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Original Article

Resveratrol-Tempeh reduce micronucleus frequencies bone marrow cells and stimulate osteocyte proliferation in aluminum chloride-induced mice

Redução de frequências de micronúcleos células da medula óssea e estímulo proliferação de osteócitos em camundongos induzidos por cloreto de alumínio através do uso de Resveratrol-Tempeh

Y. Irnidayanti[a,b*], D. G. Maharani[b], M. H. Rizky[b], M. I. Noer[a] and V. Rizkawati[a]

[a]Universitas Negeri Jakarta, Faculty of Mathematics and Natural Sciences, Department of Biology, Jakarta, Indonesia
[b]Universitas Negeri Jakarta, Faculty of Mathematics and Natural Sciences, Research Group of Structure and Animal Development, Jakarta, Indonesia

Abstract

Aluminum (Al) is widely used for water purification, cooking pots, cosmetic and pharmaceutical preparations, toothpaste tubes, and food processing industries. Although the transport in the digestive tract is very poor but if the load is high, it can be absorbed and accumulated. About 50-70% of Al accumulates in the bones and can have an impact on human health. Resveratrol (RES), isolated from tempeh as an Indonesian food ingredient, can increase cell viability and has promising cytoprotective effects. RES has the capacity to interact with oxidative stress, so it has the potential as a therapy in bone repair. Therefore, this study aimed to evaluate the effect of RES on the number of osteocytes and bone marrow cells in Al-induced mice. Swiss Webster mice were divided into four groups: (1) untreated groups, (2) $AlCl_3$-treated groups, (3) Al+Res5 treated groups, and (4) Al+Res10 treated groups. Al dose 200 mg/kg body weight was administered intraperitoneally. RES was given one hour after administration of Al, with doses of 5 and 10 mg/kg Body Weight. Al and RES administration is carried out for one month. All mice were sacrificed, and mouse bones were isolated for histological preparations and a half for genotoxic assays. Bone marrow cells were collected and stained with My Grunwald. The number of micronuclei polychromatic erythrocytes (MNPCE) was examined in 1,000 PCEs per animal. The number of PCEs is counted by at least 200 erythrocytes (PCE + NCE) per animal. The results showed that the administration of Al significantly increased the number of micronuclei (MN) but after administration of RES at doses of 5 and 10 mg/kg Body Weight significantly reduced the number of MN in bone marrow cells. A dose of RES 10 mg/kg BW stimulates proliferation and increases the number of osteocytes in bone significantly. It can be concluded that Al can cause genotoxicity in bone marrow cells and RES is anti-genotoxic and can stimulate osteocyte proliferation.

Keywords: aluminum, bone marrow cells, genotoxic, osteocytes, Resveratrol.

Resumo

O alumínio (Al) é amplamente utilizado para purificação de água, panelas, preparações cosméticas e farmacêuticas, tubos de pasta de dente e indústrias de processamento de alimentos. Embora o transporte no trato digestivo seja escasso, se a carga for alta, pode ser, todavia, absorvida e acumulada. Cerca de 50-70% do Al se acumula nos ossos e pode ter impacto na saúde humana. O resveratrol (RES), isolado do tempê indonésio como ingrediente alimentar, pode aumentar a viabilidade celular e tem efeitos citoprotetores promissores. O RES possui a capacidade de interagir com o estresse oxidativo, e por essa razão pode ser utilizado como terapia no reparo ósseo. Portanto, este estudo teve como objetivo avaliar o efeito do RES no número de osteócitos e células da medula óssea em camundongos induzidos por Al. Camundongos Swiss Webster (SW) foram divididos em quatro grupos: (1) grupos não tratados, (2) grupos tratados com $AlCl_3$, (3) grupos tratados com Al+Res_5 e (4) grupos tratados com Al+Res_{10}. Uma dose de 200 mg/kg de peso corporal foi administrada por via intraperitoneal. O RES foi administrado uma hora após a administração do Al, nas doses de 5 e 10 mg/kg de peso corporal. A administração de Al e RES foi realizada por um mês. Todos os camundongos foram sacrificados, e os ossos dos camundongos foram isolados para preparações histológicas e meio para ensaios genotóxicos. As células da medula óssea foram coletadas e coradas com *My Grunwald*. O número de eritrócitos policromáticos micronúcleos (MNPCE) foi examinado em 1.000 PCEs por animal. O número de PCEs foi contado por pelo menos 200 eritrócitos (PCE + NCE) por animal. Os resultados mostraram que a administração de Al aumentou significativamente o número de micronúcleos (MN), mas após a administração de RES nas doses de 5 e 10 mg/kg de peso corporal reduziu significativamente o número de MN nas células da medula óssea. Uma dose de RES de 10 mg/kg BW estimula a proliferação e aumenta significativamente o número de osteócitos no osso. Dessa forma, pôde-se concluir que o Al pode causar genotoxicidade em células da medula óssea e o RES é antigenotóxico e pode estimular a proliferação de osteócitos.

Palavras-chave: alumínio, células da medula óssea, genotóxico, osteócitos, Resveratrol.

*e-mail: irnidayanti@gmail.com; yirnidayanti@unj.ac.id
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1. Introduction

Aluminum (Al) has several applications in daily life, including the treatment of drinking water as a flocculant, the production of antiperspirants, and the processing, packaging, and storage of foodstuffs. Al is indispensable for human life in the twenty-first century, and it is ensured that daily exposure is accelerated, and body load continues to increase. Humans can come into contact with Al in a variety of ways, including through cookware and food additives. Al-containing drugs such as antacids and vaccine adjuvants can also increase the amount of body exposure. The main exposure in the population occurs through food consumption (Shaw and Marler, 2013). Most processed foods contain relatively high concentrations of Al compared to unprocessed (Krewski et al., 2007). Several reports indicated that the concentration of Al in foods from different countries (United States, Greece, Belgium, Southern China, and the European Union) varies widely, and there are prominent differences. Human exposure in foodstuffs is negligible, but the use of Al in household appliances for acidic or salty foods and others can increase exposure moderately.

Each day, 50-75 mg of Al enters the body through antiperspirant spray, and 0.07 mg is present in each glass of water (Crisponi et al., 2013; Sharma et al., 2015). Extensive consumption, specifically in a daily lifestyle that cannot be separated from Al, makes this industrial product's potential for humans to be exposed (Taïr et al., 2016). An increase can occur during acid rain, where more Al mineral transports to drinking water sources. It is not surprising that Al in the human body is increasing. Therefore, it is included in the priority list of hazardous substances approved by the Agency for Toxic Substances and Disease Registry (ATSDR). Daily intake of Al is very low (0.05-2.2%), and its elimination is also slow. Continuous Al intake over many years can lead to accumulation in the deep compartment (Priest, 2004).

Al accumulates in all tissues of animals, preferentially in kidneys, liver, heart, bones, and brain (Gonzalez et al., 2007). Crisponi et al. (2011) reported that 70% Al will remain in the bone, and other studies showed that 58–70% of the total human Al body burden accumulates in bone, which has a half-life of 10-20 years (Krewski et al., 2007; VanDuyn et al., 2013). The presence of Al in bone for a long time can affect osteoblasts' proliferation, differentiation, and mineralization (Yang et al., 2016; Huang et al., 2017). Loss of osteoblasts led to a dramatic reduction of bone marrow cellularity and resultant extramedullary hematopoiesis, consistent with the loss of the ability support hematopoiesis (Visnjic et al., 2004). Al can reduce the number of osteoblasts, inhibiting bone formation. In addition, a reduced number of osteoblasts can lead to peak bone mass closer to the osteoporosis threshold (Crisponi et al., 2011). Since iron deficiency, osteoblast cells take up Al, which accumulates in the bones, thereby can impair absorption of calcium and phosphorus. Disorders of calcium and phosphorus metabolism can reduce the content of minerals and trace elements. Impact on bone cause a decrease bone mineral density and lead to the destruction of bone microstructure (Li et al., 2011).

Al is also genotoxic in bone marrow cells, which is indicated by the formation of micronuclei (Al-Obaidy, 2016). Cellular interactions caused by Al can form oxygen free radicals. ROS causes an increase in lysosomal membrane permeability through inhibition of protein pumps and the release of DNase in the cytoplasm (Zatta et al., 2000). The release of the DNase enzyme, which passage into the nucleus leads to DNA fragmentation, breaks chromosomes, and interferes with the formation of the mitotic spindle in the nucleus (Lima et al., 2007). Paz et al. (2017) showed that Al has the potential as a genotoxic agent and can damage DNA even in very small concentrations. Therefore, it is urgent to find protective strategies for Al-induced bone toxicity.

Resveratrol (RES) are polyphenolic compounds from natural foods such as fruits, vegetables, and seeds. Products derived from grapes, such as grape juice, can reduce lipid peroxidation and DNA damage. However, using products with high concentrations as preservatives in wine can cause side effects from these compounds. Many studies proved that RES is a protective cell against oxidative stress (Kairisalo et al., 2011) and can act as a radioprotector and radiomitigator (Dobrzyńska and Gajowik, 2022). It is effective in reducing genotoxic damage and minimizing the number of sister chromatids and micronucleus exchange (Türkez and Şişman, 2012). It can also inhibit bone resorption, promote osteoblast differentiation (Boissy et al., 2005), and reduce nuclear DNA fragmentation (Sgambato et al., 2001). Therefore, RES in natural foods acts as important antioxidants (Leonard et al., 2003) and free radical scavengers. Trans-RES is rapidly absorbed and metabolized in the body, and metabolites excreted from RES are always conjugated with glucuronides and sulfates in urine or serum (Williams et al., 2009).

RES is one of the most studied stilbenes, and the compound has been isolated from tempeh, an original Indonesian food (Irnidayanti and Sutiono, 2019). RES-tempeh is neuroprotective because it can increase viability and inhibit neuronal cell death in vitro (Irnidayanti et al., 2022). Recent studies recommended RES and its bioactive compounds as a nutritional supplement because of positive effects on health (Moraes et al., 2021). This study is very important in nutrient supplement development because this compound has the potential as a protective agent against osteocytes and DNA damage due to aluminum induction. This study aims to determine the role of RES as an anti-genotoxic agent against bone marrow cells and stimulate osteocyte proliferation

2. Materials and Methods

This study was performed according to procedures approved by the Institutional Ethical Committee of Indonesia University, Indonesia (Protocol number KET-221/UN2.F1/ ETIK/PPM.00.02/2020).

2.1. Chemical agents

The Aluminum chloride (CAT-No:7784-13-6, Merck-Germany) was used in this study, and was diluted in aquabides at a dose of 200 mg/kg bodyweight (Guo et al.,

2001). RES-tempeh was isolated from tempeh (Irnidayanti and Sutiono, 2019). Meanwhile, RES was diluted in dimethyl sulfoxide (DMSO, Sigma) at a dose of 5 mg/kg bodyweight and 10 mg/kg bodyweight. The volume of injection for the control and treated mice was 0.1 ml/10 g bodyweight. Distilled water was used as a control, and all the solutions were prepared immediately before use.

2.2. Animal sample

Swiss Webster mice were used, and after the acclimatization period, male mice were divided into four groups as follows: (1) untreated groups, (2) Al-treated groups, (3) Al+Res5-treated groups, and (4) Al+Res10-treated groups. The range of body weight is 23.5-29.5 g, and they kept on a commercial diet and provided tap water ad libitum. The animals were obtained from the laboratory at the Food and Drug Supervisory Agency, Jakarta. They were kept in an environment of 23ºC and 27ºC, at 83% humidity, with 12 hours of light/12 hours of darkness (Rugh, 1968).

2.3. Experimental procedures

The Al solution was injected intraperitoneally in all treated groups every two days for a month (Sharma et al., 2006). One hour after Al injection, RES was injected intraperitoneally in Al+Res5 and Al+Res10 treated groups. The body weight was recorded daily until samples were collected. Subsequently, animals were sacrificed by cervical dislocation, and the femoral bones were isolated and cleaned. Half of the bones were fixed in 10% Neutral buffered formalin solution for histological observation, and the remaining were used for the in vivo micronucleus assay.

2.3.1. Paraffin methods

The femoral bones were fixed in neutral buffered formalin solution 10% overnight. Furthermore, the bones were rinsed in water for 1 h to remove the fixative. For two days, the decalcification process with a TBD-1 Decalcifier solution was conducted (Shandon, Thermo Scientific). The femur bones were cut 2mm in thickness and soaked in tap water overnight. Proceeded to dehydration in ascending ethanol concentrations (70% up to 100%). The femur bones were cleared in xylene, and paraffin infiltration was conducted in an oven at a temperature of 57-60°C for four hours. Embedding was carried out in paraffin block and was sliced by a rotary microtome at 5 µm thickness. Meanwhile, the slides were stretched and dried in a hot plate at a temperature of 42°C. To remove the paraffin, the slides were immersed in a xylene solution, hydrated in alcohol whose concentration decreased gradually, and stained with a solution of Hematoxylin-Eosin (Conn et al., 1960). Finally taped on the glass objects that Entellan had poured.

2.3.2. The in vivo micronucleus assays

The femoral bones were swabbed with 70% ethanol, and the epiphyseal portions were isolated and cleaned from the other tissues and muscles. The epiphysis portion was clamped with forceps in a vertical position over the edge of a test tube by a sterile syringe. The bone marrow cells were flushed out with 0.5 mL of fetal calf serum (Gibco) into centrifuge tubes by a 1-mL syringe fitted with a 22 G needle. The suspension was centrifuged at 500 rpm for 5 minutes, and the supernatant was discarded. Furthermore, the deposited cells were resuspension again with the remaining serum (Kasamoto et al., 2013). A small amount of the cell suspension was dropped on a glass slide and spread by pulling the material behind a polished cover glass held at an angle of approximately 45°. The slide smears were allowed to air-dry and fixed in methanol for 3 minutes. Slides stained with 3% Giemsa (Merck) for 30 min, rinse with sodium phosphate buffer and purified water, and dry. Meanwhile, it was rinsed with 0.001% citric acid solution, and purified water, before redrying. Emersion oil was used with a light microscope at a magnification of 1000 times for counting the cells. The mature erythrocytes (normochromic erythrocytes: NCE) were colored pink and have a bluish tint in the immature forms (polychromatic erythrocytes: PCE). As an indicator of cell toxicity in bone marrow, PCE was counted to at least 200 erythrocytes (PCE+NCE) per animal under a microscope (1000x). The ratio of PCE to total erythrocytes was calculated as percent. At least 1000 PCE per animal were examined, and the number of micronuclei (MNPCE) was counted accurately. Incident of MNPCE to total PCE is also calculated as percent. According to Schmid (1975), the criteria of micronuclei were identified as darkly stained (purple), round or almond shape. Micronuclei have sharp borders and are generally 5-20% of the size PCE.

2.4. Statistical analysis

The variables are the number of osteocytes and histology of the femoral trabeculae, MNPCE cell and the ratio of PCE/NCE. The data analysis used one-way analysis of variance (ANOVA) and Dunnett tests to show significant differences between the groups and to indicate in which groups at α 0.05 the differences are significant.

3. Result

The normal bone of the femur has large inner spaces known as vascular cavities, lined by bone trabeculae as walls. The bone trabecula contains collagen fibers arranged in the lamella. The bone trabecular matrix has cylindrical osteocytes, and the lacunae are clear (Figure 1A). Al exposure led to a deregulation of trabeculae compared to the untreated groups, some osteocyte cell destruction, and empty lacunae, trabecular derangement (Figure 1B). Administration of RES at a dose of 10 mg/kg body weight caused trabecular rearrangement of the femoral bone in the Al-treated group of mice (Figure 1D), but in the Al + Res5 group did not occur trabecular rearrangement (Figure 1C).

The number of osteocytes in the trabecular bone of the femur in the Al-treated group is significantly lower than in the untreated groups (Table 1). The number of osteocytes in Al+ Res 5-treated groups not differed from Al-treated groups, and both were a decrease significantly compared to the untreated group. Al caused a decrease in the number

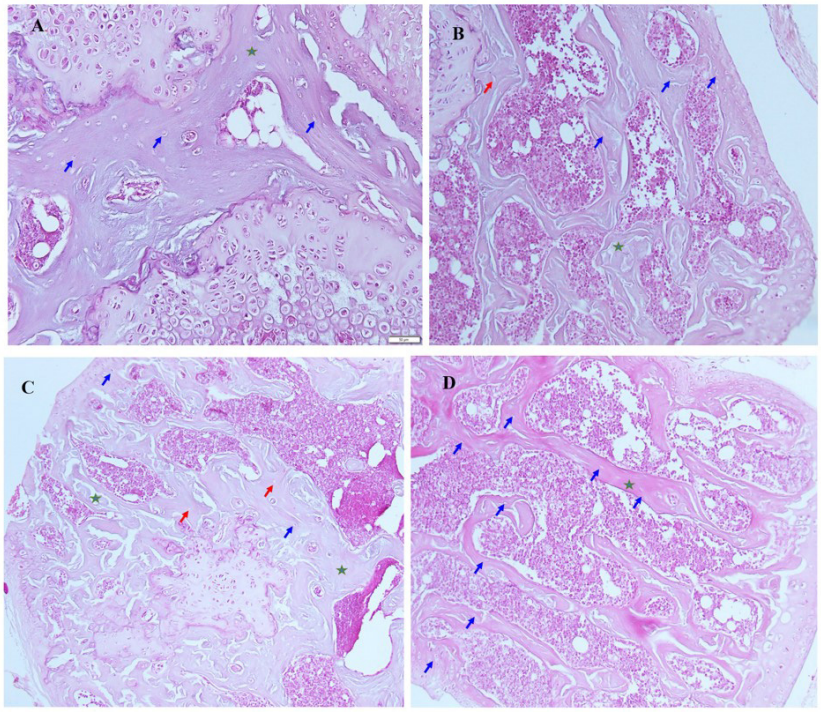

Figure 1. H&E staining of the femur bone mice. (A) untreated-group, 10X20; (B) Al-treated group 10X10; (C) Al+Res5-treated group, 10X10 and (D) Al+Res10-treated group, 10X10. Green star: Bone trabecular, Blue arrow: osteocytes, Red arrow: empety lacunae.

Table 1. Effects of RES-tempeh on PCE micronucleus formation in the bone marrow and the number of osteocytes of femur cancellous bone in AlCl3-induced mice.

Sample	No. of PCE scored for MN (Σ)	MN PCE observed $\bar{x}$ ± SD	MNPCE (%)	Osteocytes $\bar{x}$ ± SD
Control	1000	95.33±16.24[a]	9.53[a]	539.92±14.515[a]
Al-Group	1000	279.16±15.19[b]	27.92[b]	447.33±23.367[b]
Al+ Res 5-treated group	1000	174.83±16.09[c]	17.48[c]	477.74±10.62[b]
Al+ Res 10-treated group	1000	116.33±8.16[a]	11.63[a]	707.15±15.288[c]

Note: significant at P≤0.05. PCE: polychromatic erythrocyte. a, b, c, d superscript meaning significant difference from each other's.

of osteocytes and inhibited osteocyte proliferation in the trabeculae. Administration of RES at a dose of 10 mg/kg body weight significantly increased the number of osteocytes in bone mice of Al-induced. The number of osteocytes in Al+Res 10-treated groups is significantly much greater than Al+5 treated group. It means that resveratrol at a dose of 10 mg/kg body weight affects the proliferation of osteocytes in trabecular of femur bone.

Table 1 shows the effect of RES on the formation of micronucleus (MN) as an indicator of genotoxicity. The number of MN in the Al-treated group (279.16 ± 15.19) was significantly higher than in the untreated group (95.33±16.24). Administration of RES at doses of 5 and 10 mg/kg body weight was significantly lower than the Al group. However, RES 10 mg/kg body weight was not significantly different from the untreated group. This means that RES 10 mg/kg body weight is more effective than RES 5 mg/kg body weight. It can be concluded that Al causes DNA damage, so it is genotoxic and RES is anti-genotoxic because it can protect against DNA damage caused by Al.

This study demonstrated that the PCE/NCE ratio was not significant and demonstrated no bone marrow cytotoxicity observed in male rats after Al exposure compared to the untreated group (Table 2). The ratios indicated the rate of proliferation and PCE to NCE turnover, and RES administration was not significant to the PCE/NCE ratio.

4. Discussion

Femur cancellous bone has vascular cavities occupied by blood vessels, hematopoietic cells, bone marrow, and trabeculae. The trabeculae are the walls of the blood

Table 2. Effects of RES-tempeh on the ratio of PCE/NCE in Al-induced mice bone *marrow cells*.

Group	Total of erythrocytes scored	No. of PCE observed $\bar{x}$ ± SD	No. of NCE observed $\bar{x}$ ± SD	Ratio PCE/NCE $\bar{x}$ ± SD
Untreated groups	200	116.67 ± 10.17	82.17 ± 9.54	1.45 ± 0.32
Al-Groups	200	116.50 ± 13.95	81.00 ± 12.70	1.49 ± 0.42
Al+ Res5-treated groups	200	118.50 ± 10.19	79.33 ± 10.29	1.53 ± 0.35
Al+ Res10-treated groups	200	116.17 ± 9.11	81.33 ± 9.44	1.46 ± 0.29

Note: significant $P \leq 0.05$. NCE: normochromatic erythrocyte.

vessels' cavities, filled with osteocytes, the mature bone cells. The trabecular bone structure is a good predictor of bone mass, and it is more susceptible to loss due to toxic agents. A bone mineral density decrease is also caused by impaired absorption of calcium and phosphorus. The disorders of calcium and phosphorus metabolism caused by Al can reduce the content of minerals and trace elements (Li et al., 2011). The inhibition of the turnover of bone and the reduction of the differentiation activity of osteoblasts can be caused by Al (Rodriguez et al., 1990). The effect of long-term Al exposure reduces the levels of mineral and trace elements in bone and caused bone loss, particularly in cancellous bone (Li et al., 2011). Furthermore, the number of osteocytes in this trabecular area of Al-treated groups is significantly more than in the control. It is concluded that aluminum interferes with mineral resorption in bone trabeculae.

Administration of RES-tempeh at a dose of 10 mg/kg BW affected the trabecular area of Al-induced mice. This data is supported by the number of osteocytes in this trabecular area significantly more than in untreated groups and Al-treated groups. Zhao et al. (2014) showed that RES's effect can increase trabecular area values. Other studies also showed that RES could protect bone marrow mesenchymal stem cells and regulate osteogenic differentiation (Lv et al., 2018; Lei et al., 2008). Flavonoids have highly particular effects on many enzymes and can influence cellular pathways such as differentiation and proliferation (Lahouel and Fillastre, 2004). RES reduces Chromium-induced MN frequency and can repair cells. This is due to the polyphenol content and antioxidant properties of RES (Nicolás-Méndez et al., 2022). It was concluded that the dose of RES-tempeh with a dose of 10 mg/kg BW can increase the number of osteocytes. RES stimulates the proliferation of osteoblasts and the differentiation of osteoblasts into osteocytes. RES-tempeh is a promising protective agent against bone loss and has the same activity as standard RES.

The antioxidant activity, as free radical scavengers, has an essential role in protecting the cell. Al-induced genotoxicity is due to the production of free radicals and their impact on the DNA. Meanwhile, MNPCE formation has been commonly used as a sensitive biological genotoxic assay of toxic agents (Kanimozhi and Prasad, 2009). In this study, RES of 10 mg/kg BW decreased the total MNPCE value after being induced by Al. MNPCE frequency is an indicator of chromosomal structural damage in bone marrow erythrocytes, and the decrease in micronucleus formation is related to chromosomal abnormalities. This means that a decrease in DNA damage can be shown by a reduction in the frequency of micronuclei. Therefore, RES acts as an anti-genotoxic and potentially prevents damage to DNA. In a dose-response, the antioxidant activity showed a significant free radical scavenging effect on Al. Only a dose of 10 mg/kg BW is more effective in a decreased micronuclei formation.

The micronucleus test data showed no significant difference in the PCE: NCE ratio in all treated groups compared to untreated groups. Therefore, the results showed that Al and RES did not affect the ratio but had a significant effect on DNA damage, as indicated by an increase in the frequency of MNPCE. This ratio indicates PCE to NCE proliferation and turnover rates similar for healthy mice. The administration of RES in Al-induced mice did not show a cytotoxic effect but indicated an increased risk of genotoxicity.

5. Conclusion

These findings demonstrated that Al effect trabecular of femur bone was relatively thinner than the control group. However, administration of RES at a dose of 10 mg/kg BW in the Al-treated group underwent an increase in size than the trabecular of the control. The number of osteocytes in the group of mice Al+Res 10 mg/kg BW also showed a significant increase than in the Al-treated group. RES doses of 10 mg/kg BW stimulated proliferation, which increased the number of osteocytes in bone.

The effect of RES on the ratio PCE/NCE is not significant for all Al-treated and Al-Res groups. The best dose to reduce the frequency of micronuclei is 10 mg/kg BW. Due to its high phenolic content, the Res seems to have an antigenotoxic role. These studies contribute to the understanding of the potential antigenotoxic value of polyphenols such as RES and the exploration of their possible use as protective agents related to genotoxic damage.

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