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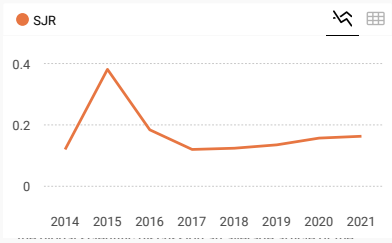

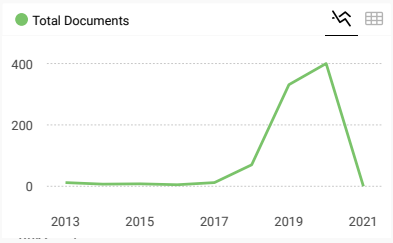

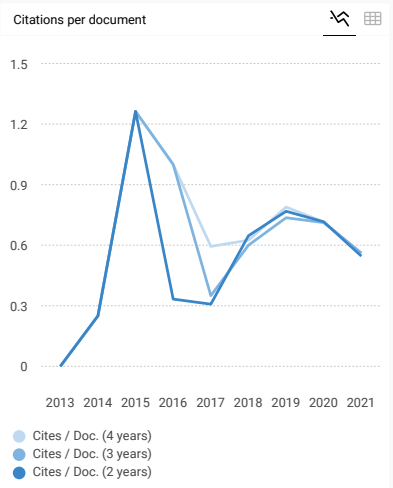

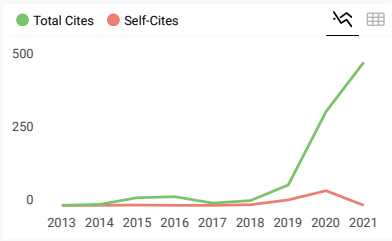

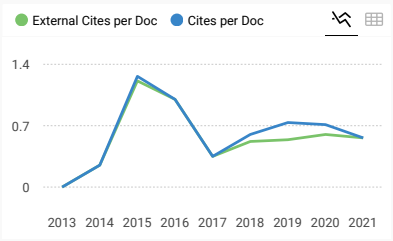

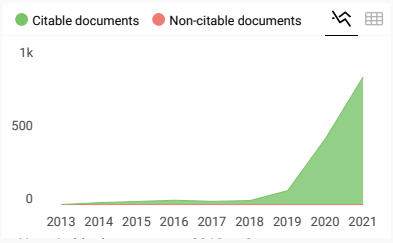

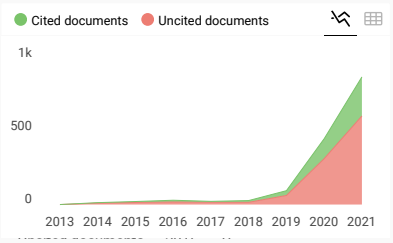

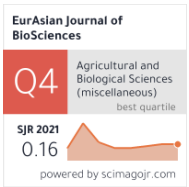

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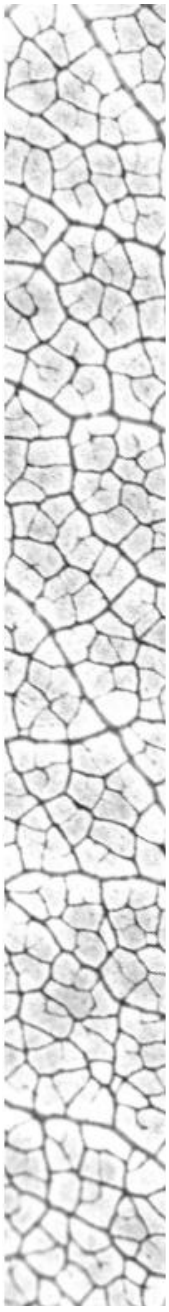

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Camellia sinensis with its active compound EGCG can decrease necroptosis via inhibition of HO-1 expression

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Abstract
Stroke is the most common neurological disorder in the word. During ischemic stroke there is increasing of oxidative stress. Green tea (*Camellia sinensis)* have antioxidant and free radical scavenger effect. In vivo study using male *Rattus Novergicus* with 5 group, control MCAO group, EGCG 10 mg/kgBW, EGCG 20 mg/ kgBW, EGCG 30 mg/kgBW, extract green tea 30 mg/kgBW. Treatment is for 7 days before sacrifice and perform brain tissue IHC examination for HO-1, TNFR1, and RIP3. There is significant different in HO-1 expression started at 10 mg/kgBW treatment ($p = 0.013$). Significant different on TNFR1 started at group EGCG 20 mg/kgBW ($p = 0.004$), there is significant different on RIP3 started at EGCG 20 mg/kgBW group ($p = 0.002$). There is correlation between HO-1 and TNFR1 ($r = 0.497$; $p = 0.000$), TNFR1 and RIP3 ($r = 0.551$; $p = 0.000$) and HO-1 and RIP3 ($r = - 0.433$; $p = 0.001$). *Camellia sinensis* with its active compound EGCG decrease RIP3 expression through down regulation of HO-1.

Keywords: MCAO, green tea extract, EGCG, HO-1, TNFR1

Machin A, Purwanto DA, Nasronuddin, Sugianto P, Aulanni'am A, Subadi I, Susilo I, Adianto Ch, Hidayati AN, Utomo B (2020) *Camellia sinensis* with its active compound EGCG can decrease necroptosis via inhibition of HO-1 expression. Eurasia J Biosci 14: 1813-1820.

INTRODUCTION

Stroke is the most common neurological disorder and top killers among the non-infectious diseases in the world (Rachmawati et al. 2019, Willey 2012). Sixty-nine percents of all stroke patients are in low and middle-income countries (Howard et al. 2016). Hypertension and dyslipidemia are some of the major risk factors for stroke which can cause blood supply to stop flowing to the brain (Arina et al. 2018, Marbun et al. 2018). Stroke is not affect in elderly, but also affects young people (Puspitasari et al. 2015). Patients who have stroke especially in transient ischemic attack (TIA) or ischemic stroke (IS) have higher risk of recurrence (Akbar et al. 2018). Most stroke patients have disabilities after stroke although 50-70% of patients return to functionally independent (Willey 2012). Standard treatment of ischemic stroke is iv-thrombolysis using r-TPA, but only 2-8.5% of ischemic stroke patients receive thrombolysis, that because many patients come to Emergency department beyond the time of thrombolysis or there is a contraindication to iv-thrombolysis (Feigin et al. 2016, Jeon et al. 2014, Willey 2012).

During acute stroke, there is decrease of cerebral blood and cause the decreases of ATP production in the neuronal cell and will impair ion homeostasis (Hang et al. 2016). Impairment ion homeostasis will produce reactive oxygen species (ROS) nitrogen species and other free radicals and accelerated neuronal death (Lewén et al. 2001, Rodrigo et al. 2013). Oxidative stress during ischemia and reperfusion will damage cellular components and play an important role during ischemia (Bereczki et al. 2018). There is a significant correlation

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between oxidative stress caused by ischemic and reperfusion and clinical outcome in stroke patients (Bereczki et al. 2018, KimS.-J. et al. 2013, Kishimoto et al. 2019).

HO-1 is the inducible form of heme oxygenase and induced by many stimuli including heavy metals, UV radiation, inflammation, oxidative stress, ox-LDL, and inflammation (Bereczki et al. 2018, Li et al. 2014). HO-1 catalyzes heme degradation and produce Fe2+, CO and biliverdin IXa and limited the iron-mediated cell injury. Up-regulation of HO-1 response to stressful stimuli, up-regulation in glia is induced in response to multitude of stimuli and tissue insult. HO-1 expression also may detriment to the brain and contribute to the overall of the neuronal damage (Panahian et al. 1999). HO-1 is express in many cells in the brain and express in many experimental models such as ischemic stroke, intracerebral hemorrhage, and traumatic brain injury. HO-1 level is higher in stroke patients than in TIA and associated with stroke prevalence. HO-1 is expressed in response to oxidative stress in various diseases (Nanloh S Jimam, Nahlah E Ismail 2019).

TNF-α is an important mediator in response to inflammation and correlates with stroke severity (Yue et al. 2009). Analysis using RT-PCR in MCAO Rat stroke model shows that expression of TNF-α mRNA increase during 3 hour after stroke and remain steady in 24 hours after stroke (Flores-Cantú et al. 2016). TNF-α expression is not only in neuronal cell, but also in astrocyte, microglia, plexus choroids, endothel an PMN that infiltrating cerebral tissue (Flores-Cantú et al. 2016, Olmos et al. 2014, Pan et al. 2007, Wajant et al. 2003). Cell culture study from atherosclerosis research Nanjing University to show the effect of epigallocatechin-3-gallate (EGCG) on HUVEC exposed with TNF-α to explore the expression of MCP-1 shows that EGCG inhibits TNF-α expression induced by mRNA MCP-1 expression. This research conclude that EGCG decreased TNF-α level through NF-κβ (Wang et al. 2014). TNF-α have a member of TNF superfamily of ligand responsible for broad variety of human disorders from inflammation to neoplasm. TNFR1 is main receptor transducing the signal from TNF-α. TNF-α induce NF-κβ and induced cell death (Pobezinskaya et al. 2012).

Necroptosis is a form of cell death induced by TNF-α thus can trigger inflammation (Linkermann et al. 2013). Necroptosis can be induced by external stimuli such as TNF-α. TNF-α stimulate TNFR1 and complex II including RIP1, TRADD, FADD and Caspase 8 that will initiate apoptosis, if cell have high RIP3 level, RIP3 will interact with RIP1 and forming necrosome and stimulate Necroptosis (ChenS. et al. 2017, Feoktistova et al. 2015, Moriwaki et al. 2013, Nogusa et al. 2016, Zhu et al. 2016). The Principle of necroptosis needs to activate RIP3 and pseudokinase lineage kinase-like (MLKL). RIP3 can be activated with various stimuli such as TNF-α. RIP1 and RIP3 are important kinases to form the necroptosis caused by TNF-α and have unique signal (Nogusa et al. 2016).

Green tea (*Camellia Sinensis)* is a source of polyphenol that known as catechin including EGCG. EGCG has been proven to have neuroprotective in some clinical condition including intracerebral hemorrhage, cardiovascular event, infection, cancer, and other diseases (Singh et al. 2015, Zhang et al. 2017). Meta-analysis study shows that people who drinking tea more than 3 cups per day have 21% lower chance to get a stroke attack than to a person who drinks less than 1 cup per day. The protective effect of EGCG is through the increasing of nNOS and eNOS and the decreasing of iNOS, inhibiting of MMP-9, improving synapse transmission and stimulating the 67LR (Adikesavan et al. 2013, Gundimeda et al. 2014, KimH.-S. et al. 2014, Lim et al. 2010, Sayal et al. 2016). EGCG also has neuroprotective effect through inhibiting inflammation by inhibiting TNF-α (Wang et al. 2014). This study aims to investigate the influence of *Camillia sinensis* and its active compound including EGCG on HO-1, TNF-α, and RIP3.

MATERIAL AND METHODS

Animal

Male *Rattus Novergicus* weight 200-275 gram from Gajah Mada university breeding center. The samples are to be conditioned for 1 week before establishing stroke model. The experiment is performed in animal laboratories of the Faculty of Pharmacy Universitas Airlangga. After the subjects are conditioned, they are to be randomized into 5 groups. The first group is MCAO control group, the second group is given EGCG 10 mg/kgBW, the third group is given EGCG 20 mg/KgBW, the fourth group is given EGCG 30 mg/KgBW, and the last group is given standardized extract green tea.

Mcao Model

The *Rattus Novergicus* are anesthetized using ketamine 80 mg/kgBW and Xylazine 10 mg/kgBW intraperitoneal. After the *Rattus Novergicus* is anesthetized we then conduct excision in the right neck until carotid communis can be seen, we differentiate the carotid artery until we found internal carotid and we clamp the artery using small bulldog clamp for 180 minutes. After 180 minutes, bulldog clamp is removed and we close back the incision on the neck and observe the rat conscious while we also observe weather the stroke model is emerged or not.

Intervention

We dilute EGCG or green tea extract with aquades with concentration 1mg/ml and give it to our samples once a day using rat sonde every morning before the samples have a meal. We examine the sample's behavior using ladder rung, FUAT, and Y-maze 1 day before establishing the MCAO model. On days 1, 3, 5,

Table 1. Effect of EGCG and green tea extract on HO-1 Expression in stroke model

Group	Mean±SEM	p
Sham	3.083±0.149	-
Control	4.818±0.122	0.000*
EGCG 10 mg/kgbb	4.230±0.122	0.013**
EGCG 20 mg/kgbb	3.917±0.149	0.001**
EGCG 30 mg/kgbb	3.333±0.142	0.000**
Green tea extract 30 mg/kgbb	3.583±0.149	0.000**

*Compared to sham
**Compared to Control stroke

Table 2. Effect of EGCG and green tea extract on TNFR1 Expression in stroke model

Group	Mean±SEM	p
Sham	2.916±0.193	-
Control	4.637±0.152	0.000*
EGCG 10 mg/kgbb	4.230±0.166	0.150**
EGCG 20 mg/kgbb	3.833±0.149	0.004**
EGCG 30 mg/kgbb	3.333±0.188	0.000**
Green tea extract 30 mg/kgbb	3.417±0.149	0.000**

*Compared to sham
**Compared to Control stroke

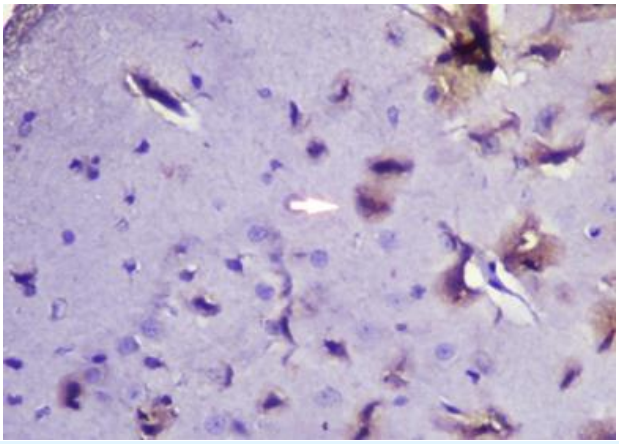

Fig. 1. HO-1 expression

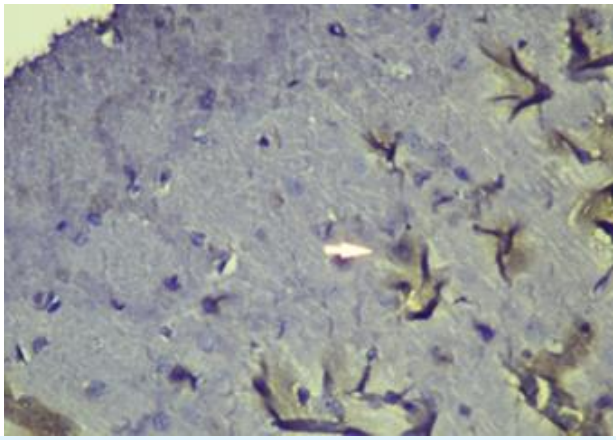

Fig. 2. TNFR1 expression

and 7 after establishing the model (we will report it in another manuscript). On day 7 we sacrifice our samples. We anesthetize our samples using Propofol 0,1 mg/100 gr of rat intravenous. We open the rat thorax and aspirate its blood using Spruit and aspirate its blood for 1 cc for ELISA examination. We cut the rat neck and get the rat brain and fix it using formalin 4% and then perform paraffin block (Jafarzadeh et al, 2018).

Immunohistochemistry

Paraffin block from rat brain is cut using microtome in 1,5 cm in front of bregma, paraffin block is cut at 5 μm thickness and placed in the slide and warming it at 65 C for 2 hours, place slide in the xylene solution three times 5 minutes each to clean up the paraffin, do rehydration with soak the slide in ethanol 100%, then ethanol 95% and ethanol 70%. Clean up peroxidase activity by soaking it in peroxide 3% solution for 3 minutes, clean up with this buffer for 3 minutes. Dilute antibody for HO-1, TNFR1, and RIP3 made by Santa Cruz Biotechnology, Inc with concentration 1:50. Give Enzyme conjugate after giving antibody and dilute it within TBS with BSA 1% and incubate it in room temperature for 1 hour then give chromogen for 10 minutes and rinse with flow water and give counterstain if needed and dried and clean it. Read in 400x microscope. IHC assessment use Scoring guideline according to DC Alfres, MD, proportion score 0: no marker is expressed in all specimen, 1: >0-1% marker is expressed, 2: >1-10% marker is expressed, 3: >10-33.3% marker is expressed, 4: >33.3%-66.6%, 5:>66.6%-100% marker are expressed and intensity score 0: expression of the marker is negative, 1: weak expression, 2: mid expression, 3: strong expression. All scores are sum between proportion and intensity score.

Statistical Analysis

We perform descriptive analysis among variables and perform 1 sample KS for normality test and if the distribution is abnormal, we perform the Kruskal-Wallis test. We perform Mann-Whitney to test the difference EGCG effect among groups and we perform a correlation test to assess correlation between variables.

RESULT

EGCG Decreases Ho-1 Expression

We perform statistical analysis to know the normality of distribution in our data using 1 sample kolmogorof-smirnov and found that our data distribution is abnormal. We perform the Kruskal-Wallis test and shows that our data have differentiation statistically significant. We perform the Mann-Whitney test according to our result on Kruskal-Wallis and compare MCAO control with remain groups and show that all groups have statistically different (**Table 1**). Our data show that treatment with Either EGCG or green tea extract decreases HO-1 expression.

EGCG Decreases TNFR1 Expression

Our study revealed that EGCG decreases TNFR1 expression as shown in **Table 2**. Among different doses, TNFR1 is beginning to statistically different compared with the MCAO control group in the dose EGCG 20 mg/kgBW. It means that it needs a bigger dose of EGCG to inhibit TNFR1 expression. Our data shows that green

Table 3. Effect of EGCG and green tea extract on RIP3 Expression in stroke model

Group	Mean±SEM	P
Sham	3.167±0.167	
Control	5.636±0.203	0.000*
EGCG 10 mg/kgbb	5.077±0.178	0.093**
EGCG 20 mg/kgbb	4.583±0.149	0.002**
EGCG 30 mg/kgbb	3.750±0.130	0.000**
Green tea extract 30 mg/kgbb	4.167±0.167	0.000**

*Compared to sham
**Compared to Control stroke

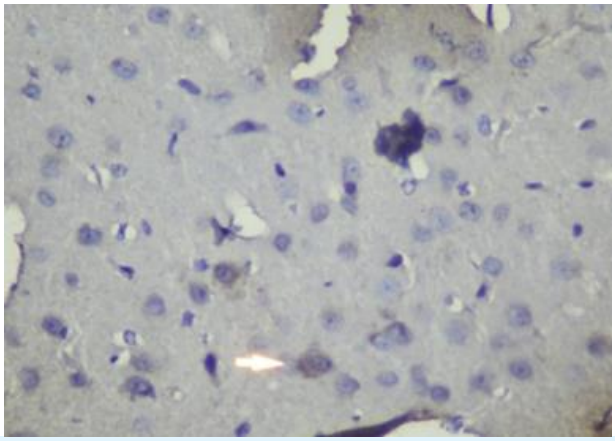

Fig. 2. RIP3 expression

tea extract has similar activity to EGCG in inhibiting TNFR1.

EGCG Decreases RIP3 Expression

We found that similar to TNFR1 expression, EGCG begins to inhibit RIP3 expression at dose 20 mg/kgBW and as the dose increase, the inhibition is stronger and the difference compared to MCAO control is more significant (**Table 3**). Our data also said that the inhibiting of RIP3 expression is dose-dependent. Our data also reveals that extract green tea at dose 30 mg/kgBW has similar activity to inhibit RIP3 expression.

We perform a correlation test with spearman correlation and find that it is statistically significant in the correlation between HO-1 and TNFR1 (r=0.497; p=0.000), there is also a correlation between TNFR1 and RIP3 (r=0.551;p=0.000) and there is also a correlation between HO-1 and RIP3 (r=-0.433;p=0.001).

DISCUSSION

Thrombolysis is the first choice for treating acute ischemic stroke, but because narrow therapeutic windows only a few numbers of stroke patients receive thrombolysis. Beyond thrombolysis treatment, there are some agents have developed as stroke treatment. Some agents can be classified as neuroprotective agents that act as anti-inflammation, antioxidant, neuron stimulant and radical scavenger (ChenX. et al. 2016, Jeon et al. 2014, Willey 2012). Green tea polyphenol has attention because beneficial effects related to its function as an antioxidant and free radical scavenger (KimH.-S. et al. 2014). Related to the issue, recently natural compound i.e green tea have gotten attention. Green tea is a kind of famous traditional drink and mostly consumed in Asian countries that produce *Camellia sinensis*. Green tea has polyphenol that acts as an antioxidant and free radical scavenger. Green tea has four polyphenol derivate according to its structure epicatechin (EC), epigallocatechin (EGC), Epicatechin gallate (ECG) and Epipallocathechin-3-gallate (EGCG) (KimE. et al. 2019, KimH.-S. et al. 2013, KimY. et al. 2016). Previous study stated EGCG acts directly on neural precursor cells to modulate adult hippocampal neurogenesis (Ortiz-López et al. 2016). During acute ischemic stroke-free radical is produced. Reactive oxygen species (ROS) can damage lipid membrane, protein, and other structures directly (Kadenbach et al. 2009, Murray et al. 2011, Srinivasan et al. 2012). The main ROS source in mitochondria, plasma membrane, reticulum endoplasm and peroxisome by enzymatic reaction and auto oxidation of some compounds such as catecholamine and hydroquinone (Lewén et al. 2001).

HO-1 is a metabolic enzyme responsible for heme metabolism (Bereczki et al. 2018). HO-1 has been used as an oxidative stress marker. Our study shows that there is inhibition in HO-1 expression induced by EGCG treatment or green tea extract treatment. Inhibition of HO-1 expression is started at 10 mg/kgBW dose, this may imply that the antioxidant effect of EGCG will inhibit HO-1 expression. Our result is different from Chengmey LV, et al study. They perform the intervention in stroke model using intravenous *α-lipoic acid* after transient MCA occlusion. Clinical neurologic assessment is performed using the Gracia score and infarct volume is measured using TTC stain. They measure oxidative stress using *Nrf-2* and HO-1 using western blot. They found increasing in HO-1 level in the control group compared to the sham group 24 hours after reperfusion (Lv et al. 2017).

Liu, et al have different results in their research to know the role of EGCG inhibiting inflammation in the smooth muscle cell and expression of IL-1β caused by oxidative stress. Liu using cell culture giving EGCG with concentration 12.5uM, 25uM, 37.5uM, and 50uM. Liu uses western blot to examine markers. They found increasing of HO-1 expression 24 hours after the intervention. Higher expression of HO-1 at 37.5uM. That study concludes EGCG decreases IL-1β expression and trigger cell culture proliferation through the HO-1 pathway. That study implies that EGCG will cause proliferation arrest in smooth cell muscle and increase ROS production as a result of an antioxidant deficiency in blood vessel smooth cell (Liu et al. 2014).

Intervention using Liraglutide 100 ug/kg every 12 hours for 7 days in the MCAO model and examination using western blot to measure Nrf2 and HO-1 found decreasing Nrf2 and HO-1 level in non-diabetics group and liraglutide. There is increasing Nrf2 and HO-1

expression in Diabetes and cerebral ischemia models (Deng et al. 2018). Our study shows decreasing HO-1 expression after EGCG or extract green tea treatment; EGCG is a radical scavenger thus decrease ROS in ischemic brain tissue. HO-1 expression is responding from the increasing ROS (Bereczki et al. 2018). Decreasing ROS in ischemic cerebral tissue will decrease HO-1 expression, and decrease in HO-1 has beneficial effect (Wu et al. 2012). Our result is similar to the role of HO-1 in intracerebral hemorrhage that has harmful effect in the model of intracerebral hemorrhage. Increasing of HO-1 induced by increasing of pro-oxidant level in brain parenchyma conclude that HO-1 is the main executor to clean up brain parenchyma damage (Aronowski et al. 2011). There are two possibilities why EGCG decreases HO-1 expression in our research: first HO-1 function as oxidants like function in Intracerebral hemorrhage or EGCG decreases oxidative stress thus can decrease HO-1 as a response to oxidative stress. Intracerebral hemorrhage can be treated with medical conservative treatment, surgical operative, and minimally invasive surgery (Muharram et al. 2019).

TNFR1 is the main receptor for TNF-α and plays a role in inflammation after ischemic cerebral infarction. The default signal of TNF-α activated after TNF-α binding to TNFR1 that will activate NF$\kappa\beta$ which responsible for cell survival and growth, but when it impairs it will induce the death cell including apoptosis and necrosis. Our study shows that there is a significant difference between an intervention and a control group. The difference is started at dose 20 mg/kgBW and also at the green tea extract group. Yang et al shows that EGCG decrease responds to TNFα and inhibits ADAM-10 at TNFR1 ectodomain and weakens the response to TNF-α (Yang et al. 2016).

TNFα first identified as necrosis factor at tumor cells and contribute to innate immunity (Wang et al. 2014, Yang et al. 2016). TNF-α at brain tissue has pathophysiological and hemostatic role, at normal brain TNF-α regulate synapse plasticity, learning, memory, sleep, and strengthening astrocyte. In pathological condition astrocyte, microglia release vast amount TNF-α as neuroinflammation response related to pathological condition (Jiang et al. 2012, Leu et al. 2013, Yang et al. 2016). EGCG also inhibit TNF-α trough inhibition NF-Kβ activity via 67LR that will trigger Nrf2-Keap1 dissociation, in another way released Nrf2 translocation in nucleus activate transcription gen that will inhibit TNF-α 2018. Our result shows that EGCG and green tea extract can attenuate inflammation in the brain tissue after ischemic stroke. Inhibition TNFR1 expression may because of antioxidant and radical scavenger features of EGCG.

Our result also shows that RIP3 have a significant difference in EGCG and extract green tea group started at dose 20 mg/kgBW of EGCG compared to the control MCAO group. Study form hyperoxic lung injury using Sprague-dewley using edavarone as intervention and exposed to 100% oxygen 250 kpa for 6 hours shows that in hyperoxic lung injury have increased of oxidative stress such as MDA and decrease of antioxidant activity such as SOD and GSH. There are increasing RIP1 and RIP3 after prolong hyperoxia ($p<0,05$). Edavaron can decrease oxidative stress markers, RIP1, RIP3 and MLKL (Han et al. 2018).

RIP3 first identified as two different groups through screening two different hybrids yeast as RIP1 binding protein along with RIP1 and RIP2 (NewtonK et al. 2016, NewtonKim et al. 2014). RIP3 gen in humans located at chromosome 14 and it mRNA encodes polypeptide with 518 amino acid. RIP3 has active domain kinase in the N terminal that will keep other RIP kinases and it has an important role in necroptosis. It is not like other kinases located at C terminal, RIP1 death domain and Caspase Recruitment Domain (CARD) at RIP2 and ankyrin repeat RIP4. C terminal responsible for regulation ant recruiting some scaffold signaling (Feoktistova et al. 2015, Wegner et al. 2017). Our study shows that either EGCG or green tea extract has beneficial effect for necroptosis inhibition through strong antioxidant effect and direct inhibition for RIP3 expression.

We also perform path analysis for all three markers and shows that HO-1 expression correlates with TNFR1 ($r=0.497$; $p=0.000$) and HO-1 also have a correlation with RIP3 ($r=0.433$; $p=0.001$), our result shows that HO-1 in our study is an oxidant that can increase TNFR1 activity through TNF-α release, HO-1 also can stimulate RIP3 directly, but not as strong as through TNFR1. Our result has contradictive results with Kim, et al study in ischemic hepatocyte. They perform analysis HO-1, TNFR1, TRADD, FADD, Caspase-8, Caspase-3 and cytochrome-C using western blot. They found increasing Ho-1 levels four times 4 hours after reperfusion. Kim also found that no significant changes on TNF-α level after reperfusion, but 4 hours after reperfusion there are significant changes in TNF-α level. Caspase-3 and Caspase-8 also increasing significantly in 4 hours after reperfusion and remain increase after 24 hours. Cytochrome C, TNFR1, TRADD, FADD expression also increase like other markers (KimS.-J. et al. 2013). Some studies show that HO-1 has a cytoprotective effect, but other study shows contradictive result (Bereczki et al. 2018, Kaiser et al. 2019, KimS.-J. et al. 2013, Li et al. 2014). Although our result has contradictive results with Kim et al study, it shows the same pathway in protective role, in other words decreasing HO-1 expression will attenuate inflammation and necroptosis in MCAO model and EGCG or green tea extract can decrease inflammation and necroptosis.

Our study has limitation include immunohistochemistry is semi quantitative methods and need other researches to quantified markers. Our study

is on the basic level for a clinical study to explore the effect of EGCG or green tea extract in ischemic stroke patients.

CONCLUSION

Camellia sinensis with its active compound EGCG decreases RIP3 expression through down regulation of HO-1.

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