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Antioxidant activity of gamma cobalt-60 irradiated chitosan and vitamin E combination to lead acetate-induced rats

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ABSTRACT. The potential of chitosan as a blood lead chelator and an antioxidant had been proven, yet its ability was still not optimal. Chitosan had a relatively large molecular size, which reduced the effectivity of its distribution towards the tissues. Gamma Co-60 irradiation technique was presumably applicable to solve the issue. The antioxidant activity of chitosan could also be optimized by the addition of vitamin E. This study aimed to analyze the antioxidant activity of the combination of Gamma Co-60 irradiated chitosan and vitamin E in lead acetate-induced rats. Twenty-four rats, which were distributed in six groups, were treated using the combination of gamma Co-60 irradiated chitosan at a dose of 150 kGy and vitamin E 1000 IU. All groups, except for the naïve group, were induced with lead acetate. The positive control group was induced with only lead acetate, while treatment group 1 had an additional treatment of irradiated chitosan. The treatment groups 2-4 were treated using the combination of irradiated chitosan and vitamin E in increasing doses respectively for forty days. Blood serum was collected for measurement of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), and Malondialdehyde (MDA). The results showed that the provided treatment increased the enzymatic activity of SOD, CAT, and GPx, and reduced the MDA level in lead acetate-induced rats. However, as the vitamin E dosage was increased, it posed several side effects. It was concluded that the combination Gamma Co-60 irradiated chitosan and vitamin E increased the activity of various endogenous antioxidant enzymes and decreased lipid peroxidation, dependent on the amount of vitamin E.

Keywords: lead intoxication; irradiated chitosan; tocopherol; antioxidant enzymes.

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Introduction

Craftsmen working in industries that use lead (Pb) as raw material become the most susceptible group for Pb exposure, which can lead to Pb intoxication. Pb is not easy to be eliminated from the body and it is prone to accumulation. Chronic Pb accumulation will pose detrimental disruptions in the nervous system, hematopoietic, kidneys, bones, and reproductive system (Carocci, Catalano, Lauria, Sinicropi & Genchi, 2016) disorders. This systemic damage occurs since Pb^{2+} ions will transform into free radicals. These Pb^{2+} free radicals will bind to sulfhydryl and nucleophilic functional groups. These newly generated chemical bonds also play a role in the emergence of oxidative stress. Excessive production of free radicals will damage the cell structures, membranes, and DNA by activating the inflammatory signal cascades (Chibowska et al., 2016; Metryka et al. 2018). Pb^{2+} free radicals also interfere with the formation of enzymes for vitamin D synthesis and the production of hemoglobin (Hb), thus triggering cell membrane damage and failure of Hb production (Rahman, Al-Awadi, & Khan, 2018; Ray, 2016).

Prolonged Pb exposure in their working environment has boosted the possibility of bodily Pb accumulation for craftsmen (Marianti, Anis, & Abdurachim, 2016). Therefore, Pb^{2+} ions must be inactivated by chelation to prevent any side effect from the free radical ions (Kim et al., 2015). One of the well-known, nontoxic, natural compounds for heavy metal chelation is chitosan. Chitosan is a type of polysaccharide made from chitin deacetylation and has six free arms on its chemical structure (Komil & Hamblin, 2016). These free arms bind free heavy metal ions which has the potential of exuding free radical effect. A study about chitosan effectivity in binding blood Pb^{2+} ions has been conducted. Marianti and Mahatmanti (2018) reported that the synergistic effect of chitosan and vitamin C combination increased chitosan capability in chelating blood Pb^{2+} ions. However, chitosan has lower chelation activity compared to disodium ethylene diamine tetraacetate

(NaEDTA) (Marianti, Anatiasara, & Ashar, 2017), which has been commonly used to neutralize Pb^{2+} ions effect in Pb toxication. Chitosan has a relatively large molecular size, which is 1 MDa. Due to its large molecular weight, chitosan also has low solubility. This issue has been suspected as the cause of chitosan's inability in chelating maximum number of heavy metal ions.

Chitosan has minimum therapeutic effects due to its low solubility. Therefore, its molecular size must be reduced to improve the therapeutic effects and oral bioavailability. Chitosan in lower molecular weight shows higher antioxidant activity than natural chitosan (Sugiyanti et al., 2018; Garcia et al., 2015). The solubility level of chitosan is highly correlated to its molecular weight. Compounds with smaller particle sizes can be delivered deeper and further into tissues and provide controlled-release therapy (Rizvi & Shaleh, 2018). Compared to large particles, nanoparticles have stronger curvature on their surface. In coenzyme Q_{10}, the dissolution velocity increases as the particle size decreases and increases its bioavailability (Sun et al., 2012). The different concentration between intestinal epithelial cells and mesenteric circulation will occur, which increases the absorbance level of the compound.

Irradiation is one of the effective methods to decrease chitosan size. A dosage of 150 kGy of cobalt-60 irradiation significantly reduces the molecular weight and viscosity of chitosan (Marianti, Anggraito, & Christijanti, 2020). This condition is mainly caused by the scission of glycosidic bond on deacetylation degree (72e75%) (Garcia et al., 2015). Irradiated chitosan has a higher bioactivity compared to nonirradiated chitosan. This is proven by a significant increase in antimicrobial activity, both in gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) bacteria (Aktar, Hasan, Afroz, Rashid, & Pramanik, 2017). As the molecular weight reduces, chitosan will have a higher chelating activity on blood Pb^{2+} ions and a higher bioactivity as an antioxidant.

The free radical activity of Pb ions inside the body can be identified from several biomarkers, which are Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and Malondialdehyde (MDA) (Hormozi, Mirzaei, Nakhaee, Izadi, & Haghighi, 2018). Marianti and Mahatmanti (2018) reported that a combination of chitosan-vitamin C was synergistic and able to increase the levels of various endogenous antioxidant enzymes (SOD, CAT, GPx) in lead acetate-induced rats.

Aside from vitamin C, vitamin E is also widely known as a beneficial antioxidant that improves cellular antioxidative defense mechanism by regenerating antioxidants. These antioxidants also affect cellular signaling and stimulate redox-sensitive regulation pathways. Vitamin E has a key role in stabilizing cell membranes. In cell membranes and lipoproteins, the antioxidant vitamin E captures peroxyl radicals and breaks the lipid peroxidation chain reaction. Vitamin E does not prevent the initial formation of free radicals which occurs in carbon in a lipid-rich environment, but it reduces the formation of secondary radicals. The function of Vitamin E is protecting cell membranes from lipid peroxidation.

Kurutas (2016) stated that there was evidence to suggest that α-tocopherol and ascorbic acid work synergistically in a cyclic type of process. During the antioxidant reaction, α-tocopherols transformed into α-tocopherol radicals by donating hydrogen to lipid or lipid peroxyl radicals so that α-tocopherol radicals can be reduced to the non-radical α-tocopherol form. This process occurs with the assistance of ascorbic acid. However, excessive dose of vitamin E may cause pro-oxidant condition stimulated by Fenton reaction. Depends on the presence of transition metal, high dose of vitamin E acts as pro-oxidant that alter the cellular redox balance. Furthermore, this condition will disrupt the number of reactive species and pose detrimental effects on many organs (Pérez-Torres, Guarner-Lans, & Rubio-Ruiz, 2017; Rutkowski & Grzegorczyk, 2012).

Lead accumulation causes various hazardous effects on health since Pb^{2+} ions will transform into free radicals inside the body. Aside from reducing the blood lead level, the oxidative activity from lead must also be reduced to prevent the detrimental effects. Chitosan and vitamin E are known for decreasing oxidative activity that occurs due to continuous free radical exposures. Therefore, this study aims to analyze the antioxidant activity of the combination of Gamma Co-60 irradiated chitosan and vitamin E in improving activities endogenous antioxidant enzymes and MDA level in lead-acetate-induced rats.

Material and methods

Animals

This study used healthy male Wistar rats aged two months with a body weight of 200 ± 10 g. Rats were obtained from the Laboratorium Penelitian dan Pengujian Terpadu (Integrated Research and Testing

Laboratory) Unit 4 Universitas Gadjah Mada, Yogyakarta, Indonesia. Rats were acclimatized for seven days in the Laboratory of Animal Physiology of Biology Department Faculty Mathematics and Natural Sciences Universitas Negeri Semarang, Indonesia. Animals were fed with standard rat pellets and water ad libitum. The ethical clearance was issued by The Medical/Health Research Bioethics Commission, Faculty of Medicine Universitas Islam Sultan Agung Semarang, Indonesia with approval number of 665/X/2019/Komisi Bioetik.

Chitosan irradiation

This research used pharmaceutical-grade Chitosan powder (CV Chimultiguna), made from the chitin of Crustacean shells, sized 80-100 Mesh, viscosity of 20-100 mPa s, with minimum degree of deacetylation at 94%. Chitosan was irradiated with Gamma Cobalt-60 ray from Gamma Chamber 400 A at the dosage of 150 kGy (Marianti et al., 2020). The irradiation process was performed in Center for Isotopes and Radiation Application (CIRA) BATAN Jakarta, Indonesia. Irradiated chitosan dissolved in 2% acetic acid.

Vitamin E

Vitamin E used in this study was D-Alpha-Tocopherol Acetate Powder (Zhejiang Medicine Co. LTD). The amount of 700 IU of Vitamin E g^{-1} was dissolved in palm oil.

Experimental procedures

This research was an experimental laboratory study that was carried out in Laboratory of Biology Faculty Mathematics and Natural Sciences Universitas Negeri Semarang, Indonesia. Twenty-four male Wistar rats (*Rattus norvegicus*) were distributed into six groups, each group consisted of four rats. The doses of irradiated chitosan and vitamin E were determined before lead-acetate induction.

All groups were induced with 175 mg kg^{-1} Body Weight (BW) of lead acetate, except for naïve group (NG). Groups induced by lead acetate were positive control group (PC); treatment group (TG) 1, treated with 64 mg kg^{-1} BW of Gamma Co-60 irradiated chitosan; treatment group (TG) 2-4, treated with the combination of 64 mg kg^{-1} Body Weight (BW) of Gamma Co-60 irradi

ated chitosan and vitamin E at the doses of 1.44 (low dose), 2.16 (medium dose), and 3 (high dose) mg kg^{-1} BW respectively (Marianti & Mahatmanti, 2018). All treatments were administered through peroral route. A complete series of treatments was carried out for 40 days. On the 41st day, 3 mL of blood was collected from the orbital sinus of each rat and was added with EDTA to prevent coagulation. Then, samples were centrifuged at 3000 rpm for 15 minutes, for blood plasm collection. Blood plasm was used to measure the enzymatic activity of SOD, CAT, GPx, and MDA level.

Lipid peroxidation (MDA) assay

MDA level was determined using the thiobarbituric acid (TBA) test (Zeb & Ullah, 2016). The test was conducted at Laboratorium Pangan dan Gizi (Food and Nutrition Laboratory) Inter University Center/IUC Universitas Gadjah Mada Yogyakarta Indonesia.

Antioxidant enzyme activities assay

The tests were conducted at Laboratorium Pangan dan Gizi (Food and Nutrition Laboratory) Inter University Center/IUC Universitas Gadjah Mada Yogyakarta Indonesia.

Enzymatic activity of SOD was determined using Colorimetric Assay (Biovision). Blood samples were placed into tubes that were previously added with EDTA. Tubes were centrifuged at 1000 rpm for 5 minutes. The blood plasm layer, formed during the process, was separated from the solid parts using a micropipette. Then, blood plasm was stored in freezer at the temperature of -80°C. The supernatant was stored at -80°C until it was ready to be analyzed. SOD activity was calculated using units (U mL^{-1}).

SOD activity was measured based on the inhibition rate of ferric cytochrome-c reduction. Ferric cytochrome-c reduction increased absorbance which can be observed at 550 nm and 25°C. Xanthine oxidase must remain cold prior to the treatment. The reaction medium was prepared by prepending 2.9 mL of solution A (0.76 m g of xanthine in 10 mL of 0.001 M NaOH, added with 1.8 mg of cytochrome c solution in 100 mL of phosphate buffer, pH 7.8 without EDTA) into 3 mL of the reaction tube. The reaction began by adding 50 µL of solution B (2.88 mg mL^{-1} of xanthine oxidase in EDTA phosphate buffer) into the solution A. The tube was then slowly mixed using a vortex. Absorbance rate changes were observed using a spectrophotometer. The phosphate buffer was used as blank solution for samples and bidistillated water was used as blank solution for the control group (Winarsi, Wijayanti, & Purwanto, 2012)

Catalase activities assay

Blood plasm was made into lysate which included 200 µL of blood plasm + 800 µL of 0.5% triton X-10 solution. Stock solution was made by dissolving 10 µL of catalase into 50 mL of phosphate buffer. Standard solution was prepared by dissolving 0.5 mL of stock solution into 9.5 mL of phosphate buffer (1/20) and 0.5 mL of stock solution into 19.5 mL of phosphate buffer (1/40). It was mixed 10 µL of lysate with 12.5 mL of phosphate buffer. The reaction started after adding 1 mL of hydrogen peroxide (H_2O_2). The solution was slowly stirred using a vortex. A spectrophotometer was used to detect decreased absorbance at wavelengths of 240 nm and the interval of 15, 30, 45, and 60 seconds. A240 value ranges from 0.02-0.10 (Winarsi et al., 2012).

Glutathione peroxidase assay

Enzymatic activity of plasmic glutathione peroxidase (GPx) was analyzed using liquefied 100 µL of plasm with 200 µL of physiological saline (0.85% of NaCl solution). From the solution 0.1 mL was mixed with 0.4 mL of 0.5% triton-X which produced hemolysate. The amount of 100 µL of hemolysate and 100 µL of Drabkin solution were hemogenized using mixer vortex, then added with 2.6 mL of phosphate buffer. The solution was slowly mixed. To the solution, 0.1 mL of NADPH, 0.01 mL of GSSG-R, 0.01 mL of NaN_3, and 0.1 mL GSH was added Then, the solution was mixed. Before absorbance reading 1 mL of H_2O_2 was added into silica cuvette. The absorbance rate was measured in the spectrophotometer at the wavelength of 340 nm. Absorbance reading was conducted at an interval of 1-2 minutes (Winarsi et al., 2012).

Statistical analysis

The resulting data were analysed using the IBM Statistical Package for Social Sciences (SPSS) Windows version 24.0. All data were represented in mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA). Statistical probability of $p < 0.05$ was considered significant. Duncan Multiple Range Test (DMRT) would be used if the results differed significantly.

Results

After being induced with lead acetate for 40 days and being treated with a combination of 150 kGy of irradiated chitosan and vitamin E in various doses, the enzymatic activity of various antioxidants (SOD, CAT, and GPx) and lipid peroxidation (MDA) from all rats were measured. The results are shown in Figure1.

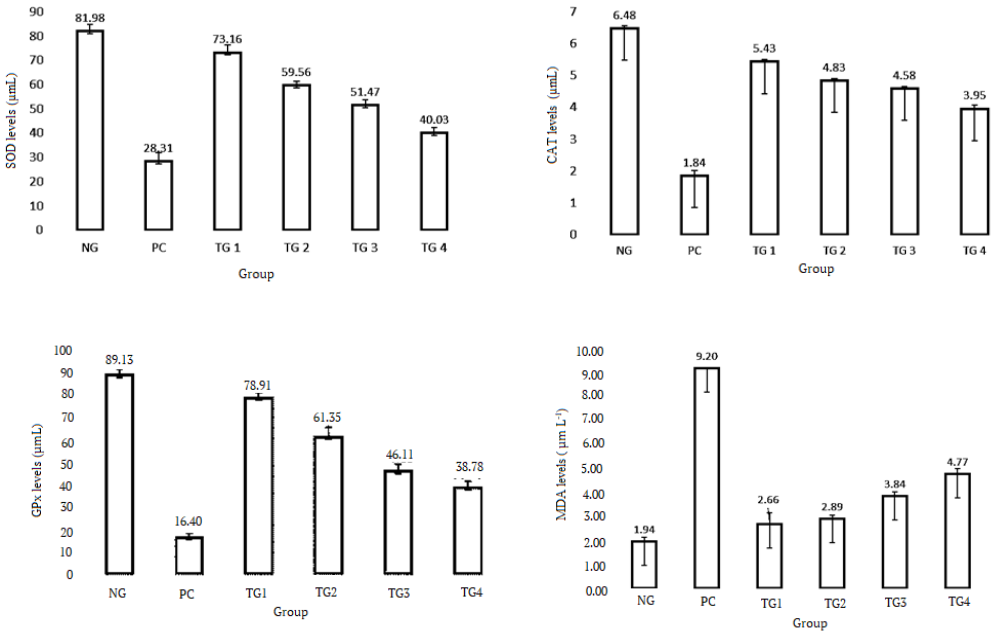

Figure 1. Levels of antioxidant enzymes (SOD, CAT, GPx) and lipid peroxidation (MDA) in lead acetate induced rats treated with Gamma Co-60 irradiated chitosan and vitamin E. (NG (Naïve Group): not subjected to any treatment; PC (Positive Control Group): induced with 175 mg kg^{-1} BW of lead acetate; TG 1 (Treatment Group 1): induced with 175 mg kg^{-1} BW of lead acetate + 64 mg kg^{-1} BW chitosan irradiated, TG 2-4 (Treatment Group 2-4): induced with 175 mg kg^{-1} BW of lead acetate + 64 mg kg^{-1} BW chitosan irradiated + vitamin E at the doses of 1.44, 2.16, and 3 mg kg^{-1} BW, respectively.

Figure1 showed that out of all groups, treatment group (TG) 1 had the highest antioxidant enzyme level, while positive control (PC) group had the lowest levels of antioxidant enzymes. The results of the highest antioxidant enzyme activity for groups treated with irradiated chitosan-vitamin E combination showed a certain trend. Groups that were treated with the combination of low dosage of Gamma Co-60 irradiated chitosan and vitamin E (TG 2-4) had the highest antioxidant enzyme activity. However, as the vitamin E got higher, the antioxidant enzyme activity decreased. This phenomenon also occured in lipid peroxide level, which used MDA as biomarker. Higher MDA levels could be found in groups with higher vitamin E doses.

One-way ANOVA results indicated that there was a very significant difference of antioxidant effect between all treatment groups in all tested parameters (Table 1).

Table 1. The result of One-way ANOVA analysis on the antioxidant activity from the Gamma Co-60 irradiated chitosan and vitamin E combination in lead acetate-induced rats.

Variable	F value	Significance (p<0.05)
SOD	169.554	.000
CAT	871.863	.000
GPx	353.245	.000
MDA	290.759	.000

Since the results showed a significant difference, DMRT analysis was then performed. The DMRT result showed that in all the parameters tested in this study there were significant difference among all treatment groups (Table 2).

Table 2. The result of Duncan Multiple Range Test (DMRT) analysis on the antioxidant activity of the combination of gamma Co-60 irradiated chitosan and vitamin E in rats induced by lead acetate.

Group	SOD (U mL^{-1})	CAT (U mL^{-1})	GPx (U mL^{-1})	MDA (µm L^{-1})
NG	81.98[a] ± 2.82	6.48a ± 0.08	89.13a ± 2.40	1.94a ± 0.18
PC	28. 31b ± 3.51	1.84b ± 0.18	16.40b ± 1.71	9.20b ± 0.43
TG 1	73.16c ± 3.18	5.43c ± 0.08	78.91c ± 2.30	2.66c ± 0.49
TG 2	59.56d ± 1.64	4.82d ± 0.07	61.35d ± 4.30	2.89c ± 0.16
TG 3	51.47e ± 2.32	4.58e ± 0.06	46.11e ± 3.18	3.84d ± 0.19
TG 4	40.03f ± 2.12	3.95f ± 0.12	38.78f ± 2.55	4.77e ± 0.22

Note: Values with different letters in the same column are different by DMRT (p <0.05).

Discussion

The results from this study show that Gamma Co-60 irradiated chitosan effectively increases the activity of the endogenous antioxidant enzyme. The results are proven in lead acetate-induced rats tested in this study. Groups of lead acetate-induced rats, which were treated with Gamma Co-60 irradiated chitosan, had the highest enzyme activity (SOD, CAT, and GPx). Those groups also presented low MDA levels. This result is presumably caused by the chemical properties of irradiated chitosan. Irradiated chitosan has low viscosity and molecular weight, which increases its solubility inside bodily fluids. By possessing higher solubility, irradiated chitosan will be widely transported throughout the tissue. Irradiated chitosan will bind free radicals from Pb^{2+} ions in bodily fluid. The results itself are in accordance with Garcia et al. (2015) and Aktar et al. (2017). Since the free radicals are bound to irradiated chitosan, the ions will be more stable and cause less oxidative effects on the body. Low MDA level, as observed in this study, also supports the theory. MDA is widely known as the lipid peroxide biomarker, which is formed during free radical activities. High lipid peroxide levels will later on damage the cells (Ayala, Muñoz, & Argüelles, 2014). Naturally, human body is equipped with systems to counter the negative effects emitted by free radicals. Endogenous antioxidant enzymes scavenge electrons that will deactivate and eliminate the free radical activities (Nimse & Pal, 2015).

As the viscosity of chitosan decreases, its effectivity as a chelating and antioxidant agent also significantly improves. Chitosan viscosity is highly correlated to its molecular weight (MW). Lower molecular weight will decrease the viscosity of chitosan. As the viscosity gets lower, the solutes will be effectively and more widely transported to the tissues by the solutions (Rizvi & Saleh, 2018).

In order to enhance the antioxidative property of chitosan, vitamin E as an exogenous antioxidant was also added as a treatment in this study. The result showed that a combination of Gamma Co-60 irradiated chitosan and vitamin E in several doses, effectively increased the antioxidant activity and reduced the MDA level.

Several studies have shown that excessive dosage of exogenous antioxidants exhibits pro-oxidant activity. This condition worsens due to the presence of several divalent metal ions such as Fe, Cu, and other transition metal ions which will reduce antioxidant activity. Antioxidants provide the most reductive power in the presence of the fenton reaction along with increased OH formations (Koçyiğit & Selek, 2016). This reaction has the potential to increase the detrimental effects from free radical activities. In this study, the presence of blood lead in experimental animals is proven to decrease endogenous antioxidant activities (SOD, CAT, and GPx) and increase MDA level, especially in the group treated with the highest dose of vitamin E compared to the group receiving lower doses.

Several *in vitro* studies have reported that increasing the administration of exogenous antioxidants (quercetin, catechin, epicatechin, epigallocatechin-3 gallate) in excessive dosage alters the chemical properties of the compounds. These antioxidants will transform into prooxidants. Quercetin has the potential to reduce ROS level in mitochondria and prevents its dysfunctions (Godoy et al., 2016).

Reactive oxygen species (ROS) plays several essential roles in the physiological system. ROS takes part in cell signaling processes, regulation of immune responses, gene expression and antioxidant activity. Fourty mammalian gene variations are activated by the presence of H_2O_2. The redox balance is important for homeostasis that regulates many biological processes and must be maintained for optimum antioxidant activity (Pérez-Torres, Guarner-Lans, and Rubio-Ruiz, 2017). On a high level, antioxidants change into prooxidant. This condition is also supported by the result of this study. In treatment group 4, which was treated with the highest vitamin E levels, the rate of lipid peroxidation increased. Increasing on lipid peroxidation rate was indicated by the increase of MDA level. During lipid peroxidation, the cell membrane loses electrons to free radicals which impairs the membrane integrity. Free radicals also take up electrons from polyunsaturated fatty acid chains, which are reactive hydrogens, from the methylene ($-CH_2-$) bridge. The final product of lipid peroxidation is reactive aldehydes, including malondialdehyde (MDA). Being known as a lipid peroxidation inhibitor, the usage of vitamin E must be sufficient. Excessive usage of vitamin E will disrupt the redox balance that poses several cellular dysfunctions.

Conclusion

The combination of Gamma Co-60-irradiated chitosan and vitamin E effectively increases the activity of endogenous antioxidant enzymes. This combination can also reduce lipid peroxide level in lead acetate-induced rats. However, increasing the dose of vitamin E will yield harmful effects. Vitamin E will convert into a prooxidant when the high-dosage treatment of the vitamin meets with a relatively high level of lead ions in lead acetate-induced rats.

Providing vitamin E in high dosage, supported by the relatively high level of lead ions in lead-acetate induced rat, will convert vitamin E into a prooxidant.

This research faced several limitations. The treatment was only conducted for 40 days, whereas the ideal duration for sub-chronic exposure was 60 days. Aside from that, there were only several parameters that can be derived from this research, due to the limited amount of blood samples collected from test animals.

Irradiated chitosan and its combination with other antioxidants (vitamin C and E) have the potential to serve as health supplement products to overcome the side effects of Pb^{2+} ions accumulation. This supplement will be beneficial for people who cannot avoid prolonged heavy metal exposure from their environment.

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