight increase in the expression of TNFα in the substantia nigra, although not significant, compared with the normal control. Previous studies have detected TNF- immunoreactive glial cells in the substantia nigra of Parkinsonian patients, but not in those of control subjects. Moreover, another study presented a significant difference in TNF receptor R1 levels in the substantia nigra compared with normal controls. Another study that observed male TNFα (−/−) deficient mice and C57BL/6 mice treated with MPTP at 4 × 15 mg/kg dose in 24 h intervals showed by real-time RT-PCR that striatal mRNA levels of TNFα were significantly enhanced after MPTP administration. In addition, the TNFα (−/−) mice showed a lower mortality compared with wild-type mice. Meanwhile, in MPTP-induced TNFα receptor 1 (−/−) and TNFα receptor 2 (−/−) knockout mice at 15/mg/kg dose for 8 consecutive days, no significant differences in striatal dopamine or dopamine transporter levels were observed between TNFR1 (−/−), TNFR (−/−), and wild-type mice, although all of them showed reduced levels. This suggests that the lack of an increase in TNFα that we observed in this study may be partly caused by the lower dose of MPTP compared with other studies. Although shown through the intense staining of the bodies of dopaminergic cells, along with TNFα-
immunopositive cells in the substantia nigra pars compacta in this particular study, the neuroinflammation may not proceed to the same extent where TNFα levels become significantly reduced.

Andrographolide significantly and dose dependently reduced LPS-induced microglial activation when given in a pretreated manner [14]. Post-treatment by andrographolide at a 2.5 μM dose up to 8 h after treatment with LPS has been shown to significantly decrease the release of proinflammatory factors, including TNFα [14]. In contrast, post-treatment at 20 h showed no significant neuroprotective effect [14]. Surprisingly, andrographolide showed no statistical significance of neuroprotection against 1-methyl-4-phenyl-pyridine-induced neurotoxicity in neuron-enriched cultures [14]. Another study also observed a marked p65 subunit translocation after 24 h of pMCAO treatment in a cerebral ischemic mice model, which was attenuated by andrographolide treatment, suggesting that NF-κB activation may be suppressed by andrographolide [15]. The present study revealed that TNFα levels in andrographolide-treated groups were reduced in comparison with both positive and negative controls, but this reduction was not significant. The reason for this may be due to the timing of the treatment, with pretreatment possibly showing a more significant effect. In addition, the time duration between the onset of the disease model and the treatment with andrographolide may have also impacted the neuroprotective effect. The observed change in TNFα levels compared with the normal group was significant, which agrees with previous studies that andrographolide may indeed have a reducing effect on TNFα. Further study is needed to determine whether the compound acts through NF-κB or other mechanisms. The change may work in a dose-dependent manner as observed in this study, where the group with the treatment of andrographolide at 50 mg/kg bw expressed lower levels of TNFα than the group treated with a 5 mg/kg bw dose.

A greater effort of research, with particular focus on a histopathological study, should be performed in order to obtain information on TNFα expression in Parkinsonism models and to determine whether andrographolide at certain doses has a neuroprotective effect against MPTP-induced Parkinsonism. More appropriate tools to conduct the cell counting of samples should also be considered to acquire better results and data.

5. Conclusions
Andrographolide administration at a dose of 5 and 50 mg/kg body weight of mice did not significantly decrease the expression of TNFα in the substantia nigra pars compacta of an MPTP-induced (20 mg/kg bw) Parkinsonism mice model, although when compared with the normal group, it did show significant changes in a dose-dependent manner. It can be concluded that the neuroprotective effect of andrographolide against Parkinsonism was not proven with the pre-determined dose of both MPTP and andrographolide treatment.

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