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Antifibrotics and Antioxidants of Chlorogenic Acid Inhibits Toll-Like Receptors-4 as Liver Fibrotic Marker

Rosdiana Naibey[1*], Widya Wasityastuti[2], Nungki Anggorowati[3], Nur Arfian[4]
[1]Faculty of Medicine, University of Papua, Papua, Indonesia
[2]Department of Physiology, Medical Faculty, Gadjah Mada University, Yogyakarta, Indonesia
[3]Department of Anatomical Pathology, Medical Faculty, Gadjah Mada University, Yogyakarta, Indonesia
[4]Department of Anatomy, Medical Faculty, Gadjah Mada University, Yogyakarta, Indonesia
*Corresponding Author. E-mail: r.naibey@unipa.ac.id, Mobile number: +62 821 3796 2379

ABSTRACT

Introduction: Chlorogenic Acid (CGA) is an antifibrotic and antioxidant for fibrotic tissues. These double roles be able to inhibit or fibrotic tissues chains because of internal and external issues. For example, virus, bacteria or other pathogens and also by drugs, alcohol, cigarettes, etc. as external factor that affect quality of body tissues. Toll-Like Receptor-4 (TLR-4) as a marker fibrotic tissues. It is a key for researcher could be find out by expression performance. The aim of this study is to reveal the CGA as a candidate of antifibrotic & antioxidant in liver fibrosis that induced by CCL_4.

Methods: This is a pure experimental research with a simple experimental design or post-test only control group design. The total 29 mices of 2.5-month-old male Swiss mices with weigh 35-40 gram divided into 6 group: 3 groups of controls (injected by natrium chloride, CGA, and CCL_4) and 3 groups of treated (injected by CGA doses 42 mg/kg, 63 mg/kg or 84 mg/kg). Liver organ was used to examine the expression of TLR-4 by rt-PCR. This research revealed that expression of TLR-4 lower than the CCL_4 control group (respectively, p=0.042; p=0.005; p=0.006; and p=0.001). Higher dose of CGA showed greater ability as anti-fibrotic through inhibit the expression of TLR-4. Some research found the expression of TLR-4 has been decreased by treatment of Clorogenic Acid (CGA).

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Email:
greenmedicaljournal@umi.ac.id

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Conclusion: To sum up, CGA has double roles to repair liver fibrotic tissues. The greater doses of CGA, the stronger inhibition of TLR-4 expression.

Keywords: CCL_4 antifibrotic; antioxidant; TLR-4; Liver; CGA

Introduction

Liver disease is one of five big killer diseases to human in the world that Liver chirrhosis, age-standardized death rates (15+), per 100,000 population [1]. Liver problem should be treated early to achieve Sustainable Development Goals (SDGs)[2]. Liver is a one of accessories organ in the human body. It does digestion process when food and water available [3]. Damaging of Liver tissue will be decreasing quality and functional of liver tissue. Liver tissue problem would be found out by roles of Toll-Like Receptors as a marker of inflammatory cytokine [4]. TLR-4 is transmembrane receptor that play roles for natural and adaptive immunity. Roles of TLR-4 could be found by Pathogen-associated molecular patterns (PAMPs) recognition. They will associate or works together to send signals and activate natural and adaptive immunity responds. TLR-4 also has play roles in liver tissue injury and as one of PAMPs family member.It would be activated by some signals from ligan of cellular compartment. The increasing of TLR-4 expression could be seen by tissue damage and matrix degradation. Then, it would be the formed damage-associated molecular patterns (DAMPs) [5].

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Methods

Type of this research was *post-test only control group design*. The samples were used Swiss male mice, which is 2,5 months old, ± 35-40 gram, and 29 in number. Every mice are healthy, active and categorized in 6 groups by Federer technique design [34]. Each group contains of 4-5 mice. Group I (S0=NaCl), II (S1=CGA), III (S2=CCL_4), IV (S3=CCL_4 0.5 mL/kg BW + CGA 42 mg/kg BW), V (S4=CCL_4 0.5 mL/kg BW + CGA 63 mg/kg BW), dan VI (S5=CCL_4 0.5 mL/kg BW + CGA 84 mg/kg BW). The mice are fed 1 gr/mouse/day dry feed in pellet form and provided with clean water that replaced every 3 days in order to protect from bacteria or any other danger that may disturb the absorption process on CGA and CCL [32]. The main substance in this research is CCl_4 (250 mL) (Merck) which is dissolved in olive oil (1:0,5), CGA (1000 mg) (Sigma-Aldrich) in a dosage of 42 mg/kg, 63 mg/kg and 84 mg/kg [32] based on human doses consumes [31] and NaCl (250 mL) [33]. The mice are measured once in a week to determine the dosage since thetreatment is based on body weight and are inducted by NaCl, CCL_4, CGA, and CCL_4+CGA for 4 week. NaCl and CGA treatments are done once in 24 hours through intragastric while CCL_4 treatment is done twice in a week through intraperitoneal.[12] This research procedure hasbeen approved by preclinical research ethic committee of LPPT-UGM Yogyakarta (No.Ref. 00002/04/LPPT/II/2017). After the treatment, next step was examined the blood serum and the liver was taken out from Linea Mediana part for RNA extraction to saw the expressions of TLR-4. The data of TLR-4 was analyzed by One Way ANOVA with significance grade $p<0,05$.

Result

1. TLR-4 Expressions in Liver Fibrotic Tissues

TLR-4 is one of transmembrane receptors that have important role in liver fibrosis. The statistical analysis shows that more significant differences between groups of treatment S1 (1.85±0.43), S2 (1.72±0.76), and S3 (1.54±0.61) than the group of control K3 (3.34±1.29). Based on what the graph shows (Illustration A) regarding the role of CGA on the expression of TLR4, group S1(CGA 42 mg/kg BW), S2(CGA 63 mg/kg BW) and S3(CGA 84 mg/kg BW) could inhibit CCL_4 (0.5 mL/kg BW) became fibrosis. Whereas, one-way ANOVA test showed some significant differences on TLR-4 between groups (p=0.005). rt-PCR showed on illustration B. Based on that finding, CGA can lower the expression of TLR-4 on liver tissues which experience fibrosis. [12] The induction of CCL_4 can strengthen the expression of

TLR-4. This research uses fibrosis model because it is inducted by CCL_4. The results showed the expression of TLR-4 which wassignificant on group S1(1.85±0.43), S2(1.72±0.76), and S3(1.54±0.61) compared to the groupof control K3(3.42±1.22). rt-PCR shows there is damaging on the liver tissue.[12]

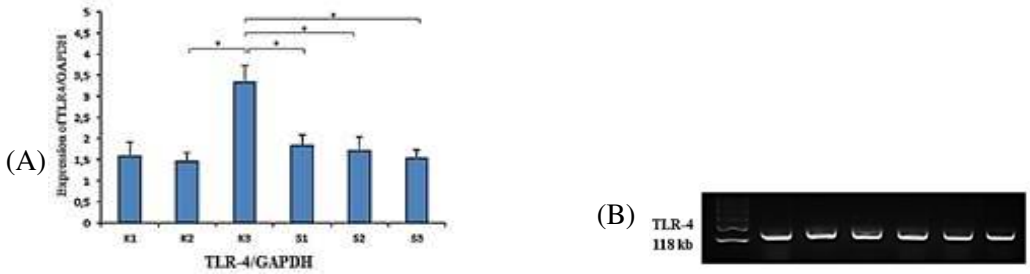

Illustration: [A] Expression of TLR-4/GAPDH; [B] Expression of TLR-4 by RT-PCR and densitometry analyzes using Image J software; Notes: K1(NaCl 0.5 mL/kgBW), K2(CGA 63 mL/kgBW), K3(CCL_4 0.5 mL/kgBW), S1(CCL_4 0.5 mL/kgBW+CGA 42 mg/kgBW), S2(CCL_4 0.5 mL/kgBW+CGA 63 mg/kgBW), and S3(CCL_4 0.5 mL/kgBW+CGA 84 mg/kgBW). (*) TLR-4(p=0.005)[12]

Discussion

1. TLR-4 Expressions in Liver Fibrotic Tissues

Increasing expression of TLR-4 happens because TLR-4 is capable to identify pathogenic activity through understanding on pathogenic ligands from damaging of molecular pattern which is like matrix and cellular damage, thus the signals of TLR-4, MyD88, and NF-κB are activated. [8-9] Then, some cytokine and chemokine proinflammatory are activated which is make free radicals turn into trichloromethyl peroxidase radical ($CCL3O^-$) then enter to cellular apoptotic pathways. Through mediator of IGF-1, PDGF, TGF-β, ET-1, ROS, then liver stellate cells are activated and then changed into myofibroblast. Next, myofibroblast contractility triggers increasing of matrix deposition and extracellular matrix which finally result in fibrotic. [15,19]

Inflammation is a normal process in which to maintain homeostatic of the body. But if the inflammation going to level of fibrotic and it will change functionality and quality of the tissue. also due to effect of the external and internal environment. For example, liver fibrotictissue. Liver inflammation tissue that progresses to liver fibrosis due to exposure of toxins, autoimmunity, ROS, and oxidative stress to B cells, NK cells, extracellular matrix, dendritic cells. These cells release pro-inflammatory cytokines to activate hepatic stellate cell (HSC) and stimulates bone marrow-derived cells, fibroblast and epithelial to-mesenchymal transition (EMT). Subsequently, myofibroblasts are activated by proliferation and migration to apoptosis. So, there is an imbalance between synthesis and degradation of collagen which causes liver tissue failure or damage [14-15]

Liver fibrotic in this research that has been done by using Carbon Tetrachloride (CCL_4). CCL_4 was used due to its toxic property to the liver. Liver damage caused depends on the large dose was given. The

main principle action that takes place in mechanism of CCL_4 in liver fibrotic cells by the presence of free radicals, lipid peroxidase and decreased activity of enzymes antioxidants. Histological appearance could be observed by fat infiltration, necrosis centrolobular then eventually to be cirrhosis. [28-29]

As mention before, damaging of liver tissue will decrease the quality and functional of itself. Liver tissue problem would be find out by the expression of Toll-Like Receptors (TLR-4) as the marker of inflammatory cytokine. TLR-4 is transmembrane receptor that play roles for naturaland adaptive immunity. Roles of TLR-4 could be found by Pathogen-associated molecular patterns (PAMPs) recognition. They will associate or works together to send signals and activate natural and adaptive immunity responds. TLR-4 also has play roles in liver tissue injury and as one of PAMPs family member. It would be activated by some signals from ligan of cellular compartment. The increasing of TLR-4 expression could be seen by tissue damage and matrix degradation. Then, its formed damage-associated molecular patterns (DAMPs) [4-5].

2. Expressions of TRL-4 after CGA treatment

However, the induction of CGA causes the expression of TLR-4 significantly decrease (p=0.005). The results showed the expression of TLR-4 was lowers significantly on the groups of S1(1.85±0.43), S2(1.72±0.76), and S3(1.54±0.61) compared to the group of control K3(3.34±1.29). Also, group of treatment S1(1.85±0.43), S2(1.72±0.76), and S3(1.54±0.61) are compared to group of control K2 (1.47±0.44), group of treatments are lower. The results showed that the expression of TLR4 fell significantly. CGA treatment be able to decrease expression of PDGF, ROS production, free radical, phosphorylation of ERK1/2, proliferation of HSC,expression of collagen I/III and TIMP, which means that fibrogenesis pathways was inhibited [5,18].

During exposure to viral hepatitis, alcohol, autoimmune diseases, and toxins on parenchymal cells (hepatocytes) and non-parenchyma (Kuffer cells, endothelial cells, and fat stores) immediately activate the TLR4 signaling pathway.[5] After binding TLR-4 with its ligand thenthe signal goes to MyD88 via modulation TIRAP and TRAM proteins which then mediate the TIR adapter protein with MyD88. Furthermore, the TRAM adapter protein connects the TIR and TRIF regions between the cell. Then the MyD88 pathway will attract IRAK and TRAF6 proteins. Protein TRAF6 activates TAK1. TAK1 activates the later IKK complex enter the NF-kB activation pathway. TAK1 also activates the MAPK pathway as well as causes the release of proinflammatory cytokines such as IL-1/10/4/6, CD40L, and radicals free . In addition, other pathways besides MyD88 are TRIF pathways. The TRIF pathway interacts with RIP1 and TRAF6. Activation of RIP1 and TRAF6 proteins further activates NF kB and MAPK. The TRIF protein also interacts with TRAF3 which also activates TBK1/IKKi and both activate other proteins, namely IRF3 and IRF7 which then triggers the release of pro-inflammatory cytokines such as INF- type 1. After that, the tissue inflammatory conditions would be appeared. Inflammatory tissue conditions also because of the

synthesis and degradation of the extracellular matrix was not balanced. [5,17-21]

3. CGA has ability (Antifibrotics and Antioxidant) to repair liver fibrotic

The results of the study clearly reveal the expression of TLR-4 was decreased by treatment of CGA. The greater doses of CGA, the stronger inhibition of TLR-4 expression. CGA was reduced the amount of PDGF, ROS production, free radicals, ERK1/2 phosphorylation, HSC proliferation, collagen I/III . expression and TIMP resulting in inhibition of fibrosis. [22-25] In addition to the large dose of CGA, need to be considered, as well as the influence of the food and drink consumed for the process repair network. Also role of nutrition is very helpful in recovery process of liver tissue injury. The double role of CGA able to inhibit formation and reaction of free radical chains with oxygen. These two molecules are dangerous to damage membranes and plasma membranes. [27,28] So, also CGA as antioxidant due to it have 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA),5-caffeoylquinic acid (5-CQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), and 4,5-dicaffeoylquinic acid (4,5-diCQA).

Additional, minor CGAs including 3-feruloylquinic acid (3-FQA), 4-feruloylquinic acid (4-FQA), 5-feruloylquinic acid(5-FQA), 3-p-coumaroylquinic acid (3-p-CoQA), 4-p-coumaroylquinic acid (4-p-CoQA), and5-p-coumaroylquinic acid (5-p-CoQA) are also present in traceable amounts in coffee beverages. These electrochemical chains as power to inhibits fibrogenesis pathways. [30] A review study reported that content of CGA e.g. 5-CQA has antioxidants activity and be able to inhibits various of disease models. The ability of 5-CQA indicate to down regulate pro-inflammatory cytokine, through modulation of key transcription factors but if high doses of 5-CQA given to rat by intravenous, it would appear inflammatory reaction. So, researcher should be more carefully to use CGA as an antioxidant. [29] Otherwise, for this research was different methods and sample for CGA treatment. It might be the reason for CGA roles as antifibrotic and antioxidants inhibit fibrogenesis pathways in liver fibrotic tissue model. By this research reveal, Phenolic compounds of CGA very important for repairing is liver fibrotic tissue. Liver tissue recovery depends on doses of CGA. The greater of doses the better recovery of the tissue. Not only to concern on doses but also adequacy for nutritional, water, and external environment exposure e.g. temperature would be helpful to maintain the good quality of the liver tissues.

Conclusion

CGA is a phenolic compound with nine (9) electrochemical powers. These powers have the ability to inhibits fibrogenesis pathway especially TLR-4. The expression of TLR-4 depends on level of liver fibrotic tissue. Also, recovery of the fibrotic tissue depends on the amount of Phenolic (CGA). The greater doses of CGA, the stronger inhibition of TLR-4 expression. Not only to concern on dosses but also adequacy of nutrition and water would be helpful for maintain the good quality of the liver tissues.

Recommendation

This research was focused on TLR-4 but it might be more helpful also for next study to see the other marker like Myd88 expression, it is also as a root of liver fibrogenesis pathways.

Conflicts of Interest

None

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References

1. WHO. 2021. Liver Cirrhosis. WHO online. Home page on-line. Available from Liver cirrhosis, age- standardized death rates (15+), per 100,000 population (who.int); internet access Agustus 1, 2021: 16.00 pm.
2. United Nation. Good Health and Well-Being. 2020. UN *online*. Home page on-line. Available from #Envision2030 Goal 3: Good Health and Well-being | United Nations Enable; Internet access Agustus 5, 2021;10.00 am.
3. Sherwood L. 2014. Human Physiology:From Cells to Systems. 9th Ed. USA:Cengage Learning;593
4. Guo J, Friedman S. Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. J.FT Biomed Central. 2010; 3:21; 1-19.
5. Yang L, Seki E. Toll-like receptor in liver fibrosis: cellular crosstalk and mechanisms. J. Front. in Physiol. 2012; 3: 1-18.
6. Seruga M, Tomac I. Electrochemical Properties of Chlorogenic Acids and Determination of Their Content in Coffee Using Differential Pulse Voltametry. Int.J.Electrochem. Sci. 2016; 11:2854- 2876.
7. Kuhnert N., Said I.H., & Jaiswal R. 2014. Assignment of Regio-and Stereochemistry of Natural Products Using Mass Spectrometry Chlorogenic Acids and Derivatives as a Case Study. J. Stud. Nat. Prod. Chem. 42; 305-339.
8. PubChem. Compound Chlorogenic Acid . PubChem online. Home page on-line. Available from https://pubchem.ncbi.nlm.nih.gov/compound/chlorogenic_acid# section=KEGG—Phytochemical- Compoun-ds; Internet Access November 22, 2016: 1.14 pm.
9. Dong L, Han N, Hou N, Li J, Yan Y. Chlorogenic Acid Enhances The Effect of 5- Fluorouracil in Human Hepatocellulear Carcinoma Cells Through The Inhibition of Extracellular Signal-Regulated Kinases. Preclinical Report : Anti-cancer Drugs. 2015; 26(5):540-546.
10. Pena MPD, Pyrzynska K, Sentkowska A, Skowron MJ. Chlorogenic Acids, Caffeine Content and Antioxidant Properties of Green Coffee Extracts: Influence of Green Coffee Bean Preparation. EurFood Res Technol J. 2016; 242:1403-1409.
11. Chemical Book. Chlorogenic Acid. Chemical Book online. Home page on-line. Available from http://www.chemicalbook.com/Chemical Product Property _EN_cb2478906.html. Internet access: November 17, 2016 ; 4.00 pm.
12. Naibey, R. Pengaruh Pemberian Asam Klorogenat (CGA) Terhadap Fibrosis Hepar Mencit Jantanyang Diinduksi dengan Karbon Tetraklorida (CCL4). Tesis S2. Oktober 2017; 32-43.
13. Yu, C.,Wang, F., Jin, C., Huang, X., Miller, D.L., Basilico, C., & Mckeehan,W.L. 2003. Role of fibroblast growth factor type 1 and 2 in carbon tetrachloride-induced hepatic injury and fibrogenesis. Am. J. Pathol. 163;1653–1662.
14. Wang F.S., Xu R., & Zang Z. 2012. Liver fibrosis: mechanisms of immune-mediated liver injury.Cell. Mol. Imm. J. 9;296-301.
15. Shi H, Shi A, Dong L, Lu X, Wang Y, Zhao J, Dai F, Guo X. Chlorogenic acid protects against liver fibrosis in vivo and in vitro through inhibition of oxidative stress. Clin. Nut, J. 2015; 35:1366-1373.
16. Guo J, Friedman S. Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. J.FTR Biomed Central. 2010; 3:21; 1-19.
17. Blouin A, Bolender RP, Weibel ER. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. J Cell Biol. 1977;72:441–55. [PMC free article] [PubMed] [Google Scholar]
18. Bataller R., & Brenner D. 2005. Liver Fibrosis. Clin. Invest. J. 2 (115);209-2118.
19. Basilico C., Yu C., Huang X., Jin C., Mckeehan W. L., Miller D.L., & Wang F., 2003. Role of fibroblast growth factor type 1 and 2 in carbon tetrachloride-induced hepatic injury and fibrogenesis. Am. J. Pathol. 163;1653–1662.
20. Bataille F., Hellerbrand C., Steiling H., Muhlbauer M., Scholmerich J., & Werner S. 2004. Activated hepatic stellate cells express keratinocyte growth factor in chronic liver disease. Am. J. Pathol. 165;1233–1241.
21. Dockal M., Niiya M., Pollak E.S., Scheiflinger F., Uemura M., Zheng X.W., Wells, R.G., Zheng,X.L., 2006. Increased ADAMTS-13 proteolytic activity in rat hepatic stellate cells upon activationin vitro and in vivo. Thromb. Haemost J. 4;1063–1070.
22. Kumar H., Kawai T., & Akira S. 2009. Toll-like receptors and innate immunity. J. Biochem. Biophysic. Res.Com. 388; 621-625.
23. Shi H, Shi A, Dong L, Lu X, Wang Y, Zhao J, Dai F, Guo X. Chlorogenic acid protects against liver fibrosis in vivo and in vitro through inhibition of oxidative stress. Clin. Nut, J. 2015; 35:1366- 1373.

24. Kim J, Jeong I, Kim C, Lee YM., Kim JM, Kim JS. Chlorogenic acid inhibits the formation of advance glycation end product and associated protein cross-linking. Arch.Pharm.Res.J. 2011; 34(3): 495-500. [25] Riufeng G, Yunhe F, Zhengkai W, Ershun Z, Yimeng L, Minjun Y. Chlorogenic acid attenuates lipopolisaccharide-induces mice mastitis by supressing TLR4- mediated NFkB signaling pathway. Euro.J.Pharm. 2014; 729:54-58.
25. Brun P, Castagliuolo I, Pinzani M, Palu G, Martines D. Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. Am. J. Physiol. Gastrointestinal Liver Physiol. 2005; 289: G571–G578.
26. Cheeseman K. H., Albano E. F., Tomasi A. and Slater T. F. (1985), Biochemical studies on the metabolic activation of halogenated alkanes. Environ. Health Perspect. 64, 85- 101.
27. Clawson G. A. (1989), Mechanism of carbon tetrachloride toxicity. Pathol. Immunopathol. Res. 8,10
28. Matei, M.F.; Jaiswal, R.; Kuhnert, N. Investigating the Chemical Changes of Chlorogenic Acids during Coffee Brewing: Conjugate Addition of Water to the Olefinic Moiety of Chlorogenic Acids and Their Quinides. J. Agric. Food Chem. 2012, 60, 12105–12115. [CrossRef] [PubMed].
29. Du, W.Y.; Chang, C.; Zhang, Y.; Liu, Y.Y.; Sun, K.; Wang, C.S.; Wang, M.X.; Liu, Y.; Wang, F.;Fan, J.Y.; et al. High-dose chlorogenic acid induces inflammation reactions and oxidative stress injury in rats without implication of mast cell degranulation. J. Ethnopharmacol. 2013, 147, 74–83.[CrossRef] [PubMed].
30. Martijo. Kesehatan dan Kemampuan Adaptasi Hewan, Universitas Gadjah Mada, Yogyakarta. 1992.
31. Ludwig I.A., Mena P.M., Calani L., Cid C., Rio D.D., Lean M.E.J., *et al*. 2014. Variation in Caffein and Chlorogenic Acid Conntents of Coffees: What are We Drinking? *Food Func. J*. 5:1718-1726.
32. Laurence D.R., & Bacharach A.L. 1964. Toxicity tests: Evaluation of drug activities. Pharmacometrics, 161. London: Academic Press.
33. Pound A.W & Lawson A.T. 1974. Protection By Carbon Tetrachloride Against The Toxic Effects of Dimethylnitrosamine in Mice. *Brit.J.exp.Path*. 56;77-82.
34. Federer T.W. 1963. Experimental Design: Theory and Application. 2nd Ed. MacMillan: Newyork