THE EFFECT OF GINKGO BILOBA (EGB) EXTRACTS ON THE EXPRESSION OF HSP 90, VEGF AND BDNF IN THE RATTUS NOVERGICUS WITH LEAD (Pb) EXPOSURE

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THE EFFECT OF GINKGO BILOBA (EGB) EXTRACTS ON THE EXPRESSION OF HSP 90, VEGF AND BDNF IN THE RATTUS NOVERGICUS WITH LEAD (Pb) EXPOSURE

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ABSTRACT

Background: the rapid industrial growth did regrettably create new problems, especially in a heavy amount of lead (Pb) waste. This may cause intoxication, that give rise to health problems specifically in the nervous system. EGb is believed to have neuroprotective effects. Despite lots of studies, the mechanism of action of EGb on repairing brain cell damage due to exposure to Pb remains unclear. The effect of EGb on neuron proteins related to apoptosis and neuronal cell death due to Pb intoxication remains unknown

Aim: to determine the effect of *Ginkgo biloba* extract on the expression of HSP 90, VEGF and BDNF on *Rattus novergicus* exposed to Pb

Method: This study was experimental by means of randomized experimental post study design. The study population used was 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, that was divided into 4 groups. The variables in this study were independent variables, dependent variables, and controlled variables. After the data were obtained and analyzed, a statistical analysis was performed.

Results: Based on the acquired data, the HSP 90 levels among groups differed significantly (p=.004), so did the VEGF (p=.030). Likewise, a significant difference among treatment groups was also recorded on the expression of BDNF (p= 004)

Conclusion: EGb can reduce HSP 90 expression in rats exposed to lead. EGb can increase VEGF expression in rats exposed to lead. EGb can increase BDNF expression in rats exposed to lead

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

INTRODUCTION

Indonesia's economic progress in the past few decades has improved the quality of life. This was also followed by increased industrial growth, purchasing power and social status of the community. Industrial growth, is encouraging, however, on the other hand, it may lead to more environmental pollution. The main concern is if the industrial waste is thrown away without being processed first. One of the waste is lead (Pb), which often pollutes the environment, especially in the locations of mining, casting and metal industries[1]. In Cinangka village, Bogor, severe Pb intoxication has occurred due to the waste from used battery smelting facilities. Study results showed that Pb levels in the blood of children ranged from 16.2 to 60 µg / dl, or exceeded the normal standards allowed by the World Health Organization (WHO). According to the American Academy of Pediatrics (AAP), the level of Pb in venous blood ≥10 ug/dl is considered as Pb intoxication[2]. Pb blood level above the save limit may cause health problems, especially in children. Pb can enter the body through the consumed food, beverages, water; or inhaled air and dust which have been contaminated with Pb[3].

Through various neurobiological mechanisms, Pb intoxication may cause neuronal cell death[4]. At the cellular level, Pb induces oxidative stress, mitochondrial dysfunction, disrupts cellular energy metabolism which ultimately leads to apoptosis and cell death[5]. Pb can inhibit the release of calcium from the mitochondria, cause the formation of Reactive Oxygen Species (ROS), and activate the apoptotic process. Oxidative stress due to Pb intoxication may cause damage to cell membrane components, with protein and fat as the main targets which ultimately lead to neuronal cell death[6]. Pb intoxication causes oxidative stress through two different pathways that occur simultaneously[7]. First is the ROS accumulation, followed by is depleted antioxidant reserves. Pb can affect ROS metabolizing enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), reducing intracellular glutathione (GSH) level[6].

The mechanism of lead toxicity is still controversial, through various 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[8]. In the Pb mitochondria, it damages the organelles, decreases energy metabolism and supports the occurrence of free radicals[9], releases cytochrome C into the cytoplasm, activates apoptosis and cell death. Within the nucleus, Pb binds to chromatin proteins, arising a carcinogenic effect[10]. Pb also binds to Nuclease Ape1 (a substance that repairs DNA damage), causing DNA damage that triggers carcinogenic substances formation[11]. Another effect of Pb is the hydrolysis of RNA, which may alter the work of antioxidants and biosynthetic enzymes9. In the endoplasmic reticulum (ER), Pb impairs the function of chaperone[9]. There are several proteins that are affected by Pb before apoptosis, including Heat shock protein (Hsp) 90, BDNF, and VEGF. HSP belongs to a protein produced by the cell as a response to

a various stress condition, such as infection, toxin, and hypoxia.[12] Besides, Pb intoxication may also increase the level of HSP[13].

BDNF is a protein associated with the Nerve Growth Factor. Neurotrophic factors are found in the brain and peripheral16. Low-dose of Pb exposure will cause pathological changes in synapses, swelling of nerve endings, and mitochondrial damage and decrease in BDNF[14].

Tropomyosin receptor kinase B (TrkB) is a family of receptor tyrosine kinases. Autophosphorylation of TrkB depends on BDNF[15]. Activation of the BDNF-TrkB pathway is important in the development of short-term memory and neuronal growth[16]. BDNF-TrkB signals significantly decreased by exposure to Pb[17].

Vascular endothelial growth factor (VEGF), is a signal protein produced by cells that stimulates blood vessel formation. VEGF is involved in vasculogenesis and angiogenesis[18]. Pretreatment of astrocyte culture with a low concentration of Pb, there will be an increase in VEGF expression and loss of motor neurons in co-culture can be prevented by neutralizing antibodies to VEGF[19]. *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 been used in traditional Chinese and Japanese cooking as well as medicine. EGb can also be found in Indonesia[20].

Ginkgolide A (GA) is an effective antagonist of Platelet Activating Factor (PAF) and reduces nerve damage due to excitotoxic ischemia and injury[21]. Ginkgolide-B (GB) is the most effective PAF antagonist from EGb constituents and has been clinically tested its efficacy in sepsis, multiple sclerosis, migraine, and ischemia. Ginkgolide C (GC) is a less observed EGb constituent, due to its small affinity and stability. Compared to GB, GC was 25 times weaker as PAF antagonist[22]. Bilobalide (BB) has anti-inflammatory properties through reducing neuronal inflammation after injury, inflammation due to induction of hypoxia and inflammatory pain. Flavonoids work directly on a variety of signal cascades, such as Akt / protein kinase B (PKB), PI3K, MAPK, and protein kinase C [23]. The most studied aspect of flavonoids is their ability as antioxidants which directly remove oxidants, free radicals[24].

EGb has a neuroprotective effect through several methods, among others, EGb increases the significant activity of SOD[25], increases circulating levels of Polyunsaturated fatty acids (PUFA), and decrease saturation index of Polyunsaturated species[26], inhibits the formation of amyloid-b fibrils which are factors of Alzheimer's disease[27]. EGb can also penetrate the Blood Brain Barrier (BBB) as evidenced by studies that measure the similarity of EGb levels in blood plasma and brain. Although there have been many studies of the clinical benefits of EGb in various diseases[28,29], the effect of ginkgo biloba extract on the expression of HSP 90, VEGF and BDNF *Rattus novergicus* exposed to Pb is remained unclear.

METHOD

This study is an experimental study by means of randomized experimental post study design. This research was conducted at the Experimental Animal Laboratory of the Faculty of Veterinary Medicine, Airlangga University for the treatment of experimental animals and for immunohistochemical examinations carried out at the Veterinary Pathology Laboratory of the Faculty of Veterinary Medicine, Airlangga University.

The animal used in this study was the Wistar strain of male rats (*Rattus novergicus*) obtained from the UGM Integrated Research Center (LPT). Prior to the treatment, the rats were put into cages for adaptation to a stable environment for a week. The samples of this study were healthy male rats fulfilling the inclusion criteria (rats aged 4-5 months, weight 140-150 grams, and healthy) and exclusion criteria (sick rats and rats weighing 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 obtained a sample size of 8 tails. So the total sample is 32 individuals.

The variables in this study are divided into three, namely: (1) Independent variables, consisting of (1) Pb acetate at a dose of 50 mg/kgBW (2) EGb at a dose of 100 mg/kg (2) Dependent variable, consisting of (1) Expression of HSP-90 (2) Expression of VEGF (3) Expression of BDNF and the last variable are (3) Controlled Variables in the condition of Rattus novergicus rats as follows: (1) Gender (2) Age (3) Weight (4) Feed and drinks (5) Health (6) Condition / characteristics of the cage (7) Maintenance (8) How to provide lead exposure (9) Method of checking parameters.

There were 4 study groups, namely, K1 (normal group), K2 (rats with lead exposures, without EGb treatment), K3 (EGB exposed groups without exposure to Pb), K4 (low dose of lead exposures + EGb group of rats). In the beginning, all the study groups were exposed to EGb 100 mg/kg for 5 days before Pb exposures for 5 days. Furthermore, terminations and surgeries were performed for brain collection and fixated. The next step is done by examining: Expression of HSP 90, VEGF, and BDNF in the hippocampus with immunohistochemistry. After the data were obtained and analyzed, the analysis was carried out with SPSS 22 software with the Mann-Whitney test ($p = 0.000$).

RESULTS

Examination Methods and Expression Examination of HSP-90

This histopathological examination is intended to determine the expression of HSP 90 in neurogenic

cells in the hippocampus area. HSP 90 expression data were obtained according to the modified Remmele method, where the Remmele scale (ImmunoReactive Score / IRS) score was the result of multiplication between the scores of the percentage of positive immunoreactive cells with color intensity scores in immunoreactive cells. Data for each sample is the IRS average value observed in 5 different areas at 1000x magnification.

Table 1: Results of HSP-90 Expression Examination

HSP_90	Treatment	N	Mean	Sum of
	K2	6	9.50	57.00
	K4	6	3.50	21.00
	Total	12		

The results of the HSP-90 expression examination above are to answer the hypothesis that EGb administration can reduce HSP 90 expression in rat with Pb exposure.

Statistically K2 (Rat with Pb exposure (50mg / kg bw), EGb Treatment) and K4 (Pb exposure + EGb) shows that HSP 90 levels differ significantly (Mann-Whitney = Z = -2,887; p = .004).

Examination methods and Examination results of VEGF Expressions

This histopathological examination is intended to determine VEGF expression in neurogenic cells in the hippocampus area. VEGF expression data were obtained according to the modified Remmele method, where the Remmele scale (IRS) score was the result of multiplication between the scores of the percentage of positive immunoreactive cells with color intensity scores in immunoreactive cells. Data for each sample is the IRS average value observed in 5 different areas at 1000x magnification.

Table 2: Examination Results of VEGF Expressions

VEGF	Treatment	N	Mean	Sum of Ranks
	K2	6	4.25	F25.50
	K4	6	8.75	52.50
	Total	12		

The results of the VEGF expression examination above are to answer the hypothesis that EGb can increase VEGF expression in rats with Pb Exposures.

Statistically K2 (Rat with Pb Exposures (50mg / kg bw), without EGb application) and K4 (Pb Exposures + EGb) showed that VEGF levels differed significantly (Mann-Whitney = Z = -2.169; p = .030).

Examination methods and Examination results of BDNF Expressions

This histopathological examination is intended to determine BDNF expression in neurogenic cells in the hippocampus area. BDNF expression data were obtained according to the modified Remmele method, where the Remmele scale (IRS) score was the result of multiplication between the scores of the percentage of positive immunoreactive cells with color intensity scores in immunoreactive cells. Data for each sample is the IRS average value observed in five different areas at 1000x magnification.

Table 3: Examination Results of BDNF Expressions

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

The results of the BDNF expression examination above are to answer the hypothesis that administration of EGb can increase BDNF expression in rat with Pb exposures).

Statistically, K2 (Rat with Pb Exposures (50mg / kg bw), without EGb treatment) and K4 (Pb Exposures + EGb) showed that BDNF levels differed significantly (Mann-Whitney = Z = -2,892; p = .004).

DISCUSSION

In this study, the results of the examination of HSP-90 expression showed a significant difference between K2 (Pb exposure, without EGb treatment) and K4 (Pb Exposures, with EGb treatment). Also, a significant decrease in HSP-90 after the group exposed to this Pb was treated EGb.

As a chaperone protein that helps other proteins folding process and other cell functions such as: stabilizing proteins against heat, helping intracellular transport, cell maintenance, and cell signaling, HSP-90 levels will increase, especially after exposure to various types of cellular stress[30]. This can be proven in this study by comparing K1 (Normal Rat Control Group) of 2.73 with K2 of 6.20. Increased HSP-90 levels here, can be considered as a compensation mechanism for repairing cellular damage due to PB.

The high production of HSP-90 is triggered by exposure to various types of environmental stress. Some of them are infections, inflammation, exercise, toxins, hunger, hypoxia, nitrogen deficiency, or lack of water. As a result, HSP-90 is also called protein stress[12]. Several studies have shown that misfolded proteins and ER play an important role in the development of Alzheimer's disease. It has also been reported that ER is considered a common mediator of apoptosis in

neurodegenerative disorders such as Alzheimer's disease[31]..

Increased HSP-70 expression can suppress neurotoxicity due to protein misfolding. The study by Liu et al. showed that pretreatment with EGb761 can overcome the neurotoxicity of Aβ1-42 oligomers by increasing the expression of HSP-70, Grp78, IRE1α, pAkt and significantly reducing apoptotic-related protein expression. The results of this study indicate that the neuroprotective effects of EGb761 are related to ER stress activation and increased expression of HSP-70[31]. The discussion above is the rationale for proving that HSP-90 expression in this study shows significantly different results between K2 and K4.

In this study, the examination of VEGF expression showed significantly different results between K2 and K4. There was a significant increase in VEGF expression after the group exposed to this Pb was treated EGb. VEGF is a signal protein produced by cells that stimulates blood vessel formation. VEGF is involved in the process of vasculogenesis and angiogenesis. If the network oxygen supply is lacking, VEGF will fix it[32].

Brain injury due to inorganic Pb is considered the most important concern regarding child health and environmental hazard in the world. Brain microvasculature of developing children is susceptible to high levels of Pb toxicity in the form of Pb encephalopathy characterized by cerebellar hemorrhage, increased blood-brain barrier permeability, and vasogenic edema. Pb induces vasogenic edema through the Flk-1-dependent mechanism[33].

In this test, a strong induction of angiogenesis by FGF and induction was found to be completely inhibited by EGb. FGF and VEGF endothelial cells activate ERK. Furthermore, ERK stimulates angiogenesis through proliferation, migration and endothelial cell formation. EGb can inhibit smooth muscle cell migration by reducing ERK activation and superoxide formation[34]. EGb has a strong anti-angiogenic effect. The results of this study form the basis of EGb therapy for tumor prevention and adjuvant therapy as anti-angiogenesis. In conclusion, EGb can increase the regeneration of peripheral nerves and vascularization of blood vessels[13]. The discussion above is the rationale for proving that VEGF expression in this study shows significantly different results between K2 and K4.

In this study, the examination of BDNF expression showed significantly different results between K2 and K4. There was a significant increase in BDNF expression after the group with Pb exposures was treated EGb. BDNF is a protein that is a family of neurotrophic growth factors and is associated with Nerve Growth Factor[35]. BDNF supports the survival of existing neurons and promotes growth and differentiation of neurons, neurogenesis and new synapses.

In conclusion, low-dose perinatal Pb exposure causes pathological changes in nerve endings associated with changes in key synaptic protein levels. All of these changes can cause synaptic dysfunction, which is expressed by damage to the mechanism of secretion and thus abnormalities in

neurotransmission and neuronal dysfunction[14]. Among neurotrophins (proteins that help stimulate and control neurogenesis), BDNF is the most active. Oto phosphorylation of TrkB with BDNF is important in regulating neuronal plasticity after hypoxic injury, including because of PB intoxication. The binding of BDNF to TrkB-FL will induce receptor dimerization and phosphorylation, which causes activation of the intracellular signaling pathway involved in cell viability[15].

BDNF plays an important role in the pathophysiology of Tardive dyskinesia (TD). Through increasing BDNF, EGb has antioxidant potential and is neuraoprotective. The discussion above is the rationale for proving that BDNF expression in this study shows significantly different results between K2 and K4.

CONCLUSION

EGb can reduce HSP 90 expression in rats with Pb exposures. EGb can increase VEGF expression in rats with Pb exposures. EGb can increase BDNF expression in rats with Pb exposures.

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