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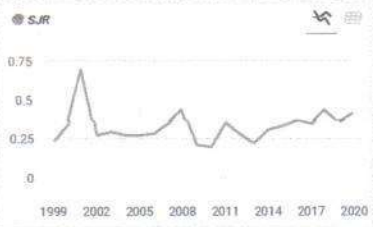


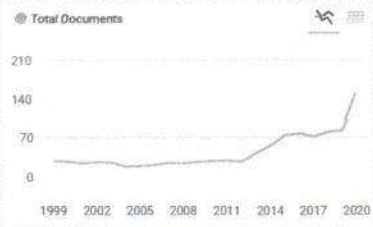


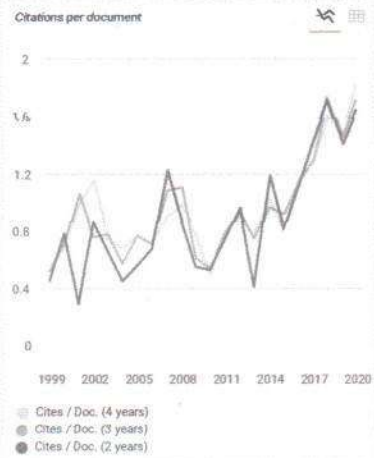


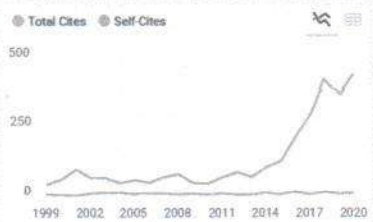


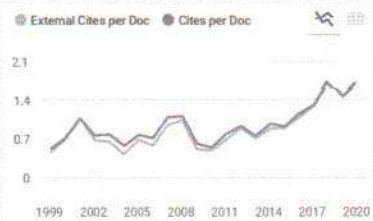


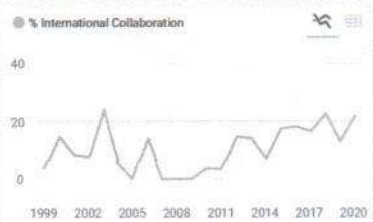


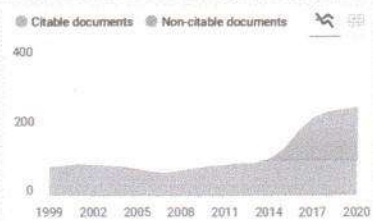


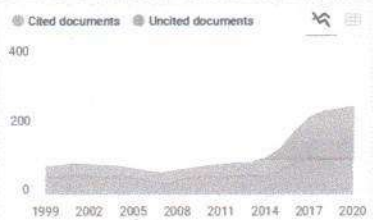



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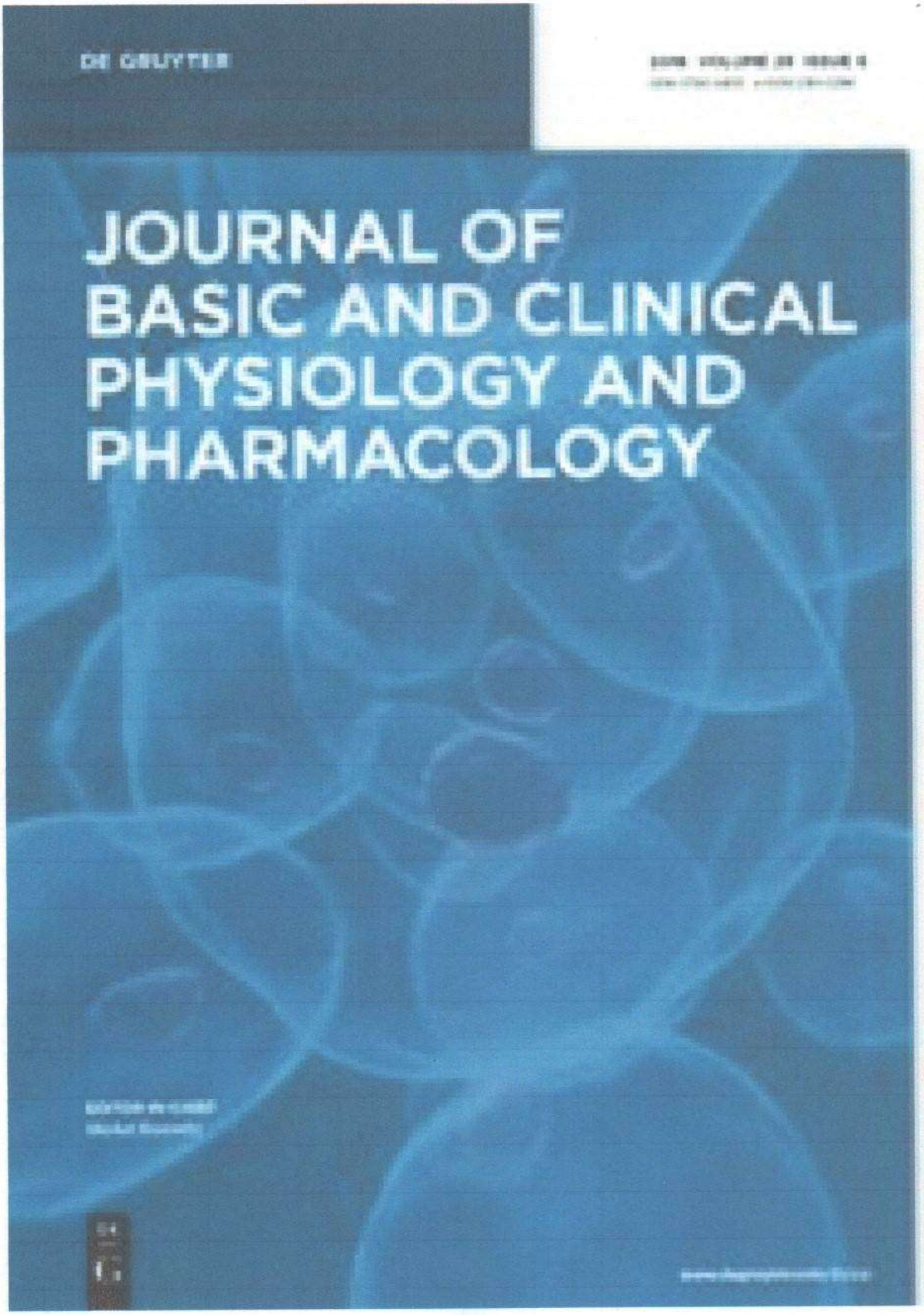
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## Case Report

Abdulloh Machin, Ramidha Syaharani*, Imam Susilo, Muhammad Hamdan, Dyah Fauziah and Djoko Agus Purwanto

# The effect of *Camellia sinensis* (green tea) with its active compound EGCG on neuronal cell necroptosis in *Rattus norvegicus* middle cerebral artery occlusion (MCAO) model

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**Abstract**

**Objectives:** To determine the inhibition effect of *epigallocatechin gallate* (EGCG) and green tea extract on neuronal necroptosis based on necroptosis morphology.
**Methods:** *In vivo* study was performed on male *Rattus norvegicus* middle cerebral artery occlusion (MCAO) model divided into five groups, MCAO-control groups, EGCG 10 mg/kg BW/day, EGCG 20 mg/kg BW/day, EGCG 30 mg/kg BW/day, and green tea extract 30 mg/kg BW/day for 7 days treatment. MCAO model was made by modification method using Bulldog clamp. After 7 days of treatment, all *R. norvegicus* were sacrificed. After that, examination using Hematoxylin–Eosin stain was conducted to look at necroptosis morphology in each group.
**Results:** We found that there are significant differences between control group and the other three groups (EGCG 20 mg/kg BW/day, EGCG 30 mg/kg BW/day, and green tea extract ($p<0.05$). There is a significant correlation between the number of neuron cell necroptosis and both EGCG and green tea extract ($p<0.05$). The correlation is negative, which means both EGCG and green tea extract will decrease the number of neuron cell necroptosis. EGCG will decrease neuron cell necroptosis starting from the dose of 20 mg/kg BW/day. EGCG 30 mg/kg BW/day produces the best result compared to other doses.
**Conclusions:** *Camellia sinensis* (green tea) with its active compound EGCG decreases neuronal necroptosis morphology in MCAO models.

**Keywords:** *Cammelia sinensis*; EGCG; green tea; necroptosis; neuron.

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## Introduction

Stroke is characterized by abrupt neurological deficit due to focal brain injury in the brain by vascular etiology [1]. Based on WHO Global Health Estimates 2016, stroke is on the second place in the list of noncommunicable diseases that cause death. The national prevalence of stroke in Indonesia in the population above 15 years old is 10.9 per mil. The highest prevalence is found in East Kalimantan with 14.7 per mil. On the other hand, the lowest prevalence is found in Papua with 4.1 per mil. The incidence of stroke increases with age. It mostly occurs in the population above 75, which is 50.2%, followed by 65–74 year old group with 45.3% [2].

Ischemic stroke is a specific type of stroke commonly found in patients rather than hemorrhagic stroke, by percentage of 85% for ischemic stroke and 15% for hemorrhagic stroke [3]. Ischemic stroke happens when there is occlusion in brain vasculature generating obstruction in the brain blood vessel which results in reduced blood flow in the brain [4]. Standardized therapy given to patients with stroke is thrombolysis therapy. The only drug approved by FDA (Food and Drug Administration) to be utilized 3 h after the onset of stroke is intravenous recombinant tissue plasminogen activator (rTPA) [5]. Prior research has showed that the first generation of thrombolytic drugs such as Streptokinase and Urokinase were not effective for treating patients with ischemic stroke [6].

Necroptosis is programmed necrosis and caspase-independent cell death. The main features of necroptosis are organellele swelling and rupture of cell membrane and wall mediated by the death signal pathway [7]. Based on

previous research, necroptosis plays a role in middle cerebral artery occlusion (MCAO) rat *in vivo* process, and its mechanism can be distinguished with apoptosis [8].

Ischemic stroke process initiated by adenosine triphosphate (ATP) derivation in the brain leads to lactic acid accumulation and dysfunction of the intracellular pump [9, 10]. Interferon-gamma (INF-γ), interleukin 1 beta (IL-1β), IL-6, and tumor necrosis factor (TNF-α) activated by the death of astrocyte leads to the increasing number of lactic acid [10, 11]. TNF- α pathway will activate necroptosis process [12]. Dysfunction of intracellular pumps produces ROS (reactive oxygen species), which leads to escalating necroptosis process through some pathways such as autophosphorylation receptor-interacting protein 1 (RIP1) [13].

The second most consumed drinks in the world is green tea [14]. The catechin major component of green tea is epigallocatechin gallate (EGCG) [15]. EGCG can used as an antioxidant, which reduces reactive oxygen species (ROS) and increases antioxidant enzyme. Based on ferric reducing antioxidant power (FRAP), positive correlation is shown by the antioxidant substance of green tea. Green tea has a better antioxidant activity than oolong and black tea [16]. Early therapy of 0.5% green tea extract in 3 weeks will produce inhibition effects towards brain ischemia processes such as peroxidation lipid, level of DNA oxidative damage, neuronal cell death, and infarct in the brain [17].

The information above shows the role of green tea with its active compound EGCG in inhibiting neuronal cell death through necroptosis pathway. Further research on *Camelia sinensis* (green tea) with its active compound EGCG in inhibiting neuronal cell death through necroptosis pathway needs to be done to identify the effect of green tea in decreasing neuronal cell necroptosis in ischemic stroke.

## Materials and methods

This study was designed as randomized posttest only MCAO-control group design true experimental. The study done by using *Rattus norvegicus* middle cerebral artery occlusion (MCAO) model treated with green tea and its active compound EGCG with the approval of Research Ethics Committee of Health Faculty, Faculty of Medicine, Universitas Airlangga. This research was conducted in animal laboratories at the Faculty of Pharmacy, Airlangga University for animal treatment. The morphological examination of neuron cell necroptosis was carried out at the Pathological Anatomy Laboratory of FK UNAIR for 4 months.

The sample of this study included 55 healthy male *R. norvegicus* MCAO models with weight of 200–275 g that met the inclusion and exclusion criteria. The sample was divided into three groups: MCAO-control groups, EGCG 10 mg/kg BW/day, EGCG 20 mg/kg BW/day, EGCG 30 mg/kg BW/day, and green tea extract 30 mg/kg BW/day for 7 days. Treatment was done by simple random sampling with the assumption that all the subjects were treated in a similar method, from taking research subjects to work and laboratory conditions. One group contained 11 rats with a total of 55 rats in all groups.

Before making the MCAO model, male *R. norvegicus* were adjusted to the new environment for 7 days. MCAO model was made by modification method using bulldog clamp to occlude cerebral media artery for 180 min. After the anesthesia stopped, we grabbed the rats by the tail 1 m above the floor and evaluated if there is flexion movement of two front leg toward contralateral of the hemisphere which indicated that the ischemia process was successful, that will cause necroptosis. Next, green tea treatment was done once a day for 7 days. We introduced the green tea treatment by dissolving green tea with aqua bidest with concentration of 1 mg/mL and giving it to the rats by gavage feeding needle. EGCG was obtained and analyzed by Xi'an rongsheng biotechnology and we used pure EGCG 98.7% (HPLC analysis document number 2019070630). Green tea extract used was labeled Meditea (IDM000580138), containing 2.5% of EGCG in 50 g sample analyzed by Angler BioChemlab on HPLC analysis, with certificate number 183689.

After that, all the rats were sacrificed using decapitation method to acquire the brain tissue. After being sacrificed, the brain tissue was taken from the hemisphere that had an infarction of 1.5 cm in front and behind the bregma and was used for histopathological examination. The brain tissue was stained by Hematoxylin eosin. The proportion of necroptosis morphology was examined and classified based on D.C Allred M.D guideline of scoring, proportion classified in score 0–5 [18]. All histopathological examination was carried out directly by pathologist Imam Susilo.

Descriptive analysis and normality test of Kolmogorov–Smirnoff were conducted for each group data. Because data distribution was abnormal, Kruskal–Wallis test was performed, followed by Mann–Whitney test to distinguish EGCG and green tea extract effect toward neuronal cell necroptosis morphology. Kruskall–Wallis test was used to compare necroptosis differences of all group. The analysis was then followed by Mann Whitney test to compare necroptosis differences of each two group after we were sure that there were differences between all groups in Kruskal Wallis test. Lastly, we performed a Spearman correlation test to find out the correlation of EGCG and green tea extract toward necroptosis morphology in MCAO model.

## Results

First, we conducted a descriptive analysis (to evaluate minimum–maximum, mean and standard deviation of our data), followed by a normality test for each group. Our data distribution is abnormal. So, we performed the Kruskal–Wallis test, and the results showed that data of the five groups is significantly different ($p<0.05$). Further, we conducted Mann–Whitney test to differentiate necroptosis morphology between each two groups presented in Table 1. We found that there are significant differences between the normal-control group and MCAO-control group ($p<0.05$), which indicates that the MCAO process happened as shown by the differences of neuronal cell necroptosis.

**Table 1:** Comparison of EGCG and green tea extract effect on neuronal cell necroptosis in MCAO model.

| Group | Mean ± SEM | n | p-Value |
|---|---|---|---|
| Normal control group | 0.44 ± 0.527 | 11 | |
| MCAO control group | 1.80 ± 0.422 | 10 | 0.00[a] |
| EGCG 10 mg/kg BW | 1.5 ± 0.522 | 12 | 0.254[b] |
| EGCG 20 mg/kg BW | 1.31 ± 0.480 | 13 | 0.049[b] |
| EGCG 30 mg/kg BW | 1.08 ± 0.289 | 12 | 0.003[b] |
| Green tea extract 30 mg/kg BW | 1.27 ± 0.467 | 11 | 0.043[b] |

[a]Compared to normal control group, [b]Compared to MCAO-control group. If p-value<0.05 considered statistically significant.

As Table 1 indicates, there are significant differences of neuronal cell necroptosis morphology between the MCAO-control group and the other three groups (EGCG 20 mg/kg BW, EGCG 30 mg/kg BW, and green tea extract) with $p<0.05$. There is a significant correlation between neuron cell necroptosis morphology and both EGCG and green tea extract ($p<0.05$). EGCG 10 mg/kg BW did not decrease neuron cell necroptosis morphology. As found in EGCG 20 mg/kg BW and EGCG 30 mg/kg BW group, EGCG can decrease neuron cell necroptosis. However, based on the p-value, 30 mg/kg BW dose is more effective than 20 mg/kg BW dose.

If we compared EGCG 30 mg/kg BW and green tea extract group, both significantly decreased neuronal cell necroptosis. However, EGCG is more effective as there is an increasing mean value in green tea extract group as presented in Table 1. The correlation is negative, which means an EGCG/green tea extract will decrease neuron cell necroptosis morphology. All of our data indicate that EGCG and green tea extract play a role in decreasing neuronal cell necroptosis during ischemic process. EGCG effect is dose-dependent as increasing EGCG dose increases the decreasing process of neuron cell necroptosis. The differences of each group in histopathological examination can be seen and compared in Figure 1, the necroptotic cell marked as green arrow with pale, fade, nonprominent, pyknotic and karyorrhexis nucleus. EGCG 30 mg/kg BW produced the most significant effect compared to the other groups as can be seen in statistical analysis in Table 1 and Figure 1.

## Discussion

Thrombolysis therapy is the only available therapy for stroke currently and has 4–5 h to its therapy window. While it is too long from the onset, intracranial hemorrhage is commonly found as a major complication of thrombolysis therapy [19]. Necroptosis described as programmed necrosis and apoptosis are regulated cell death mechanisms that happen during neurological damage process in stroke [20]. During its pathway, necroptosis will cause oxidative stress as result of an increasing number of reactive oxygen species. Mitochondrial respiratory chain primarily produces reactive oxygen species, xanthine oxidase and NADPH oxidases which leads to imbalance because of the spontaneous reperfusion or reperfusion caused by administration of pharmacological agents [21].

Green tea is commonly found in 30 countries and it is the most commonly consumed beverage worldwide [22]. Green tea is also used as a herbal plant in India and China [23]. The major catechin component of green tea is EGCG out of four catechin found in green tea [24]. Green tea antioxidant effect is already *in vivo* and *in vitro* approved. Its antioxidant effect shows similarity to antioxidant effect to a-tocopherol. Green tea also contains five times antioxidants effect compared to black tea [22].

Inhibition process of necroptosis was done by decreasing ROS by antioxidant effect produced by EGCG. EGCG will inhibit ROS produced by inhibiting some mechanisms in the necroptosis pathway. First, damaged mitochondria produce ROS, which then stimulate RIP1 and receptor-interacting protein 3 (RIP3) oxidation in three sites of cysteine(c257, c268 and c586) which also promote autophosphorylation of RIP1 and RIP3 at Ser161 so that necroptosis pathway is activated [13]. The second is inhibition toward positive feedback of ROS to increase production of necrosome in necroptosis [25]. Third, EGCG is capable of activating caspase 3 and 8 after 8 h of green tea therapy [26, 27]. Activation of caspase 8 leads to the discontinuation of necroptosis pathway. EGCG also inhibits synthesis of some inflammatory mediators: TNF-α, IL-6 and IL-8 [28, 29]. Fourth, EGCG is able to decrease expression of tumor necrosis factor receptor 1 (TNFR1) and RIP3. TNFR1 and RIP3 start to decrease in administration of 20 mg/kg BW EGCG in rat with MCAO model [30].

Neuron cell undergoing necroptosis is shown as an arrow in Figure 1, neuron cell as pale, faded, nonprominent nucleus. By comparing the intensity of necroptosis cell, our study shows that there is reduction in neuronal cell death necroptosis morphology during histopathological examination. The administration of EGCG 20 mg/kg BW, EGCG 30 mg/kg BW, and green tea extract 30 mg/kg BW shows statistically significant reduction in neuronal cell necroptosis morphology. The most significant dose is EGCG 30 mg/kg BW. The EGCG effect is dose-dependent as there is an increase of EGCG effect as we elevated the dose.

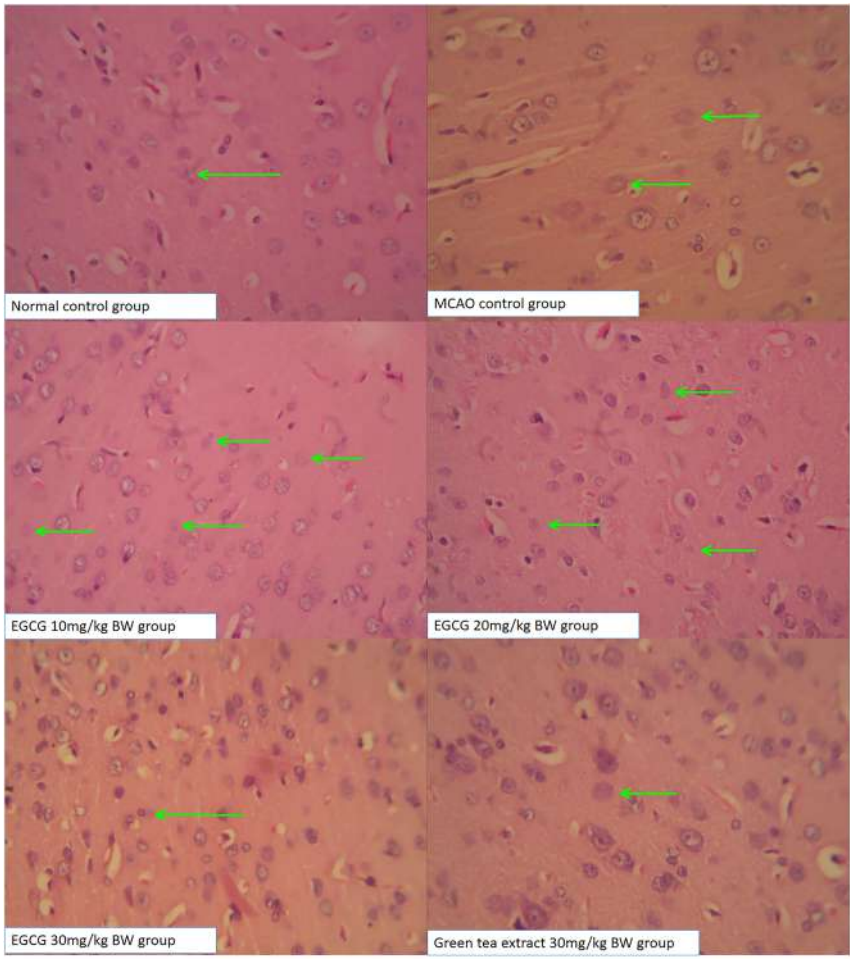


**Figure 1:** Histopathological examination result of hemisphere area in three groups (left to right): MCAO-control group, EGCG group and green tea extract group. Necroptotic neuronal cell showed in green arrow by pale, fade, nonprominent, pyknotic karyorrhexis nucleus. Necroptotic cell count can be seen decreasing in green tea extract group and EGCG group compared to MCAO-control group.

Thirty milligram per kilogram body weight dose EGCG is more effective for decreasing neuronal cell necroptosis rather than green tea extract. This is because one sachet of green tea extract only consists of 2.5% EGCG. It can be concluded that 30 mg/kg BW dose of green extract contains 0.75 mg/kg BW EGCG. As shown by p-value and mean value of proportion score, green tea extract 30 mg/kg BW produces the same effect as EGCG 20 mg/kg BW. This is due to other catechin contents such as (ECG, EC, EGC) also protein, amino acid, fiber, fat, and pigment found in green tea extract. The other catechin contents of green tea mentioned before produce synergic effect that make same antioxidant effect as EGCG of green tea extract achieved in lower dose. So, to produce the same significant effect as EGCG 30 mg/kg BW, we need a dose of green tea extract of 45 mg/kg BW (Using the ratio of 30 mg/kg BW green tea extract (0.75 mg/kg BW EGCG) which produces the same effect as pure EGCG 20 mg/kg BW).

If we applied to humans, the dose of 30 mg/kg BW/day of EGCG in rats is equal to 4.8 mg/kg BW/day EGCG. If we used a standard weight of 70 kg, we need 336 mg of EGCG each day to decrease neuronal cell necroptosis. Using the same method, we need 504 mg of green tea extract each day. Generally, people consume three cups of green tea a day; 240 ml in each cup of green tea contains 187 mg of EGCG. So, each day people consumed 560 mg of EGCG [31]. As mentioned before, we need 336 mg of EGCG or 504 mg of green tea extract each day, and the average daily consumption of three cups a day is enough to achieve the dose of EGCG/green tea extract.

## Conclusions

*Camellia sinensis* (green tea) with its active compound EGCG decreases neuronal cell necroptosis morphology in MCAO models. EGCG effect is dose-dependent starting from 20 mg/kg BW and significantly reduces neuronal cell necroptosis in 30 mg/kg BW dose and 45 mg/kg BW for green tea extract.

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