ORIGINAL ARTICLE

The Effect of Citrus Peel Extract (*Citrus Reticulata*) on Hippocampal Histopathological Appearance in Wistar Rats Induced by *Trimethyltin Chloride*

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ABSTRACT

Introduction: Oxidative stress and chronic inflammation play an important role in neurodegenerative disease. Flavonoids in citrus peel have antioxidant and antiinflammation activity which contribute as neuroprotective agent. The aim of this study is to determine the effect of citrus peel extract (*Citrus reticulata*) to hippocampal histopathological in Wistar rats were induced by *trimethyltin chloride*. **Methods:** 30 male Wistar rats were divided into five groups those were C1, C2, P1, P2 and P3. The C1 group was administered standard diet. The C2 group was injected intraperitoneally by *trimethyltin chloride* with a single dose (8 mg/kgBW). P1, P2, and P3 groups were injected intraperitoneally by *trimethyltin chloride* with a single dose (8 mg/kgBW) and administered orally by citrus peel extract after 48 hours injection of trimethyltin chloride with doses 56.25; 112.5; and 225 mg/kgBW/day until 14th day. On 15th day, samples were terminated and hippocampal tissues were collected for microscopic preparations. **Results:** The number of degenerative neurons of hippocampus in C2 (35.80 ± 13.084) was significantly higher than P1 (19.20± 9.230; p=0.049), P2 (15.80 ± 4.919; p=0.013) and P3 (19.60 ± 4.929; p=0.032). There was a significant difference of the number of degenerative neurons of hippocampus between C1 (19.40 ± 7.232) and C2 (35.80 ± 13.084; p=0.040). **Conclusion:** Administration of citrus peel extract (*Citrus reticulata*) gave an effect for hippocampal histopathological appearances in Wistar rats induced by *trimethyltin chloride*.

Keywords: Citrus peel extract, *Trimethyltin chloride*, Neuroprotective agent, Hippocampal histopathological

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INTRODUCTION

Dementia is neurodegenerative disease that progressively attacks two or more cognitive functions. According to data from World Health Organization (WHO), there were estimated 35.6 million people worldwide living with dementia in 2010 and are expected will increase doubly every 20 years, reaching 65.7 million in 2030 and 115.4 million in 2050 (1). The most common form of dementia is Alzheimer's Disease (AD), accounting for around 60-70% of cases. The incidence of AD increases with age, it is around 3% at the age of 65-74 years, 19% at the age of 75-84 years and 47% at the age of more than 84 years (2).

Neurodegenerative disease is a progressive degeneration process and gradually attacks the nervous system. Oxidative stress and chronic inflammation have big contribution in the process of neurodegenerative diseases. In physiological conditions, the levels of free radicals are maintained and regulated by enzymatic and non-enzymatic antioxidant activities. Excessive levels of *reactive oxygen species* (ROS) or *reactive nitrogen species* (RNS) result in an inflammation process in neuron (3). High metabolic activity and low antioxidant defense ability cause neurons in the brain to be more susceptible to oxidative stress, especially in neurons of aging brain.(4) The pathogenesis of Alzheimer's disease begins with the formation of Aβ deposits in the brain due to the mutation of *amyloid precursor proteins* (APP). Accumulation of Aβ in the brain results in oxidative damage to neurons (5).

Nowadays there is no effective therapy for neurodegenerative diseases, and mostly the marketed drugs are available for symptomatic therapy. Therefore, a lot of study had been done to find natural ingredients which are proposed as neuroprotector agents, one of them is citrus peel (3). Polymethaloxyflavones (PMFs) is the most abundant flavonoid in citrus peel which play a great contribution as an antioxidant and

neuroprotective agent.(3) The observation was made on rat hippocampus which is part of the cerebrum temporal lobe. The hippocampus is one part of the limbic system (6).

MATERIALS AND METHODS

Animals

Thirty male Wistar rats (aged 2-3 months, weight 200-300 grams) were purchased from the nutrition laboratory of Gadjah Mada University, Yogyakarta. All rats were provided with food and water ad *libitium* throughout the experimental period. Rats were house in cages according to their own groups. Rats were adapted for seven days before treatment, then they were randomly devided into five groups those were C1, C2, P1, P2 dan P3.

Citrus Peel Extraction

One kg of citrus peel was cleaned with water. Then the citrus peel was dried in the oven for 48 hours at a temperature of 40°C. After that, the citrus peel was crushed with blender until smooth, then filtered through a 24-mesh sieve. Citrus peel powder macerated with ethanol (ratio 1:6) for 48 hours, then filtered through *Buchner* funnel. The filtrate was evaporated by rotatory evaporator at 40oC with a pressure of 100 mBar until all the solvents evaporate (7).

Induction with *Trimethyltin chloride*

Treatment has been done for 14 days. Rats were given TMT intraperitoneally with a single dose (8 mg/kgBW) to induce AD in rats. Based on previous study, rats were injected with TMT on the 14th day started showing cell death in the hippocampus as signaling of degenerative change in the brain (8).

Experimental Protocol

Rats were randomly were devided into five groups with six rats in each group. Group C1: healthy normal control group was administered orally by aquadest; Group C2: rats were injected intraperitoneally by TMT with a single dose (8 mg/kgBW); Group P1: rats were injected intraperitoneally by TMT with a single dose (8 mg/kgBW) and administered orally by citrus peel extract with dose 56.25 mg/kgBW after 48 hours TMT injection; Group P2: rats were injected intraperitoneally by TMT with a single dose (8 mg/kgBW) and administered orally by citrus peel extract with dose 112.5 mg/kgBW after 48 hours TMT injection; Group P3: rats were injected intraperitoneally by TMT with a single dose (8 mg/kgBW) and administered orally by citrus peel extract with dose 225 mg/kgBW after 48 hours TMT injection.

Histopathological Materials

Histopathological observation was done for finding out the effect of citrus peel extract in inhibiting pathological changes in hippocampus of Wistar rats. Termination of rats were done on the 15th day. Rat brains were removed and placed in 10% neutral buffer formalin (NBF) for 24 hours. The brains were cut transversely in one third on the back of brains a thick at 3-5 mm thicknesses to get cortex, parietal lobe, temporal lobe and hippocampus, then stained with *hematoxilin and eosin* (HE) (8).

RESULTS

This study was conducted to assess the effect of citrus peel extract as an neuroprotective intervention to hippocampal histopathological appearances of rats wistar were induced by TMT. This study was started by inducing rats in C2, P1, P2, P3 groups with TMT injection intraperitoeally with single dose (8 mg/kgBW) on the first day. After 48 hours of TMT injection, rats in P1, P2, P3 groups were adminstered orally by citrus peel extract with dose 56.25 mg/kgBW, 112.5 mg/kgBW, 225 mg/kgBW. Citrus peel extract was administared in the morning to optimize absorption of citrus peel extract in digestive tract.

Microscopic examination was performed in Corpus Amnion of hippocampus. Observation of the entire field is done at 100x magnification, then for counting the number of neurons at 400x magnification. Based on the results of microscopic observation of the hippocampus showed the differences in the number of degenerative neurons in each group. Degenerative neurons marked with pyknosis of the nucleus and more eosinophilic colored cytoplasm (8). Fig. 1 shows microscopic appearances of rats hipoccampus on C2 group, P1 group, P2 group, P3 group.

Table I shows the number of hippocampal degenerative neurons in study groups. Microscopic appearance of C2 group showed the largest number of hippocampal degenerative neurons (35.80 ± 13.084). Microscopic appearance of C1 group showed the distribution of normal neurons and few degenerative neurons (19.40 ± 7.232) which are a physiological process of normal cells as known as apoptosis and normally occurs as an aging process of cells. Microscopic appearances of P1 and P3 groups mostly showed the number of degenerative neurons closed to the number degenerative neurons of C1 group (19.20 ± 9.230; 19.60 ± 4.929). P1 and P3 groups had the number of hippocampal degenerative neurons smaller than C2 group. Microscopic appearance of P2 group showed the smallest number of hippocampal degenerative neurons (15.80 ± 4.919). This result showed that administration of citrus peel extract could decrease degeneration of neurons caused by injection of TMT.

Statistical test showed no significant differences among the doses of administration, but they showed trend of decreasing the number of degenerative neurons in each study group which were given citrus peel extract (*Citrus reticulata*) that can be seen in Fig.2. There was significant differences ($p<0.05$) of the number of

degenerative neurons between C1 group and C2 group (*p=0.040*), between C2 group and P1 group (*p=0.049*), between C2 group P2 group (*p =0.013*), and between C2 group and P3 group (*p=0.032*) which can be seen in Table II.

Table I :The number of hippocampal degenerative neurons in study groups.

Groups	Number of Degenerative Neurons (Mean ± SD)	Median (min-max)
Normal control (C1)	19.40 ± 7.232	19.00 (9.00-28.00)
TMT (C2)	35.80 ± 13.084	40.00 (20.00-49.00)
Treatment 1 (P1)	19.20 ± 9.230	18.00 (8.00-32.00)
Treatment 2 (P2)	15.80 ± 4.919	14.00 (11.00-22.00)
Treatment 3 (P3)	19.60 ± 4.929	22.00 (11.00-23.00)

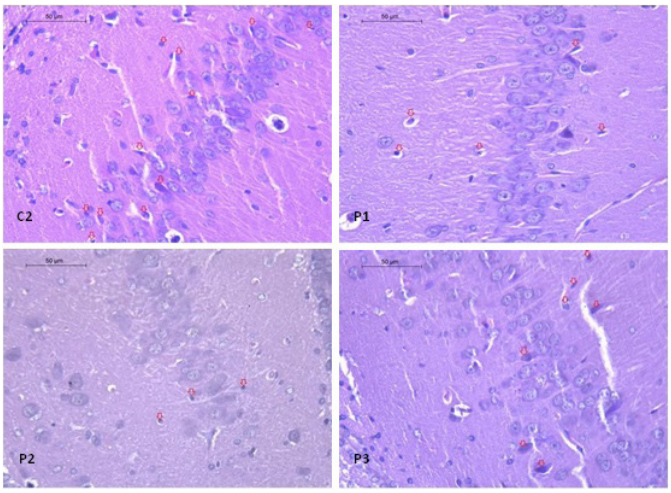

Fig. 1 : Hippocampal degenerative neurons in C2, P1, P2 and P3 groups. Degenerative neurons marked with pyknosis of the nucleus and more eosinophilic colored cytoplasm.(8). In C2 group, the result of microscopic observation showed the most abundant of degenerative neurons. Degenerative neurons on hipocampus are shown by red rows.

Table II : The differences of the number of hippocampal degenerative neurons among study groups

	C1	C2	P1	P2	P3
C1	-	*p=0.040**	*p=0.971*	*p=0.384*	*p=0.961*
C2		-	*p=0.049**	*p=0.013**	*p=0.032**
P1			-	*p=0.488*	*p=0.934*
P2				-	*p=0.257*
P3					-

*Mann Whitney tets, significant p<0,05

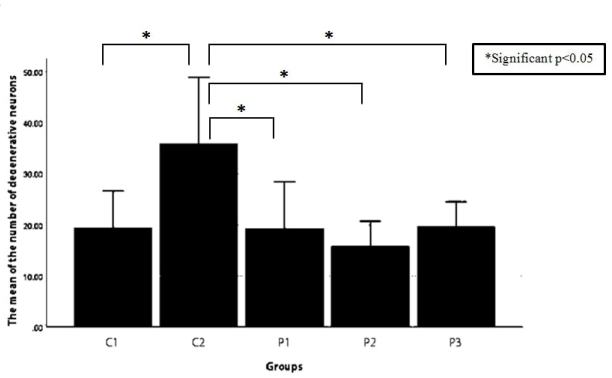

Fig. 2 : The comparative analysis of the number of degenerative neurons between groups. The comparative analysis between groups was assessed using *Mann Whitney* test. There were significant differences of the number of degenerative neurons between C1 group and C2 group, between C2 group and P1 group, between C2 group P2 group, and between C2 group and P3 group. Graph shows trend of decreasing the number of degenerative neurons in study group which were given citrus peel extract (*Citrus reticulata*).

DISCUSSION

Based on the result of this study, in the C2 group showed that the rats injected with a single dose (8 mg/kgBW) of TMT intraperitoneally on day 14 showed a highest number of degenerative neurons in hippocampus compared to the C1 group (*p = 0.040*). The microscopic apperarance showed that degenerative neurons were indicated by the picnotic nucleus and cytoplasm were more eosinophilic in color. This result is appropriate with the theory from previous study by *Kristianingrum* et al. (2016) (8). C1 group also showed degenrative neurons, but it is still in minimal numbers, this happened because of the apoptosis process which is physiologically experienced by all normal cells, where every cell in the body will experience an aging process which ends with cell death then will be replaced with new cells through the regeneration process (2).

Trimethyltin chloride (TMT) is a chemical compound that has neurotoxic activity which can cause the death of neurons in the limbus and hippocampus system in humans and animals (17). TMT can easily pass through the blood vessel barrier of the brain and enter the brain because this compound is dissolved in water and fat so that it has the potential to cause damage to the brain (8). TMT produce oxidative stress in brain neurons by increasing the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (10). Physiologically, free radical levels are maintained and regulated by enzymatic and non-enzymatic antioxidant activity. Disruption of the balance between the production and elimination of oxidizing chemical species can cause oxidative stress that leads to cellular damage. Excess of ROS and RNS levels cause inflammatory processes in neurons. High metabolic activity and low antioxidant defense capabilities make neurons in the brain more susceptible to oxidative stress, especially in aging brain neurons (2).

Antioxidant and anti-inflammatory activities play an important role in neuroprotective mechanisms. Based on the previous study, it is proven that flavonoids in citrus peel have bioactivity that can improve health, such as antioxidant, anticarcinogenic, antiviral, anti-inflammatory, antitumor and *antiatherosclerosis*. Flavonoids contained in citrus peel are *flavanones, flavones,* and *polymethoxyflavones*. The main flavonoids contained in citrus peel such as hesperidin, neohesperidin, and *hesperitin* have antioxidant activity and are able to pass through the brain barrier, so that flavonoids in citrus peels have the potential to inhibit neurodegeneration and improve brain function (4, 12).

In this study, it was found that the administration of citrus peel extract (*Citrus reticulata)* with dose 56.25 mg/kgBW; 112.5 mg/kgBW; 225 mg/kgBW showed better microscopic appearance compared to the C2 group that was given TMT with singledpse (8 mg/kgBB) intraperitonelly, where most of the neuron cells in the hippocampus were in good condition and only few neurons were degenerated. The improvement of these microscopic appearance are due to the administration of the orange peel extract which has a neuroprotective effect. Extract of orange peel (Citrus reticulata) has a neuroprotective effect, because it has the ability to act as an antioxidant that can counteract the bad effects of free radicals caused by TMT injection. The nuroprotective effect on orange peels has been proven from previous studies on the potential of orange peels as antioxidants, such as in the study conducted by *Hwang et al.* (2012)(4).

In this study, a stratified dose of citrus peel extract (*Citrus reticulata*) were given in the treatment groups, dose of 56.25 mg/kgBW; 112.5 mg/kgBW; 225 mg/kg. Based on the result, the dose 112.5 mg/kgBW was the best dose to reduced the number of degenerated neurons in the hippocampus. The dose of 56.52 mg/kgBW showed a better decreasing in the number of degenerated neurons than the dose of 225 mg/kgBW. Although there was meaningful difference among the doses after statistical tests, there was a visible trend of decreasing the number of degenerated neurons in each study groups. So these results can showed neuroprotective effect of flavonoids in the extract of orange peel (*Citrus reticulata*).

CONCLUSION

Administration of citrus peel extract (*Citrus reticulata*) with doses 112.5 mg/kgBW, 56.25 mg/kgBW dan 225 mg/kgBW for 14 days reduced the number of degenerative neurons in hippocampus of rats induced by TMT (single dose 8 mg/kgBW). This study proves citrus peel extract (*Citrus reticulata*) has neuroprotective effect in hippocampal hitopathological appearances in rats induce by TMT. Further study is needed to prove that rats have Alzheimer's disease after TMT injection use complex staining for histology of rats brain.

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