Antioxidant and Anti-Inflammatory activity of Chlorogenic Acid on Lead-Induced Fibroblast Cells

E Girsang¹*, I N E Lister¹, C N Ginting¹, S L Nasution¹, S Suhartina¹, U Z Munshy², R Rizal² and W Widowati³

¹Universitas Prima Indonesia, Jl. Belanga No. 1, Medan 20118, North Sumatera, Indonesia
²Biomolecular and Biomedical Research Center, Aretha Medika Utama, Jalan Babakan Jeruk II No. 9, Bandung 40163, West Java, Indonesia
³Faculty of Medicine, Maranatha Christian University, Jl Surya Sumantri No. 65, Bandung 40164, West Java, Indonesia

*Corresponding author’s email: ermigirsang@unprimdn.ac.id

Abstract. Lead (Pb) can be a cause of oxidative stress, because it is a toxin found in the environment and can have a harmful effect on the body. Increased reactive oxygen species (ROS) and nitrogen species (RONS) levels can cause cellular aging and inflammation. Natural compounds found in plant are one of the sources of antioxidant and anti-inflammatory agents that has ability to prevent aging including chlorogenic acid (CA). Cytotoxicity assay of CA against fibroblast cells (BJ) was conducted using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). The intracellular ROS levels were detected by flow cytometry using a DCF-DA fluorescent probe. BJ cells were incubated at 37°C, 5% CO₂, treated by 25 and 6.25 µg/ml CA for 4 h and followed by 400 µg Pb for 3 days. The anti-inflammatory potential was determined using ELISA to measure IL10 and TNFα. CA at 3.13-25 µg/ml were nontoxic to the BJ cells. CA treatment at 6.25 and 25 µg/ml was capable to reduce the accumulation of ROS in lead-induced BJ cells. CA at 6.25 and 25 µg/ml increased IL10 and reduced TNFα compared to positive control (lead-induced cells). This study shows that chlorogenic acid has the potential as protective effect through suppression of ROS levels and have anti-inflammatory properties related to Pb poisoning.

Key words: lead, reactive oxygen species, chlorogenic acid, IL10, TNFα

1. Introduction

Lead (Pb) can be a cause of oxidative stress, because it is a toxin found in the environment and can have a harmful effect on the body. Oxidative stress is a condition where there is an imbalance between the levels of ROS and antioxidant defenses, and causes oxidative damage [1]. Oxidative stress caused by lead can be induced through reactive oxygen species (ROS) and nitrogen species (RONS) generation, which has become important mechanism underlying lead toxicity [2], moreover a decrease in the activity of the antioxidant system can also lead to lead-induced oxidative stress. ROS is a term used to indicate the presence of free radicals originating from O₂ or for non-radical species [3].

In cells, the generation of ROS exists in equilibrium with a variety of antioxidant defenses. Low ROS levels are often found in organism with long life span [4]. Conversely, excessive ROS generation can primed senescent cells proliferate in aging, as a stress response [5]. ROS plays an important role in the...
induction of apoptosis, oxidative stress can cause cellular apoptosis through extrinsic cell death receptor pathways and also intrinsic cell death pathways [6]. The accumulation of ROS which induces apoptosis is then an important contributor to several diseases and aging [7]. In aging process, the accumulation of ROS can leads to loss of skin elasticity and cause formation of wrinkle, brown spots, uneven pigmentation, and even skin cancer [8]. Moreover, production of reactive oxygen species (ROS) have an important role to the advancement of many inflammatory disease [9].

Natural compound especially phytochemicals are eminent for its biological activities including antioxidant, antiaging, and anti-inflammatory [8,10,11]. Chlorogenic acid (CA), one of the most abundant polyphenol compounds in the human diet, CA is a group of phenolic secondary metabolites produced by certain plant species [12]. Most natural products containing CA displayed anti-inflammatory effects, indicating that CA may be a potential antioxidant, antiaging, and anti-inflammatory agent [13]. Here, we studied the antioxidant and anti-inflammatory effects of CA on lead-induced fibroblast cells (BJ) as aging role model.

2. Materials and Methods

2.1. Fibroblast Cells Cytotoxicity assay

Human fibroblast cell line (BJ) (ATCC® CRL-2522™) was obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. We observed to determine the maximum tolerance concentration of CA on BJ cells and to determine the optimal oxidative damage concentration of lead(Pb) for the following experiments. BJ Cells were cultured in MEM (Biowest, L0416-500) supplemented with 10% fetal bovine serum (FBS) (Biowest, S1810-500), 1% Antibiotic/antimycotic (ABAM, Biowest, L0010100), 1% Nanomycopulitine (Biowest, L-X16-100), 1% Amphotericin B (Gibco, 1%), 0.1% Gentamicin (Gibco, 15750045). Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. After that, 80% of cells confluency, 5000 cells were seeded in each well of 96-well plate. After 24 h incubation, the cells were treated with CA at various concentrations (3.13, 6.25, 12.5, 25, 50, and 100 µg/mL) for 24 h. To determine cell viability, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA) was used. MTS was added to each well at a ratio of 1:10 [8,14] The plate was incubated in 5% CO2 at 37°C for 4 h. Absorbance was measured at 490 nm on a microplate reader. The data are presented as the percentage of viable cells (%) and data were analyzed using ANOVA and continued by Tukey post hoc test.

2.2. Measurement of intracellular reactive oxygen species

The ROS levels were detected by flow cytometry using a DCF-DA fluorescent probe (invitrogen) according to the method of Jie et al [10,15,16] with minor modification. After 7 days of culture, BJ cells were digested with trypsin-EDTA and 105 cells were incubated with 10 µM DCF-DA at 37°C for 30 min, after that incubated with CA (25 and 6.25 µg/ml) for 4 h and followed by 400 µg Pb for 3 days. The intracellular ROS levels were measured using Miltenyi Flow Cytometer (MAQS quant). BJ Cells treated with Pb without CA treatment showed as controls. The measured fluorescence values were expressed as a percentage of control.

2.3. Quantification of IL10 and TNFα level

Quantification of Interleukin 10 (IL10) and Tumor necrosis factor-α (TNFα) were assessed using ELISA Kit, IL10 (Elabsci, E-EL-H0103) and TNFα (Elabsci, E-EL-H0109). The procedure was in accordance with manufacturer protocol. Sample absorbances were read at 450 nm using spectrophotometer (Multiskan GO, ThermoScientific). Color changes of samples are observed then read immediately at 450 nm wavelength and the IL10, TNFα level can be calculated based on a protein standard curve [17–19].
3. Results and Discussion
Skin aging is a natural process due to environmental factors such as lead exposure. The repetitive exposure to lead toxicity cause accelerated physical changes in the skin and connective tissue through the cell contents and ROS [8], increased generation of ROS can reduce antioxidant system activity or changes in both can lead to lead-induced oxidative stress. Different antioxidant enzymes and molecules have been evaluated to analyze lead-induced oxidative stress in both clinical and experimental studies [20].

3.1. Fibroblast Cells Cytotoxicity Assay
Fibroblasts have been used as a standard cell line for many cell biological studies. A member of the connective-tissue cell family, fibroblasts are responsible for the synthesis and metabolism of most connective-tissue components and also play an active role in the body’s general inflammatory/immune responses. During inflammation, fibroblasts are key cells in granulation tissue and scar formation. In this study we describe the cytotoxicity of CA toward fibroblast cells, based on the results CA suppressed cell growth only at the highest concentration (100 µg/ml), the results obtained are in concordance with the result of previous study [21].

The solution used in this assay contains a tetrazolium compound 3-(4,5-dimethylthiazol-2-yI)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). The MTS compound is bio-reduced by cells into a coloured formazan product due to conversion by dehydrogenase enzymes in metabolically active cells [14].

![Figure 1](image)

* The histograms are presented as mean ± standard deviation. The data were analyzed with ANOVA and continued with Tukey post hoc test. Different letters (a,ab,b,c,bcd,cd,de,e) on figure A and (a,ab,b,c) on figure B indicate significant differences among treatment. Negative Control: cells without any treatments; Vehicle Control: cells with DMSO 10% treatment.
* Negative Control: cells without any treatments; Vehicle Control: cells with DMSO 10% treatment.

Number of viable fibroblast cells (BJ) were decreased in CA concentration dependent manner, (Figure 1). The lower number of cell viability was obtained from the highest concentration of CA. The data of viable cell number were served in percentage of viable cell. Based on the results of cytotoxicity assay and Tukey post hoc test results, the safe concentration that can be used for further experiments and not damaging cells are found in CA concentrations 6.25 and 25 µg/ml.
3.2. Intracellular ROS level

The accumulation of ROS which induces apoptosis is then an important contributor to several diseases and aging [7]. In aging process, the accumulation of ROS can lead to loss of skin elasticity and cause formation of wrinkle, brown spots, uneven pigmentation, and even skin cancer [8]. Our results in accordance with previous study which state that lead exposure can increase the ROS levels [22]. Addition of CA can reduce ROS levels, previous study state that CA can reduce ROS levels approximately 20.3% after exposure with hydrogen peroxide [23].

ROS production can be seen based on level of fluorescence intensity as an indicator. Based on figure 2, ROS levels increased significantly (approximately 25%) in cells induced lead (400 μM) for 3 days compared to negative controls (Cells stained using DCF-DA). The results of treatment using chlorogenic acid with concentrations of 25 and 6.25 μg/ml can significantly reduce ROS levels (approximately 13% and 8%). The most optimal CA concentration in reducing ROS content was 6.25 μg/ml, but the concentration did not differ significantly from CA concentrations of 25 μg/ml based on Tukey post hoc test.

**Figure 2.** Effect CA toward ROS production on lead-poisoned fibroblast cell.

* The histograms are presented as mean ± standard deviation. The data were analyzed with ANOVA and continued with Tukey post hoc test (p<0.05). Different letters (a,ab,b,c) indicate significant differences among treatment.
* Negative Control: cells without any treatments; Vehicle Control: cells with DMSO 10% treatment; Positive Control : cells with Pb; CA 6.25: cells treated Pb + CA 6.25 μg/mL treatment; CA 25: cells treated Pb + CA 25 μg/mL treatment.
Figure 3. The representative of dot blot of various concentrations of CA treatment on lead-poisoned fibroblast cell toward ROS level.
TBHP = tert-butyl hydroperoxide, DCFDA = 2′,7′-dichlorofluorescin diacetate

3.3. Level of IL10 and TNFα

Increases in cellular ROS production can result in an increase in the expression of NF-κB, leading to the upregulation of factors involved in inflammation. During aging process, TNFα are potent releasers of IL6 which is one of the first cytokines to be linked to the aging process [24]. The effect of TNFα can turn triggers effects that increase inflammation that can indicate with increased ROS levels [25]. In contrast, IL10 is a cytokine that acts as an anti-inflammatory and works by stimulating antagonist proteins against TNFα as pro-inflammatory cytokine [26].

IL10 and TNFα are cytokines that acts during inflammation, TNFα is a member of the pro-inflammatory cytokine group [27], while IL10 is a member of anti-inflammatory cytokine [24]. Figure 3 showed the results of TNFα and IL10 levels using the ELISA method. It can be seen that fibroblast cells induced with lead has the highest levels of TNFα among others, while addition CA shows a significant decrease in TNFα. In the other hand, fibroblast cells induced with lead has the lowest levels of IL10 and addition of CA can elevate the IL10 levels. CA at 25 and 6.25 µg/ml can reduce the TNFα levels but no significant, same as in IL10 levels, both CA concentrations can increase IL10 levels but the results are not significant based on post hoc test.
4. Conclusion
This study shows that chlorogenic acid as natural compound has the potential as protective effect through suppression of ROS levels and have anti-inflammatory properties to decrease TNF-α level and increase IL-10 on lead-poisoned human fibroblast cells.

5. Acknowledgement
We are gratefully acknowledge the financial support of the Research Center and Service Community, Universitas Prima Indonesia, Medan, North Sumatera, Indonesia for research grant 2018. This study also was funded, facilitated and supported by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia. We are thankful to Wahyu Setia Widodo, Satrio Haryo Benowo Wibowo, Hanna Sari Widya Kusuma, Annisa Amalia, Ni Luh Wisma Ekyanty, Ika Adhani Sholihah, Dewani Tediana Yusepany, Dwi Surya Artie from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia for their valuable assistance.

References
[1] Redza-Doutordoir M and Averill-Bates DA 2016 Activation of apoptosis signalling pathways by reactive oxygen species *Biochim. Biophys. Acta. Mol. Cell. Res.* 12 2977–92
[2] De Voogt P 2015 Reviews of Environmental Contamination and Toxicology *Rev. of Env. Cont. Toxicol.* 1–297
[3] Halliwell B and Gutteridge J M C 2015 Free Radicals in Biology and Medicine (USA: Oxford University Press)
[4] Davalli P, Mitic T, Caporali A, Lauriola A and D’Arca D 2016 ROS, cell senescence, and novel molecular mechanisms in aging and age-related diseases *Oxid. Med. Cell Longev.* 2016 1–18
[5] Kuilman T, Michaloglou C, Mooi W J and Peeper D S 2010 The essence of senescence *Genes. Dev.* 22 2463–79
[6] Sinha K, Das J, Pal P B and Sil P C 2013 Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis *Arch. Toxicol.* 7 1157–80
[7] Orr W and Sohal R 1994 Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster *Science.* 5150 1128–30
[8] Widowati W, Fauziah N, Herdiman H, Afni M, Afifah E, Kusuma H S W, Nufus H, Arumwardana
S and Rihibiha D D 2016 Antioxidant and anti aging assays of oryza sativa extracts, vanillin and coumaric acid. J. Nat. Remedies. 3 88
[9] Mittal M, Siddiqui M R, Tran K, Reddy S P and Malik A B 2014 Reactive oxygen species in inflammation and tissue injury Antioxid. Redox Signal. 7 1126–67
[10] Widowati W, Darsono L, Suherman J, Yellianty Y and Maesaroh M 2014 High performance liquid chromatography (HPLC) analysis, antioxidant, antiaggregation of mangosteen peel extract (Garcinia mangostana L.) Int. J. Biosci. Biochem. Bioinforma. 458–66
[11] Widowati W, Widianto R M, Husin W, Ratnawati H, Laksmitawati D R, Setiawan B, Nugrahenny D and Bachtiar I 2014 Green tea extract protects endothelial progenitor cells from oxidative insult through reduction of intracellular reactive oxygen species activity Iran J. Basic Med. Sci. 9 702–9
[12] Olthof M R, Hollman P C H and Katan M B 2001 Chlorogenic acid and caffeic acid are absorbed in humans J. Nutr. 1 66–71
[13] Francisco V, Costa G, Figueirinha A, Marques C, Pereira P, Miguel Neves B, Celeste Lopes M, Garcia Rodriguez C, Teresa Cruz M and Teresa Batista M 2013 Anti-inflammatory activity of Cymbopogon citratus leaves infusion via proteasome and nuclear factor-kB pathway inhibition: Contribution of chlorogenic acid J. Ethnopharmacol. 1 126–34
[14] Novilla A, Djahmuri D S, Nurhayati B, Rihibiha D D, Afifah E and Widowati W 2017 Anti-inflammatory properties of oolong tea (Camellia sinensis) ethanol extract and epigallocatechin gallate in LPS-induced RAW 264.7 cells Asian Pac. J. Trop. Biomed. 11 1005–9
[15] Jie G, Lin Z, Zhang L, Lv H, He P and Zhao B 2006 Free radical scavenging effect of pu-erh tea extracts and their protective effect on oxidative damage in human fibroblasts cells J. Agric. Food Chem. 21 8058–64
[16] Prahastuti S, Hidayat M, Hasiana S T, Widowati W, Amalia A, Qodariah R L, Rizal R, Kusuma H S W and Khoiriyah Z 2019 Ethanol extract of jati belanda (Guazuma ulmifolia L.) as therapy for chronic kidney disease in in vitro model J. Reports. Pharm. Sci. XX XX
[17] Widowati W, Widianto H, Murti H, Laksmitawati D, Sari H, Kusuma H S W, Rizal R, Afifah E, Sumitro S, Widodo M A and Bachtiar I 2017 Interleukins and VEGF secreteme of human wharton’s Jelly mesenchymal stem cells-conditioned medium (hWJMSCs-CM) in different passages and oxygen tensions Biosci. Res. 14
[18] Widowati W, Afifah E, Mozef T, Sandra F, Rizal R, Amalia A, Arinta Y, Bachtiar I and Murti H 2018 Effects of insulin-like growth factor-induced wharton jelly mesenchymal stem cells toward chondrogenesis in an osteoarthritis model Iran J. Basic Med. Sci. 7 745–52
[19] Noverina R, Widowati W, Ayuningtias W, Kurniawan D, Afifah E, Laksmitawati D R, Rinendya P, Rilianawati R, Faried A, Bachtiar I and Wirakusumah F F 2019 Growth factors profile in conditioned medium human adipose tissue-derived mesenchymal stem cells (CM-hATMSCs) Clin. Nutr. Exp. 24 34–44
[20] Wang C, Liang J, Zhang C, Bi Y, Shi X and Shi Q 2007 Effect of ascorbic acid and thiamine supplementation at different concentrations on lead toxicity in liver Ann. Occup. Hyg. 6 563–9
[21] Koganov M M, Dueva O V and Tsrorin B L 1999 Activities of plant-derived phenols in a fibroblast cell culture model J. Nat. Prod. 3 481–3
[22] Lopes A C B A, Peixe T S, Mesas A E and Paoliello M M B 2016 Lead exposure and oxidative stress: a systematic review 193–238
[23] Hoelzl C, Knasmüller S, Wagner K-H, Elbling L, Huber W, Kager N, Ferk F, Ehrlich V, Nersesyan A, Neubauer O, Desmarchelier A, Marin-Kuan M, Delatour T, Verguet C, Bezençon C, Besson A, Grathwohl D, Simic T, Kundi M, Schilter B and Cavin C 2010 Instant coffee with high chlorogenic acid levels protects humans against oxidative damage of macromolecules Mol. Nutr. Food Res. 12 1722–33
[24] Morley J E and Baumgartner R N 2011 Cytokine-related aging process J. Gerontol. Ser. A. Biol.
Marcu K B, Otero M, Olivotto E, Borzi R M and Goldring M B 2010 NF-kB signaling: multiple angles to target OA *Curr. Drug Targets.* **5** 599–613

Wojdasiewicz P, Poniatowski Ł A and Szukiewicz D 2014 The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis *Mediators Inflamm.* **2014** 1–19

Laksmitawati D R, Prasanti A P, Larasinta N, Syauta G A, Hilda R, Ramadaniati H U, Widyastuti A, Karami N, Afni M, Rihibiha D D, Kusuma H S W and Widowati W 2016 Anti-inflammatory potential of gendarusa (Gendarussa vulgaris Nees) and soursoup (Annona muricata L) extracts in LPS stimulated-macrophage cell (RAW264.7) *J. Nat. Remedies* **2** 73