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Protective Mechanism of α-Tocoferol and Zinc Sulfate on The Cytochrome C and Caspase-3 Cottactic (*rattus novergicus*) Exposed to Timbal Asetate ($pb(c_2h_3o_2)_2$)

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Abstract

THIS investigation has an evaluation objective of potential protective effects of α-Tocopherol (a form of vitamin E) and zinc sulfate against alterations in the levels of Cytochrome C and Caspase-3, which are crucial biomolecules involved in apoptosis (programmed cell death), within the brain tissue of Rats with albinism mutations (Rattus norvegicus) subjected to lead acetate (Pb(C2H3O2)2) exposure. This type of research is experimental research with a posttest-only control group design. The samples used were 25 female Wistar white rats with body weight of 250-300 grams and 4 months old which were divided into 5 treatment groups. The control group was given distilled water and corn oil, the P0 group was given corn oil, distilled water, and lead acetate at a dose of 1.5 mg/head, the P1 group was given α Add in 0,5 ml corn oil and Tocopherol at a dose of 100 mg/head distilled water 0.5 ml. The P2 group was given zinc sulfate 0.54 mg/KgBW add in distilled water and, corn oil 0.5 ml, and lead acetate 1.5 mg/KgBW, the P3 group was given zinc sulfate 0.54 mg/KgBW, 100 mg/KgBW α-Tocopherol, and 1.5 mg/KgBW lead acetate orally for 21 days. Based on the results of research and data analysis, it can be concluded that the combination of zinc sulfate and α-tocopherol can reduce the expression of Cytochrome C and Caspase 3.

Keywords: Antioxidants, Apoptosis, Caspase 3, Cytochrome C, Lead.

Introduction

The ever-evolving industrial technology has a negative impact on the environment, one of which is the emergence of the hazardous substance lead (Pb) in heavy metals. Anthropogenic activities since antiquity have facilitated the widespread distribution and environmental mobility of lead, resulting in elevated non-essential exposure [1]. While atmospheric lead emissions have markedly decreased in most industrialized nations, the toxicological profile has shifted. Acute lead toxicity incidents have diminished; however, chronic low-level lead exposure persists as a substantial public health concern, necessitating continued surveillance and intervention strategies [2]. Lead is a potent industrial waste due to its extensive use and can cause toxicity and damage many tissues in the human and animal body. Some types of lead that endanger health are lead acetate, lead monoxide, leadtetraoxide (red lead), lead sulfide, and lead carbonate (white lead), which is one form of lead that often causes poisoning [3]. Oxidative stress caused by heavy metals can interfere with the antioxidant defense system. Cadmium (Cd) and lead (Pb) are the most abundant pollutants in the environment and does not decompose. These two metals are able to bind to sulfhydryl glutathione (GSH) and interfere with their cell function as antioxidant producers [4]. Cadmium (Cd) and lead (Pb) also showed a high affinity with the sulfanyl groups (−SH) and affecting the molecule functionality also suppress the effects of specific antioxidant activity such as glutathione peroxidase (GPx), catalase (CAT), glutathione transferase (GST), and superoxide dismutase (SOD) [5]. The effects of lead (Pb) exposure cause neurological damage such as acute encephalopathy, seizures, confusion, cerebral palsy [6], and in severe conditions it can cause hemorrhage, schizophrenia

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[7], alzheimer diseases and parkinson disease [8]. Oxidative stress can interfere with the performance of the hypothalamus thereby reducing the secretion of FSH and LH. Dysregulated secretion of FSH and LH can impede neurodevelopmental processes, including neurogenesis, neuronal migration, differentiation, and synaptogenesis, thereby hindering optimal brain growth and maturation [9].

Lead induces oxidative stress through the formation of free radicals that can disrupt the antioxidant balance. There are 2 main pathways of apoptosis namely the stress pathway (intrinsic) and the death receptor pathway (extrinsic). The stress pathway and the death receptor pathway will cause apoptosis by activating caspase-3, caspase-6, and caspase-7. Caspase-3 is an active form of procaspase 3 in mammals that until now has a specific role in apoptosis [10]. Caspase-3 is the main executor in cells undergoing apoptosis, which can be activated through extrinsic and intrinsic pathways. In the intrinsic pathway due to chemical compounds or loss of growth factors induced by apoptosis, mitochondria will be disrupted and cytochrome C will be detached from the mitochondrial intermembrane [11]. Cytochrome C a hemoprotein that carries electrons during mitochondrial oxidative phosphorylation. Cytochrome c oxidase leaves the membrane and combines with the cytoplasmic protein Apaf-1 when it stops functioning. Cytochrome c interacts with Apaf-1 and moves into the cytoplasm, leading to the formation of the Caspase Recruitment Domain (CARD). For apoptosomal formation, some CARD fuses and then joins pro-caspase-9. Caspase-9 is produced by a complex that joins Pro-Caspase-9. Caspase-3 functions as a regulator of cell death pathways that will be converted to pro-caspase-3 by caspase-9. Oxidative stress-causing free radical compounds are reciprocally counteracted by endogenous antioxidants such as SOD, CAT, and GPx, but the body's ability to deal with free radicals is limited, so it requires supplements from outside that are antioxidants [12]. Antioxidants play an important role in reducing the harmful negative effects of lead [13]. The lipid-soluble isomer α-tocopherol exhibits potent antioxidative properties, functioning as a scavenger of reactive oxygen species and thereby mitigating oxidative stress-induced cellular damage [14]. Physically, α-Tocopherol is a fat-soluble antioxidant found in lipoproteins or lipid membranes [15]. Administration of α-Tocopherol can protect against the effects of drugs, chemicals, and heavy metals that cause free radicals [16]. Zinc is an antioxidant that is very effective in protecting the body from free radical damage. Zinc can induce the formation of proteins that can neutralize ROS and can work synergistically with α-Tocopherol [17]. This study aims to prove the protection of α-Tocopherol and zinc sulfate against levels of Cytochrome C and Caspase-3 in the brain of white rats (Rattus norvegicus) exposed to lead acetate ($Pb(C_2H_3O_2)_2$). This type of research is experimental research with a post-test only control group design.

Material and Methods

Ethical Considerations

Animal ethics has been carried out with certificate number 502/HRECC.FODM/VIII/2022. Animal ethics testing is required to assure that all acts on experimental animals are in accordance with the standard operating procedures.

Preparation of the animal experiment

Preparation start provision cage, food, and drink container for 25 rat for randomly divided 5 group. The rats have weighed and get adaptation period is 7 days before treatment. Drinking water was given ad libitum, and commercial feed with 511-BRAVO was given sufficiently.

Time and Location

Implementation of research and data collection was located in Experimental Animal Laboratory, Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga, Banyuwangi from October to December 2022. Histopathological examination as well as measurement of cytochrome C and Caspase 3 levels were conducted at the "Satwa Sehat" Research and Diagnostic Laboratory Clinic Malang.

Experimental Design Treatment

The samples used were 25 female Wistar white rats with a body weight of 250-300 grams and 4 months old which were divided into 5 treatment groups. The control group was given distilled water and corn oil, group P0 was given corn oil, distilled water, and lead acetate at a dose of 1.5 mg/head, group P1 was given α-Tocopherol at a dose of 100 mg/head, Add in 0.5 ml corn oil and 0.5 ml distilled water. The P2 group was given zinc sulfate 0.54 mg/KgBW, corn oil 0.5 ml, and lead acetate 1.5 mg/KgBW, the P3 group was given zinc sulfate 0.54 mg/KgBW, 100 mg/KgBB α-Tocopherol, and 1.5 mg/KgBW lead acetate orally for 21 days. Based on the results of research and data analysis, it can be concluded that the combination of zinc sulfate and α-tocopherol can reduce the expression of Cytochrome C and Caspase 3.

The control group received 0.5 ml aquadest (distilled purified water) on day 1, then 0.5 ml aquadest and 0.5 ml corn oil on day 4. P0 dosed with half millilitre, of purified water with the same amount of maize oil on day 1, then 1.5 mg/kgBW lead acetate dissolved in 0.5 ml distilled water on day 4. P1 received 100 mg/head α-tocopherol in 0.5 ml corn oil and 0.5 ml purified water on day 1, then 1.5 mg/kgBW lead acetate in half millilitre purified water on day 4. P2 received 0.54 mg/head zinc sulfate in half millilitre of purified water and the

same amount of maize oil on day 1, then 1.5 mg/kgBW lead acetate in 0.5 ml distilled water on day 4. P3 received 0.54 mg/head zinc sulfate in 0.5 ml distilled water, and 100 mg/kgBW α-tocopherol in 0.5 ml corn oil on day 1, then 1.5 mg/head lead acetate in 0.5 ml distilled water after 4 hours. All treatments were given orally daily for 21 days.

Euthanasia of Experimental Animals

On day 22, final body weights of female white rats were recorded for homogeneity and normality. Rats were then euthanized with ketamine-xylazine (ketamine HCl 70-100 mg/kg BW, xylazine 2 mg/kg BW, i.m.) [16]. Cytochrome C and caspase 3 expression were evaluated by scoring color intensity (0-3) and quantifying percentage of positive cells in one 400× field of view.

Immunohistochemical Examination

Brain sections were made in glass slide and deparaffinized in xylol, rehydrated through graded alcohols concentration (100%, 90%, 80%, 70%, and 30%) to distilled water, washed in PBS (pH 7.4, 3 x 5 min), quenched in 3% H_2O_2 (5-10 min), blocked in 1% BSA/PBS (10-30 min, room temperature), and incubated with biotin-labeled cytochrome C and anti-caspase 3 primary antibodies (60 min, room temperature). After PBS washes (3 x 5 min), sections were incubated with Streptavidin-HRP (30-60 min, room temperature) [18]. Sections were washed in PBS (3 x 5 min) with pH 7.4, developed with 3,3-Diaminbenzidine tetrahydrochloride (DAB) chromogen around 10-20 minutes, rinsed using purified water (3 x 5 minutes, RT), counterstained with methylene blue/green (3 min), mounted, and observed under a trinocular microscope (40-1000x magnification). Cell counting was performed in 5 fields of view.

Data Analysis

Data were gathered and analyzed using SPSS software (version 20) for windows, with one-way ANOVA ($p<0.05$) and LSD post-hoc tests evaluated treatment effects and inter-group differences.

Results

Table 1 The data illustrates the degree of reduction in caspase expression levels subsequent to the supplementation of lead acetate, zinc and α-tocopherol. The analysis of the P-value related to Caspase-3 expression showed that the treatment group Control 1.00a ± 0.00 is not significantly different from P3 1.20a ± 0.24. P2 1.56b ± 0.16 was not significantly different from P1 1.64b ± 0.167. P0 2.24c ± 0.16 is significantly different from Control, P1, P2, and P3.

The results contained in the scoring table explain that brain Caspase-3 expression shows that all treatment groups have significant differences with the P0 group, with a P-value <0.05. The group without treatment (P0) had the highest increase in Caspase-3 expression compared to treatment group one (P1), treatment group two (P2), and treatment group three (P3) which showed improvement through a score figure close to that of control group (C). The score table shows the variation of brain Caspase-3 expression which varies from severe to mild. The control group that was not exposed to lead showed the lowest score with a mean value of 1.00 showing the least damage. The untreated group (P0) showed the highest score with an average score of 2.24. Treatment group one (P1) showed the second highest score with an average score of 1.64. Treatment group two (P2) showed the third lowest score with an average score of 1.56. Treatment group three (P3) showed the second lowest average score with an average score of 1.20.

Table 2. also shows the score of the degree of damage to cytochrome expression after the administration of lead acetate, zinc and α-tocopherol. The treatment of cytochrome expression value P with C 1.36 ± 0.16 was not significantly different from P3 1.28 ± 0.22. P2 1.72 ± 0.22 was not significantly different from P1 1.68 ± 0.22. P0 2.52 ± 0.17 was significantly different from C, P1, P2, and P3.

The results contained in the scoring table explain that cytochrome expression shows that all treatment groups have significant differences with the P0 group, with a P-value <0.05. The group without treatment (P0) had the highest increase in cytochrome expression compared to treatment group one (P1), treatment group two (P2), and treatment group three (P3) which showed improvement through a score figure close to the score value control group (C). The score table shows the variation of cytochrome expression. Treatment group three (P3) showed the lowest score with an average value of 1.28 indicating the least damage. The untreated group (P0) showed the highest score with an average score of 2.52. Treatment group one (P1) showed the second highest score with an average score of 1.72. Treatment group two (P2) showed the third lowest score with an average score of 1.68. The control treatment group (C) showed the second lowest average score with a score of 1.36.

The caspase results contained in the table explain that the control is significantly different from P0, P1 P2, and P3. P1 is not significantly different from P2 but significantly different from P3, P0, and P2 control. There is no statistically significant difference versus P1 and P3. However, P2 has a significant difference versus both P0 and the control group. This rewording elucidates the relationships with P2, in comparison to the other groups (C, P0, P1, and P3), quite clearly with regard to statistical significance, therefore avoiding repetitions and increasing clarity. P3 was not significantly different from P2 but

significantly different from P0, P1, and control. P0 is significantly different from the control, P1, P2 and P3.

The cytochrome results contained in the table explain that P1 is not significantly different from P2 and control but significantly different from P3 and P0. P2 is not significantly different from P3 and control, but significantly different from P0. P0 is significantly different from the control, P1, P2 and P3. P3 was not significantly different from the control and P2 but significantly different from P0. The control group showed no significant difference compared to P3, P2, and P1 groups. However, it differed significantly from the P0 group.

Discussion

In general, α-Tocopherol and zinc sulfate protection against Cytochrome C and Caspase-3 levels in the brain of white rats (Rattus norvegicus) subjected exposure to lead (II) acetate ($Pb(C_2H_3O_2)_2$) is very influential. Approximately 95% of Pb in the blood is bound by erythrocytes, which will be distributed to hard tissues (such as teeth, nails, hair, and bones) and soft tissues (such as bone marrow, nervous system, kidneys, and liver) [19]. On soft tissues some Pb is stored in the aorta, liver, kidneys, and brain, Pb that is in these soft tissues will later be toxic. Furthermore, Pb compounds enter the brain granulosa cells through passive diffusion to produce by-product O_2^- and trigger increased oxidation increasing ROS (Reactive Oxygen Species). The nature of Pb^{2+} ions can replace endogenous metalloenzyme ions causing enzyme inactivity, and the oxidative stress that occurs can damage the body's homeostasis and induce apoptosis through intrinsic pathways. Apoptosis is caused by various signalling pathways and regulated by complex extrinsic and intrinsic ligands [20].

There are two main pathways of apoptosis, namely pathways involving caspase function and without caspase. Mitochondria act as crosstalk organelles that are organelles that play a role in both different apoptotic pathways. The apoptotic pathway is divided into two, namely caspase-independent and dependent. Apoptotic signalling through the caspase-dependent pathway can be triggered within the cell (intracellularly) or outside the cell (extracellularly) [21]. The extrinsic (extracellular) pathway is initiated by activation of death receptors, while the intrinsic pathway begins with the release of signaling molecules from the mitochondria inside the cell. Intrinsic apoptotic route induced by oxidative stress causes disruption of the mitochondria and the release of cytochrome c from the mitochondrial intermembrane. Cytochrome c, a heme-containing metalloprotein, functions as an essential electron shuttle in the mitochondrial electron transport chain, facilitating oxidative phosphorylation. Upon cessation of its role as the terminal electron donor to cytochrome c oxidase, this protein translocates from the intermembrane space to the cytosol, where it interacts with the apoptotic protease activating factor 1 (Apaf-1), initiating the intrinsic apoptotic cascade [22]. This protein will activate the caspase-9 initiator in the cytoplasm [23]. This protein exits the mitochondria after a change in electrochemical potentiation in the membrane which causes the opening of a nonspecific channel in a permeable membrane, consisting of two inner membrane proteins, the Adenine Nucleotide Translocator (ANT) and the outer protein, the porin; Voltage Dependent Anion Channel (VDAC) [24]. These proteins act in tandem, with the outer and inner sides in contact. These channels can pass through substances that have a molecular weight of less than 1500. Alterations in the proton electrochemical gradient across the mitochondrial inner membrane modulate the efficacy of oxidative phosphorylation, potentially leading to a shift in the redox state and ATP synthesis capacity of the organelle in the mitochondria and changes in ions cause swelling of the matrix [25]. The mitochondrial inner membrane exhibits extensive invaginations, forming cristae that significantly augment its surface area relative to the outer membrane [26]. Mitochondrial matrix swelling, induced by various apoptotic stimuli, can compromise the integrity of the outer membrane [27]. This permeabilization facilitates the translocation of intermembrane space proteins, notably cytochrome c and apoptotic protease-activating factor 1 (Apaf-1), into the cytosolic compartment, thereby initiating the intrinsic apoptotic pathway. This pathway is commonly activated in response to other lethal stimuli such as DNA damage, oxidative stress, and hypoxia [28].

The mechanism of apoptosis is not easy but very complex and complicated. Apoptosis involves four stages. Start with death signal, signal transduction, pro-apoptotic gene expression, and phagocytosis. Triggers range from hormones to environmental toxins [29]. The last stage is phagocytosis or elimination by macrophages, dendritic or cells adjacent to apoptotic cells. The apoptotic cascade is characterized by distinct morphological and biochemical alterations, including nuclear chromatin condensation, cytoplasmic compartmentalization and subsequent formation of membrane-bound apoptotic bodies, and internucleosomal DNA fragmentation resulting in oligonucleosomal-length chromatin cleavage products [30]. Lead exposure disrupts the cell cycle to impairs mitochondrial function. It changes the balance of key proteins in apoptosis induction and induces the expression of enzymes responsible for programmed cell death. Cellular changes associated with a pathological increase in calcium uptake, decrease in DNA synthesis, oxidative stress, mitochondrial dysfunction, and altered gene expression [31].

The initiation of the apoptotic cascade is characterized by the suppression of anti-apoptotic proteins, primarily members of the Bcl-2 and Bcl-XL subfamilies, concomitant with the activation of pro-apoptotic proteins, notably Bax and Bak from the Bcl-2-associated X protein subfamily. This shift in the balance between pro-survival and pro-death factors triggers the subsequent steps of the programmed cell death pathway [32]. The entry of Bax and Bak into the mitochondrial membrane will stimulate the release of cytochrome C substrates into the cytoplasm and form CARD (Caspase Recruitment domain). Several CARDS then combine to form an apoptosome complex, which then binds and activates procaspase 9 to caspase 9. In the intrinsic pathway of programmed cell death, caspase-9 serves as a critical enzymatic component. This enzyme has the capability to directly trigger the executioner caspases, specifically caspase-3 and caspase-7 [33].

Antioxidants needed to prevent the effects of Pb free radicals are lipophilic antioxidants that can enter the cell membrane and cytosol of granulosa cells. Zinc sulfate and α-Tocopherol are non-enzymatic antioxidants that can prevent oxidative stress and prevent brain granulosa cell damage. Zinc and α-Tocopherol can release hydrogen ions from the hydroxyl group of the ring structure into lipid peroxidation free radicals to prevent brain granulosa cell damage [34]. α-Tocopherol is thought to be able to neutralize peroxidase intermediates and prevent damage to important molecules by converting radicals into hydroperoxides. Through the direct reaction of free radical peroxidation and lipid α-tocopherol to produce stable and completely oxidized tocopheryl quinones to resist lipid peroxidation chains so that there is inhibition or disconnection of the lipid peroxidation chain so that there is no damage to adenocytes. The antioxidant activity of α-tocopherol shows anti-apoptotic reactions, and protective and therapeutic effects against lead exposure-induced gonadal dysfunction through stimulation of anterior pituitary gonadotropic cell proliferation, and increased hormone levels. Zn is also a component of superoxide dismutase, an enzyme that can protect cells from oxidative stress damage. The use of antioxidants such as α-tocopherol can increase SOD (superoxide dismutase) activity in brain cells and prevent brain tissue damage. Zinc functions as a cofactor of the SOD enzyme that can protect cells from oxidative stress damage and activate enzymatic systems that can neutralize free radicals.

In the same study, the administration of lead acetate to female rats (Rattus norvegicus) caused an increase in the expression of cytochrome C and Caspase 3 which are signs of apoptosis. This is due to the production of ROS that damage DNA and trigger an increase in the expression response of p53, Bax, and Bcl-2. α-tocopherol has anti-apoptotic effects as well as protective and therapeutic effects through stimulation and increase in hormone levels [35]. The results of the study in Table 1, P3 given 0.54 mg/KgBW zinc sulfate, 100 mg/KgBW α-Tocopherol, and 1.5 mg/KgBW lead acetate is not significantly different from P2 containing zinc sulfate on caspase. While the P3 cytochrome was significantly different from P0 which did not use zinc and α-Tocopherol. Other studies have shown that zinc modulates mitogenic activity and can induce the synthesis of metallothionein, which is an effective protein in reducing hydroxyl radicals and absorbing reactive oxygen species (ROS) generated in stressful situations.

Conclusion

Considering the findings of the current of research and data analysis, it can be concluded that the combination of zinc sulfate and α-tocopherol can reduce the expression of Cytochrome C and Caspase 3 in the brain of rats exposed to lead acetate because there is a synergy between the two antioxidants in preventing an increase in the expression of Cytochrome C and Caspase 3.

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Conflict of interest

The authors have disclosed that they do not hold any conflicts of interest related to the publication of this article.

Ethical approval

The procedure performed in this study was approved by the Faculty of Veterinary Medicine Universitas Airlangga Ethic Committee and has been carried out with certificate No. 502/HRECC.FODM/VIII/2022.

Table 1. Mean percentage and standard deviation caspase expression of brains of white rats (Rattus norvegicus) exposed to lead acetate ($Pb(C_2H_3O_2)_2$).

Treated Animal Group	Brain (Mean ± SD)
C	$1.00^{bcd} \pm 0.00$
P0	$2.24^{bc} \pm 0.16$
P1	$1.64^{abd} \pm 0.167$
P2	$1.56^{ad} \pm 0.16$
P3	$1.20^{acd} \pm 0.24$

*Mean values of logarithmic count for different products with different superscript letters in the same rows are significantly different at ($P<0.05$).

Table 2. Mean percentage and standard deviation cytochrome of brains of white rats (Rattus norvegicus) exposed to lead acetate ($Pb(C_2H_3O_2)_2$).

Treated Animal Group	Brain (Mean ± SD)
C	1.36 ± 0.16
P0	2.52 ± 0.17
P1	1.68 ± 0.22
P2	1.72 ± 0.22
P3	1.28 ± 0.22

*Mean values of logarithmic count for different products with different superscript letters in the same rows are significantly different at ($P<0.05$).

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