Sari *et al*., Afr., J. Complement Altern Med. (2020) 17(2): 1-7
https://doi.org/10.21010/ajtcan.v17i2.1

Centella Asiatica (GOTU KOLA) TREATMENT ATTENUATES PRO-INFLAMMATORY MEDIATORS IN LIVER OF RATS WITH ELECTRICAL FOOT SHOCK STRESS MODEL

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Article History
Received: March 14th 2019
Revised Received: June 6th 2020
Accepted: July 1st 2020
Published Online: Nov. 18th 2020

Abstract

Background: Stress induces secretion of cathecolamines and glucocorticoids, which may produce liver injury. Followed by the production of inflammatory mediators, it causes apoptosis. *Centella asiatica* (CeA) has anti-inflammatory and hepatoprotective effects. The present study aims to determine the role of CeA in the attenuation of liver pro-inflammatory mediator expression in rats with electrical foot shock stress model.
Materials and Methods: Twenty-four Sprague-Dawley rats were randomized into four groups consisted of six rats each: (1) Control group, (2) CeA-treated group, (3) Stress group, and (4) CeA + stress group. Reverse transcriptase PCR of inflammatory and apoptosis markers as well as Real-Time PCR of β2-adrenergic receptor were performed from liver tissues.
Results: Electrical foot shock stress induced up-regulation of *NFκB* and *TNF-α* mRNA expressions as pro-inflammatory mediators, compared to control group. This alteration was followed by up-regulation of *BAX* and *β2-adrenergic receptor*, as well as the down-regulation of *BCl2* compared to control. CeA treatment prevented enhancement of *NFκB, TNF-α, TLR-4* and *β-adrenergic receptor* mRNA expressions, which was followed by down-regulation of *BAX* and up-regulation of *BCl-2,* compared to stress group.
Conclusion: CeA prevents secretion of pro-inflammatory chemokines and cytokines as well as apoptotic markers in liver cells through the activation of β2-adrenergic receptor.

Keywords: Apoptotis; *Centella asiatica*; Inflammatory cells; Liver injury; stress.

Abbreviations: NFκB (Nuclear factor of kappa-light-chain-enhancer of activated B cells), TNF-α (Tumor Necrosis Factor-α), TLR-4 (Toll-like Receptor 4), BAX (B-Cell lymphoma Associated X factor), BCl-2 (B-Cell lymphoma.

Introduction

Numerous conditions that lead to stress, such as restrain-induced stress, electrical foot shock-induced stress, and cold temperature-induced stress, stimulate transcriptional factor of corticotropin-releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus (Imaki *et al.*, 1995). It activates hypothalamus-pituitary-adrenal (HPA) axis that is followed by extrication of glucocorticoid and cathecolamines, such as epinephrine and norepinephrine (Black, 2002). Glucocorticoid is a primary stress hormone that regulates various physiological processes (Imaki *et al.*, 1995). Both hormones have an important effect on hepatic cells during stress. They initiate a response that is characterized by the production of cytokine and acute phase reactants as in an inflammatory state (Black, 2002).

Liver is an organ with complex physiological function, which responses to the various stresses through the up-regulation of glucose, fatty acid and certain amino acids. Stresses could activate glucocorticoid that acts through its receptor, glucocorticoid receptor, to stimulate specific target genes within target cells (Black 2002; Wong *et al.*, 2010).

Stresses could also increase cathecolamines with activation of adrenergic receptor to modulate inflammatory responses (Sorrells and Sapolsky, 2007; Grisantic *et al.*, 2010). Elevation of catecholamines during stress induces hepatocellular dysfunction and gut-derived norepinephrine, then leads to the production of pro-inflammatory cytokines and chemokines through β2-adrenergic signaling pathway (Aninat *et al.*, 2008). Experiments using restraint-stress have already proven to induce liver injury in mice, which is followed by elevation of liver enzymes and apoptosis of hepatocytes (Zhu *et al.*, 2014).

Centella asiatica (CeA), also known as Gotu kola or pegagan, in some countries, is a creeping plant that is found in tropical climate such as Indonesia, India and other Asian countries. There are four active compounds in CeA in the form of saponin (triterpenoid), namely: asiatic acid, madecasic acid, asiaticoside, and madecasicoside (Rao *et al*., 2006; Orhan, 2012). CeA and its active compounds, have been widely reported to show numerous biological activities for human health such as neuroprotective (Lokanathan *et al.*, 2016), anti-nociceptive (Huang et al. 2011), antioxidant and anti-hyperlipidemic (Kumari et al., 2016), anti-cancer (Siddique *et al.*, 2016), as well as anti-inflammatory and hepatoprotective (Tang *et al.*, 2012; Choi *et al.*, 2016; Oyenihi *et al.*, 2017) properties. Other studies have also reported that CeA attenuates matrix metalloproteinase-9 and mitochondrial dysfunction as well as cytochrome-C that plays an important role in cell death program in cerebral ischemic rats (Krishnamurthy *et al*., 2009; Lee *et al.*, 2013).

Some studies that showed protective effects of CeA in liver injury models demonstrated the mechanisms through its antioxidant and anti-inflammatory properties. Oyenihi *et al.* (2017) showed that methanolic extract of CeA may protect diabetes-induced oxidative liver tissue damage by improving the oxidant/antioxidant status and the level of pro-inflammatory cytokines. The ethanol extract of CeA administration in dimethylnitrosamine-induced liver injury reduces liver injury, antioxidant properties, and pro-inflammatory cytokines (Choi *et al.*, 2016). The hepatoprotective effect of CeA as anti-fibrotic properties via TGFβ/Smad signaling pathway in carbon tetrachloride-induced liver injury showed decrease of extracellular matrix, such as collagen1 both mRNA and protein (Tang *et al.*, 2012). Therefore, in this study, we investigated the hepatoprotective effect of CeA ethanolic leaf extract in liver injury that is caused by electrical foot shock, particularly in attenuating the inflammation process, apoptosis or programmed cell death, and β-adrenergic receptor expression.

Materials and Methods

Animal experiment and chronic electrical stress model

This study was conducted according to the established guidelines for animal care of Universitas Gadjah Mada and approved by the Ethical Committee of Medical Research and Health of Faculty of Medicine Universitas Gadjah Mada (No. KE/FK/0600/EC/2017). The experiments were performed using twenty-four male rats (Sprague Dawley strain) obtained from Experimental Animal Care Unit (UPHP) of Universitas Gadjah Mada. The rats used in this study were male, weighed between 150-170 grams at the beginning of the study. A group consists of three rats were housed inside a glass cage, under the following conditions: room temperature 25-30˚C, 50%-60% humidity, a dark-light cycle of 12:12 hours, and fed with a standard pellet diet and water *ad libitum.*

The rats were randomized into four groups consisting of six rats in each group. These groups were (1) control group, (2) CeA-treated group, (3) stress group, and (4) Stress + CeA (SCeA) group. The control group received only distilled water with no electrical stress administration or CeA treatment. The CeA group received 600 mg/kg body weight of oral CeA ethanolic leaf extract treatment daily for 28 consecutive days. The stress group received chronic electrical stress. Electrical foot shock was administered daily for 28 consecutive days to create chronic electrical stress to the rats. For each electrical foot shock, a rat was placed in a plexiglass box with an electrical current of 0.8 mA connected to the base of the box that shocked the rat in the foot intermittently (three times per minute, 5 seconds per shock continued with 15 seconds rest), for a total of 10 minutes. The rats were fully supervised throughout the procedure to prevent any undesired effects. The SCeA group received both 600 mg/kg body weight of oral CeA leaves ethanolic extract treatment as well as chronic electrical stress for 28 consecutive days. Electrical stress was given 30 minutes after oral CeA ethanolic leaf extract administration. All rats were weighed on days 0, 14, and 28. On day 28, the rats were anesthetized before they were sacrificed using intraperitoneal injection of Ketamine Ivanes® (Ikapharmindo, Jakarta, Indonesia). Abdomen and thorax were surgically opened, then perfusion was carried out from left ventricle of the heart. Livers were dissected out, left lobe was used for RNA extraction and kept in RNA Later® (Ambion AM7021, Thermo Fisher, MA, USA).

Ethanolic Extraction of *Centella asiatica*

Centella asiatica (CeA) leaves were obtained from commercial herb manufacturer (Merapi Farma Herbal, Kaliurang, Sleman, Special District of Yogyakarta, Indonesia). The plant was identified and authenticated by a certified botanist at the Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. To produce the ethanolic extract of CeA, the CeA leaves were macerated in 70% ethanol at the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada.

RNA Extraction and cDNA synthesis

Total RNA was extracted from the left lobe of liver the tissue using Genezol RNA Solution (GENEzol™, Cat. No. GZR100, Geneaid, New Taipei City, Taiwan) according to the manufacturer's instruction. The total RNA concentration was quantified using nanodrop. An amount equal to 3000 ng of RNA underwent cDNA synthesis using Revertra Ace kit (Toyobo, Cat. No. TRT-101), random primer (Takara, Cat. No. 3801, Shiga, Japan), and deoxyribonucleotide triphosphate (dNTP) (Takara, Cat. No. 4030). PCR condition was as follows: denaturation at 30°C for 10 min, annealing at 42°C for 50 min and extension at 99°C for 5 min.

Reverse Transcriptase (RT)-PCR of inflammatory markers and apoptotic markers

Reverse Transcriptase (RT)-PCR was performed to examine the following genes with specific primer; *B-Cell lymphoma Associated X factor / Bax* (F: 5′-AGACAGGGGCCTTTTTGTTAC-3′ ,R:5′-GAGGACTCCAGCCAGCCACAAAGAT-3′), *B-Cell lymphoma 2 / BCl2* (F: 5′-TTGTGGCCTTCTTTGAGTTCG-3′ ,R: 5′-TACTGCTTTAGTGAACCTTTTT-3′), *Nuclear factor of kappa-light-chain-enhancer of activated B cells / NFκB* (F: 5′-CTGGCCATGGACGATCTGTT-3′ ,R: 5′-TGATCTTGATGGTGGGGTGC-3′), *Tumor Necrosis Factor-α / TNF-α* (F: 5′-TTGCCACTTCATACCAGGAGAA-3′ ,R: 5′-TCACAGAGCAATGACTCCAA-3′) and *Toll-like Receptor 4 / TLR-4* (F: 5′-AGCTTTGGTCAGTTGGCTCT-3′ ,R: 5′-CAGGATGACACCATTGAAGC-3′). PCR conditions were as follows: denaturation at 94°C for 10 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min (35 cycles), and final extension at 72°C for 10 min. The RT-PCR was performed by mixing cDNA, Taq master mix (Cat. No. S101705, Bioron, Germany) and primers. The PCR products were analyzed on 2% agarose gel along with a 100bp DNA ladder (Cat. No. 306009, Bioron, Germany). The expression of the genes was quantified with a densitometry analysis using the ImageJ software. A housekeeping gene, *Glyceraldehyde 3-phosphate dehydrogenase / GAPDH*, was used to normalize the expression.

Real-time PCR of β2-adrenergic receptor

Real-time PCR using BioRAD CFX 96 machine was performed to assess mRNA expression of liver *β2-adrenergic receptor* during electrical foot shock stress-induced liver injury. The cDNA was amplified using *β2-adrenergic receptor* primers set (F: 5′-ATGGAGGCTACAAGCGACAC-3′ and R: 5′-CCCTCAGCCACACTCCAAAT-3′) and *β-actin primers* set (F: 5′- GCAGATGTGGATCAGCAAGC -3′ and R: 5′ -GGTGTAAAACGCAGCTCAGTAA-3′) to normalize the expression.

Statistical Analysis

SPSS 22 software for windows was used for data analyses. Data normality test was conducted using Shapiro-Wilk and One-Way ANOVA for normal data distribution. A p value less than 0.05 (p <0.05) was considered as statistically significant.

Results

Proinflammatory cytokines genes expression

The effects of electrical foot shock-induced liver injury through the activity of pro-inflammatory mediators using RT-PCR, were examined. There was no significant difference between control and CeA group in *TNF-α* and *TLR-4* mRNA expressions. However, CeA group had significantly lower *NFκB* mRNA expression. There was a significant difference in *NFκB* mRNA expression between groups (p<0.01, p=0.000). RT-PCR analysis revealed significantly higher mRNA expressions of *NFκB*, *TNF-α* and *TLR-4* in the stress group compared to the control group (p<0.01, p=0.000). Furthermore, SCeA group demonstrated lower mRNA expressions of *NFκB*, *TNF-α* and *TLR-4* compared to the stress group (Figure 1).

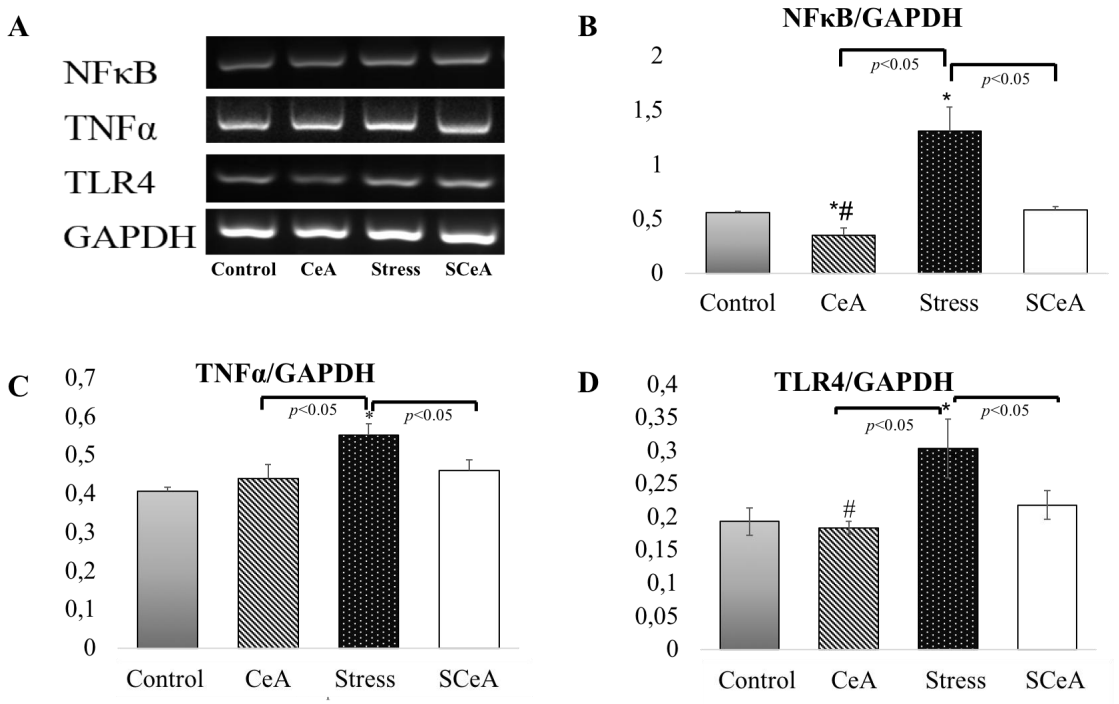

Figure 1: Inflammatory genes mRNA expression. A. Gel electrophoresis figures of RT-PCR of *NFκB, TNFα*, *and TLR4*. *GAPDH* was used as internal control. B. Bar charts showing the abundance of *NFκB* mRNA expression relative to *GAPDH*. C. Bar charts showing the abundance of *TNFα* mRNA expression relative to *GAPDH*. D. Bar charts showing the abundance of *TLR-4* mRNA expression relative to *GAPDH*. *=p<0.05 vs Control, #=p<0.05 vs SCeA.

Effect of CeA treatment on BAX and BCl2 mRNA expression

B-Cell lymphoma Associated X factor (BAX) and *B-Cell lymphoma 2 (BCl2)* mRNA expressions that contribute in apoptosis pathways were also evaluated. Under stress condition, the expression of *BAX* in the stress groups was higher than the control group. This alteration was accompanied by the lower expression of *BCl2* in the same group. *Centella asiatica* treatment during stress enhanced a significantly higher expression of *BCl2* mRNA expression (p<0.05) compared to the control group and accompanied by the lower expression of the *BAX* mRNA expression compared to the control group (Figure 2).

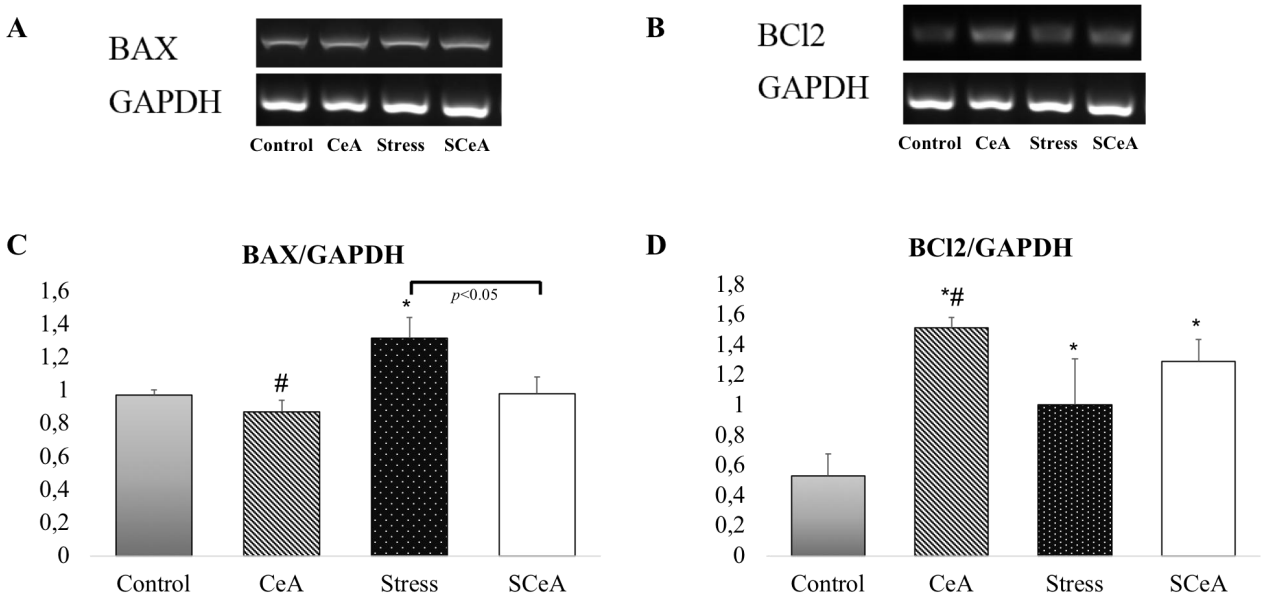

Figure 2: The alteration of both *BAX* and *BCl2* mRNA expressions in the liver after electrical foot shock-induced stress. A. Gel electrophoresis figures of RT-PCR of *BAX*. *GAPDH* was used as internal control. B. Gel electrophoresis figures of RT-PCR of *BCl2*. *GAPDH* was used as internal control. C. The bar charts showing the relative abundance of the mRNA expressions of *BAX* to *GAPDH*. D. The bar charts showing the relative abundance of the mRNA expressions of *BCl2* to *GAPDH*. *=p<0.05 vs Control, #=p<0.05 vs Stress.

Effects of CeA on liver injury due to β2 receptors

Here, we focus on β2-adrenergic receptor mRNA expression in CeA, Stress and SCeA groups. We used CeA group as control because we observed improvement in CeA group based on our previous results. Our Real-Time PCR results showed that the mechanism of electrical foot shock stress-induced liver injury was mediated by β2-adrenergic receptor through the increase of the *β2-adrenergic receptor* mRNA expression in the stress group compared to the control group (Figure 3). Electrical foot shock stress for 28-days was capable of significantly elevating *β2-adrenergic receptor* mRNA expression in the stress group ($p<0.05$, $p=0.009$) compared to the CeA group. The *β2-adrenergic receptor* mRNA expression in SCeA group was significantly lower compared to the stress group ($p<0.05$, $p=0.002$).

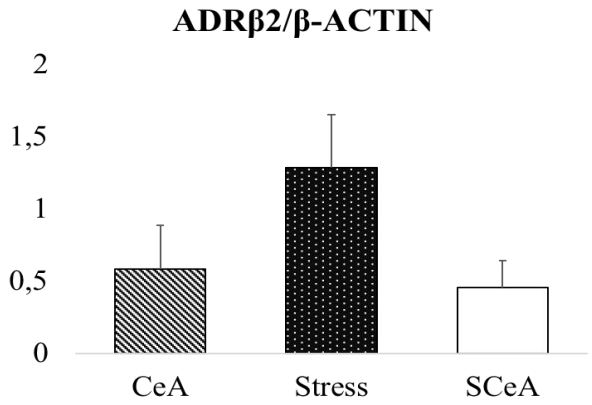

Figure 3: A. The bar charts showing the relative quantification of *β2-adrenergic receptor* mRNA expression to *β-actin*. ADRβ2= *β2-adrenergic receptor.* *=$p<0.05$ vs CeA, #=$p<0.05$ vs Stress.

Discussion

Our results revealed that CeA treatment inhibits the upregulation of proinflammatory mediators under electrical foot shock stress-induced liver injury. It has been known that stress induces the activation of sympathetic nervous system involving stress hormones such as glucocorticoid and catecholamines (Black, 2002). Catecholamines, which exert the regulator effects on cardiovascular, hepatic, skeletal muscles and immune system, could increase the risks to acquire physical and psychiatric disorders. In accordance to the cytokines' stimulation, the liver metabolisms increase under the stress condition. Sympathetic activation can stimulate or modulate production of the inflammatory cytokines (Aninat *et al*., 2008; Zhu *et al.*, 2014; van Der Poll, 2008). Activation of the sympathetic nervous system contributes to the liver injury. Research conducted by (Iwai *et al.* 1986) showed that the plasma glutamate-pyruvate transaminase was significantly lower under foot-shock stress compared to sham group after hepatic denervation. During hepatic inflammation, the mRNA expressions of *TNF-α*, *IL-6*, and *NFκB* were upregulated. Whereas corticosterone perfusion into isolated rat liver induce the increase of TNF-α and IL-6 levels (Black, 2002; van Der Poll, 2008; Swain, 2000), including under electrical foot-shock stress condition.

Social disruption stress activates adrenal gland and induces secretions of cathecolamines. They exert the immune system that is mediated by α2-adrenergic and β2-adrenergic pathways (Hankea *et al.*, 2012). Activation of β2-adrenergic in macrophage cells alters the expression of proinflammatory cytokine and chemokine including IL-1β and IL-6 (Hankea *et al.*, 2012; Cole and Sood, 2012). The stimulation of the β2-adrenergic receptor was activated by lipopolysaccharide. TLR-4 is a pattern recognition receptor that has a function as lipopolysaccharide sensor and whose activation results in the production of pro-inflammatory cytokines. The human monocyte cell line THP-1 that was challenged by lipopolysaccharide showed an increment level of the TNF-α and IL-1β. This result was reinforced by the treatment with isoproterenol, an agonist of β-adrenergic receptor, and followed by the increase of pro-inflammatory cytokines expression (Grisantic *et al.*, 2010; Soares *et al.*, 2010).

TLR-4 is actively involved in the response of hepatic injury caused by viral infection, alcoholic and non-alcoholic liver disease, autoimmune liver disease, and drug-induced liver disease (Tsung *et al*., 2005). It usually presents in the surface of hepatic stellate cells and mediates the inflammatory cells. Interaction of TLR-4 with its receptors, MD2 and CD14, activates downstream of the IκB kinase and mitogen-activated kinase (MAPK) which lead to the activation of the NFκB and activator protein (AP-1) (Tsung *et al*., 2005). These transcription factors regulate the expression of pro-inflammatory cytokines and chemokines that might mediate fibrogenesis (Guo and Friedman, 2010). A study also showed that under the lipopolysaccharide stimulation, reduction of inflammation and hepatic injury in WT mice implanted with TLR mutant mice bone marrow. Inflammation was shown by TNFα, IL-6, and MCP1. It was followed by an increase of functional liver enzyme, ALT, and the liver histopathological condition (Tsung *et al*., 2005). It reveals that TLR family play role in inflammation and hepatic injury.

Stimulation of the β2-adrenergic can induce the activation of the G protein/adenylyl cyclase (cAMP)-dependent PKA pathway. When the α-subunit and βγ-subunit were dissociated, it stimulates protein kinase A (PKA) and activates cyclic adenosine monophosphate (cAMP). Then it activates cAMP receptor element binding domain

(CREB) allowing it binds to the CRE (cAMP-responses elements) sites. The activation of the promotor element in the region of IL1β and IL-6 underlies the β2-adrenergic receptor-mediated cytokine regulation. The β2-adrenergic receptor-mediated increase of production of NFκB non-canonical pathways, which in turn activates IKKα complexes that phosphorylates C-terminal residues NFκB2p100. Phosphorylation of the NFκB2 p100 leads to its ubiquitination and proteasomal processing to NFκB2 p52. This creates transcriptionally competent NFκBp52/Rel B complexes that translocate to the nucleus and induce various target gene expression (Tan *et al.*, 2007). We inferred that the increase of NFκB was associated with the increase of TNF-α.

Stress could induce necrosis and apoptosis through activation of the β2-adrenergic receptor. During stress, the expression of the BCl2 as anti-apoptosis was depleted. On the contrary, the expression of the BAX, which is known as pro-apoptotic marker, became augmented. After *Centella asiatica* treatment, the expressions of both anti-apoptotic and pro-apoptotic marker were in reverse. Communal *et al.* (1999) provided the evidence that myocytes that were treated with norepinephrine for 24 hours showed the increase of myocyte apoptotic cells, and the number of apoptotic myocytes decreased after being treated with the antagonist of norepinephrine.

The β2-adrenergic acts through Gs- and Gi-coupled receptor which ensued by the activation of p38α pathway. Under the stimulation of the β2-adrenergic, the p38 kinase plays the role of pro-apoptotic component (Peter *et al.*, 2007; Amin *et al.*, 2011). The β2-adrenergic transgenic mice generated an enhancement of the cardiac left ventricular ejection fraction (LVEF) and development of cardiomyopathy which are characterized by myocyte hypertrophy, fibrosis, and apoptosis. The apoptotic-myocyte cells were reduced as mating the transgenic mice with the double negative (DN) p38α MAPK (Bisognano *et al.*, 2000; Peter *et al.*, 2007; Amin *et al.*, 2011). These studies support our findings that there is an upregulation of the pro-apoptotic marker and downregulation of the anti-apoptotic marker in liver under stress condition which was induced by electrical foot shock stress.

Conclusion

We concluded that electrical foot shock stress induced secretion of the pro-inflammatory chemokines and cytokines followed by an increase of the apoptotic cells in the liver through activation of the β2-adrenergic receptor. Oral treatment of *Centella asiatica* ethanolic leaf extract could improve these conditions by attenuating the secretion of pro-inflammatory chemokines and cytokines, as well as improving cellular apoptosis in the liver via β2-adrenergic signaling pathway.

Declaration of conflict of interest: The authors declare there is no conflict of interest regarding the publication of this paper. All authors read and approved the final version of this manuscript.

Acknowledgements

This study was funded by the University Leading Research Grant *(Penelitian Unggulan Perguruan Tinggi)* from The Indonesian Ministry of Research, Technology, and Higher Education. The authors wish to extend the greatest gratitude to Prof. Soedjono Aswin, M.D, Ph.D for the valuable advice regarding the establishment of electrical foot shock-induced stress animal model in this study and Mr. Mulyana for his assistance during experiments in the animal facility. This study was funded by the University Leading Research Grant for Basic Science *(Penelitian Unggulan Perguruan Tinggi)* year 2018-2019 from The Indonesian Ministry of Research, Technology, and Higher Education. Some of the data were used for completing final assignment of the undergraduate program of R.N. Mainichi, G.M. Dara and R. Kemalasari.

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