INFLUENCE OF GINKGO BILOBA (EGB) EXTRACTS IN APOPTOSIS INDEX AND NUMBER OF NEURONS AT RATTUS NOVERGICUS WITH LEAD (Pb) EXPOSURE

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ABSTRAK

Background: One of the wastes that often pollutes the environment is lead (Pb). Pb intoxication can cause damage to various organs in humans, one of which is in the nervous system. EGb is known to have neuroprotective effects. The study of the clinical benefits of EGb on various diseases has been carried out, but the mechanism of EGb in the repair process of brain cell damage due to Pb exposure is still in doubt, one of which is the effect of EGb on neuron proteins that affect apoptosis and neuronal cell death due to Pb intoxication.

Objective: to determine the effect of ginkgo biloba extract on the apoptotic index and the number of neurons in Rattus novergicus with lead exposure

Method: This study is an experimental study by means of randomized experimental post study design. The study uses test animal with several criteria, as follow; male rats, aged 4-5 months, weighing 140-150 grams. The sample of this study was healthy male rats fulfilling the inclusion criteria and exclusion criteria. The sample was divided into 4 groups. The variables in this study are divided into three, namely: (1) Independent variables (2) Dependent variables (3) Controlled variables. After the data is obtained and analyzed, then statistical analysis is carried out, namely (1) Test for normality (2) ANOVA test to prove the difference in mean of the dependent variable in this study.

Results: in the examination method and the expression of apoptotic index results showed that administration of EGb can increase BDNF expression in rats with lead exposure. Statistically K2 (Rat with lead exposure (50mg/kgBW), without EGb treatment) and K4 (Pb exposure + EGb) showed that the Apoptosis Index was significantly different (Mann-Whitney = Z = -2,887; p = .004). Furthermore, the results of the examination of the number of neurons showed that administration of EGb can increase the number of neurons in rats with lead exposure. The results of the examination of the number of neurons from the Anova calculation found a value of F = 24,532 and p = .000.

Conclusion: Application of EGb can increase BDNF expression and the number of neurons in rats with lead exposure.

Keywords: Ginkgo biloba extract, hippocampal neuron cell damage, lead exposure

INTRODUCTION

Progress and growth of the industrial sectors have a negative impact on environmental pollution, especially on residual production or waste produced by the industrial sector. Lead (Pb) is one type of industrial waste that often pollutes the environment.1 Pb intoxication occurs if the level of Pb in venous blood is ≥ 10 ug/dl. Pb intoxication above the normal limit will cause health problems, especially in children

Pb enters the body through consumption of food, drinks, air, water, and dust contaminated with Pb3, then absorbed mainly through the gastrointestinal tract, inhalation or absorbed by the skin.

In the digestive system, about 15% of inorganic Pb is absorbed by the body. The absorbed Pb is higher in children, pregnant women, and people with calcium, zinc, or iron deficiency. Even absorption can reach 50% if swallowed by infants and children. In adults, about 35-40% of PB dust is inhaled and then stored in the lungs. About 95% enter the bloodstream4. This blood serves as the initial storage place after absorption, then lead will be distributed throughout the body. Lead interferes with calcium homeostasis, where lead and calcium compete in the plasma membrane in the transport system, so the lead can penetrate the blood-brain barrier (BBB) [5].

Pb intoxication causes physiological, biochemical and behavioral dysfunction. Pb intoxication can cause neurological, cardiac, kidney, liver, bone, intestinal and reproductive abnormalities3,4. Since the last decade, much attention has been paid to the hippocampus, which is the target of Pb exposure, which causes cognitive impairment, learning, and memory. The direct effects of Pb on the CNS can cause morphological and pharmacological disorders. Morphological effects cause disruption of migration, differentiation of neurons, disruption of synapse formation. The pharmacological effects of Pb interfere with the release of neurotransmitters, the function of the GABAergic, dopaminergic, cholinergic systems. NMDA ion channels, cell membranes, energy metabolism, and mitochondria. As a result, Pb triggers the formation of reactive oxygen species (ROS), mitochondrial damage and stimulates apoptosis. At high doses, the most severe neurological effect of Pb exposure is encephalopathy [3,6–8].

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Pb intoxication causes oxidative stress through two different pathways that occur simultaneously. First is the emergence of the Radical Oxygen Species (ROS) generation, and the second is the depletion of antioxidant reserves[3,9]. Pb can affect the ROS metabolizing enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), reducing intracellular glutathione (GSH) levels [10].

The mechanism of lead toxicity is still controversial, through many mechanisms. In cell membranes, Pb causes peroxidative damage to lipids and proteins, which in turn stimulates the formation of free radicals and disorders of the antioxidant mechanism[11]. In the Pb mitochondria, it damages the organelles, decreases energy metabolism and supports the occurrence of free radicals [5], releases cytochrome C into the cytoplasm, activates apoptosis and cell death[12]. Inside the Pb,

nucleus binds to chromatin proteins so that it has a carcinogenic effect. [13]. Pb also binds Nuclease Ape1 (a substance that repairs DNA damage), DNA damage arises that triggers carcinogenic substances 14. Another effect of Pb is the hydrolysis of RNA so that it damages the work of antioxidants and biosynthesis enzymes [5]. In the endoplasmic reticulum (ER), Pb decreases the function of this protein chaperone [15]. There are several proteins that are affected by Pb before the occurrence of apoptosis, including Hsp 90, BDNF, and VEGF. Ginkgo Biloba (EGb) is a Complementary and Integrative Health (CIH) group or complementary means in health services. EGb is mainly obtained from the Ginkgo Biloba tree, the Ginkgo tree has a long history that is used in traditional Chinese and Japanese cooking and medicine. Egb is also found in plants in Indonesia[16].

In modern medicine, Egb is used solely from leaf extract. The constituents contained in EGb are 24% flavone glycosides (mainly quercetin, kaempferol, and isorhamnetin), 6% terpene lactones (consisting of 2.8% -3.4% ginkgolides A, B, and C, and 2.6 - 3,2% bilobalide) and 0.5 ginkgolide acid16. The mechanism of EGb therapy is related individually to constituents who have their own working mechanism, but pharmacologically cause synergy with one another in one formula[17].

GA is an effective antagonist of Platelet Activating Factor (PAF) and reduces nerve damage due to ischemia and excitotoxic injury[18,19]. Ginkgolide-B is the most effective PAF antagonist from EGB constituents and has been clinically tested for success in sepsis, multiple sclerosis, migraine, and ischemia16. Ginkgolide C (GC) is a less observed EGb constituent. This is due to the small affinity and stability that they have. When compared with GB, GC is 25 times weaker as PAF antagonist[20]. Bilobalide (BB) has anti-inflammatory properties through reducing neuronal inflammation after injury, inflammation due to induction of hypoxia and inflammatory pain21. Flavonoids work directly on various signal cascades, such as Akt / protein kinase B (PKB), PI3K, MAPK, and protein kinase C21. The most studied aspect of flavonoids is their ability as antioxidants which directly remove oxidants, free radicals[22].

EGb has a neuroprotection effect through several methods, among others, EGb increases the significant activity of superoxide dismutase (SOD), can increase circulating levels of Polyunsaturated fatty acids (PUFA), and decrease saturation index of Polyunsaturated species, that can inhibit the formation of amyloid-b fibrils which are factors causes of Alzheimer's disease. EGb can also penetrate BBB as evidenced by studies that measure the similarity of EGb levels in blood plasma and brain [17,23,24].

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antagonist20. Bilobalide (BB) has anti-inflammatory properties through reducing neuronal inflammation after injury, inflammation due to induction of hypoxia and inflammatory pain[21]. Flavonoids work directly on various signal cascades, such as Akt / protein kinase B (PKB), PI3K, MAPK, and protein kinase C21. The most studied aspect of flavonoids is their ability as antioxidants which directly remove oxidants, free radicals[22].

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METHOD

This study was a randomized experimental post study design. This research was carried out with the treatment of test animals and immunohistochemical examination. The experimental animal used in this study was a male rat (Rattus novergicus) Wistar strain, aged 4-5 months, weighing 140-150 grams. For one week, rats were put into cages for adaptation to a stable environment, before being used for research testing.

The sample of this study was healthy male rats (Rattus novergicus) fulfilling the inclusion criteria, namely: 4-5 months old, weighing 140-150 grams, and healthy. The exclusion criteria are sick rats and mice that weigh less than 140 grams. The sample was divided into 4 groups and the minimum number of replications in this study was determined based on Federer's formula, which amounted to 6 tails, with the possibility of dying by 20%, so each group had a sample size of 8 tails. So, the total sample is 32 individuals.

The variables in this study are: (1) Independent variables, consisting of Pb acetate at a dose of 50 mg / kg BW and EGb at a dose of 100 mg / kg (2) Dependent variables, consisting of Apoptosis Index and number of neuronal cells and the last variable (3) Controlled variables consist of: sex, age, weight, feed and drink, health, condition / characteristics of the cage, treatment, how to provide exposure to Pb and parameters measurement method.

There were 4 study groups, namely, K1 (normal mouse group), K2 (a group of rats with exposure lead, without EGb treatment), K3 (Without Lead Exposure, Treated with EGB), K4 (low

dose of Lead exposure + EGb). In the beginning, all the study groups were treated EGb 100 mg/kg for 5 days before the lead (Pb) administration for 5 days. Furthermore, terminations and surgeries were performed for brain collection and then fixated. The next step is done by examining: (1) Apoptosis Index with Tunnel Assay (2) Number of neurons with Hematoxylin Eosin staining. After the data is obtained and analyzed, then statistical analysis is carried out, namely (1) Test for normality (2) ANOVA test to prove the mean differences of the dependent variable on the study.

RESULTS

Examinations Method and Apoptosis Index Examination Results

Histopathological examination of neurogenic cells in the hippocampus area is intended to examine the apoptotic index. Meanwhile, the apoptotic index is the number of positive apoptotic cells found in each field of view at 400x magnification and the apoptotic index data is the total number of cells observed in five different viewpoints.

All of these examinations use an ordinary Nikon H600L light microscope equipped with a Fi2 300-megapixel digital camera and Nikkon Image System image processing software.

Table 1: Apoptosis Index Examination Results

Apoptosis	Treatment	N	Mean	Sum of Ranks
	K2	6	9.50	57.00
	K4	6	3.50	21.00
	Total	12		

The results of the Apoptosis Index examination above are to answer the hypothesis that administration of EGb can increase BDNF expression in rats with lead exposure.

Statistically K2 (Rat with lead exposure (50mg / kg bw), without EGb treatment) and K4 (Pb exposure + EGb) showed that there were significant differences in terms of apoptosis index (Mann-Whitney = Z = -2,887; p = .004).

Examination Method and Examination results of the number of neurons

Examination of the number of neurons is done by calculating the number of pyramid cells in the hippocampus of the test animal. Data for each sample is the mean value of the total number of neuron cells found in five different areas in the cornu ammonic area (CA) at 400x magnification.

All of this examination uses an ordinary light microscope Nikon H600L equipped with a 300-megapixel Fi2 DS digital camera and cell count and micrometer that have been calibrated on Nikon Image System software.

The examination results of the number of neurons above are to answer the hypothesis that

EGb administration can increase the number of neurons in rats that exposed to Pb.

The examination results of the number of neurons from the Anova calculation above (between groups and in groups) showed F = 24,532 and p = .000. This shows that the number of neurons is significantly different.

DISCUSSION

In this study, the examination of the Apoptosis Index showed significant differences between K2 and K4. There was a significant decrease in the Apoptosis Index after the exposed to Pb group was treated with EGb.

Pb intoxication affects the central nervous system (CNS) directly or indirectly. The influences on the CNS include apoptosis, excitotoxicity, decreased cellular energy metabolism, biosynthetic disorders, oxidative stress, liquid peroxidase, and several other effects. Among these effects, which are considered to have the most role in Pb intoxication are oxidative stress and apoptosis [5,9].

The direct effects of Pb on the CNS can be classified as morphologically or pharmacologically. In cells, Pb interferes with the release of calcium from the mitochondria, thereby triggering the formation of reactive oxygen species (ROS), triggering mitochondrial damage and stimulating apoptosis[6].

Through various neurobiological mechanisms, Pb intoxication can cause neuronal cell death. Pb can inhibit calcium release from mitochondria, causing the formation of ROS, accelerate the destruction of mitochondria through the formation of the permeability transition pore, and activate the process of programmed cell death or apoptosis[7]. At high exposure, Pb can cause permanent brain damage and death. The progressiveness of this mechanism makes brain cells highly vulnerable to oxidative stress[9]. At the cellular level, Pb induces oxidative stress, mitochondrial dysfunction, interferes with cellular energy metabolism which ultimately leads to apoptosis and cell death[28].

Oxidative stress due to Pb can cause damage to cell membrane components, with protein and fat as the main target which eventually leads to neuronal cell death[10]. EGb 761 has antiapoptotic effects through the protection of mitochondrial membrane integrity, inhibition of mitochondrial cytochrome c release, increased transcription of antiapoptotic proteins, and reduction of caspase transcription and DNA fragmentation[29]. Other effects are a reduction of oxidative stress (which has been linked to the occurrence of vascular, degenerative and proliferative diseases), coupled with the induction of phase II-detoxifying and cellular defense enzymes by activation of NRF2 / ARE. Other activities are modulation of transcription factors, such as CREB, HIF-1α, NF-κB, AP-1, and p53, which are involved in the apoptotic process[29]. The discussion above is the rationale for proving that the apoptotic index in this study shows significantly different results between K2 and K4.

Furthermore, in this study, the number of neurons examination showed significantly different results between K2 and K4. There was a significant increase in the number of neurons after the Exposed to Pb group was treated with EGb. Pb exposure has a serious effect on cognition and behavior. The study conducted by Schneider et al. Aimed to examine the effects of various levels of Pb exposure during perinatal (pregnancy/lactation) and postnatal (up to 45 days after birth) periods in hippocampal transcriptomes in male and female Long Evans rats[5].

Pb exposure has a serious effect on transcription factors and various other pathways or tissues which have a substantial effect on plasticity and adaptability. Pre and postnatal Pb exposure can damage the short-term and long-term memory abilities of young rat and hippocampal ultrastructure[5,8].

Substantial evidence suggests that the accumulation of peptides containing beta-amyloid and free radicals can contribute to the etiology and/or development of Alzheimer's disease. The EGb761 extract contains two groups of major constituents, namely flavonoids and terpenoids. The substance is seen as a polyvalent agent with the possibility of treating neurodegenerative diseases due to various factors[30].

A study conducted by Bastianetto et al., Was investigating the potential effectiveness of EGb761 on toxicity induced by beta-amyloid peptides in cells of hippocampal primary cultures, this area is greatly affected in Alzheimer's disease. The results of this study indicate that the neuroprotective effect of EGb761 is partly related to its antioxidant properties. The effectiveness of EGb761 in neurodegenerative diseases is through inhibition of toxicity induced by beta-amyloid and cell death.

The discussion above is the rationale for proving that the number of neurons in this study showed significantly different results between K2 and K4.

CONCLUSION

EGb can increase BDNF expression in rats with lead exposure. Furthermore, the administration of EGb can increase the number of neurons in rats that exposed to Pb.

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