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# The Effect of Green Tea with EGCG Active Compound in Enhancing the Expression of M2 Microglia Marker (CD206)

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## Abstract

**Background:** Stroke is a neurological deficit due to vascular disorders. Microglia are the first line of defense against brain injury. Anti-inflammatory cytokines activate M2 microglia, which upregulate CD206. EGCG is abundant in green tea, which has an anti-inflammatory effect. **Objective:** To know the effect of green tea with its active compound EGCG on CD206 expression. **Settings and Design:** True experimental trial design. **Material and Methods:** *Rattus Novergicus* were divided into six groups: a negative control group (Sham), a positive control group (P0), MCAO mice given 10 mg/kg BW EGCG (P1), 20 mg/kg BW EGCG (P2), 30 mg/kg BW EGCG (P3), and 30 mg/kg BW standardized green tea extract (P4). CD206 expression was measured using immunohistochemistry and scored according to the Allred scoring guidelines. **Statistical Analysis Used:** Descriptive test, Levine test, Kolmogorov–Smirnoff test, Independent sample *t* test, Pearson correlation test **Results:** We discovered that there is a significant difference in CD206 expression between the Sham and P0 groups ($P < 0.05$). In addition, there are significant differences in expression between the sham group and the other two groups (P1 and P2) ($P < 0.05$). Furthermore, when we compared the P0 group with each treatment group, we found that CD206 expression between P0–P2, P0–P3, P0–P4 are significantly different. There is a significant correlation between green tea with its active compound EGCG and CD206 expression enhancement. The correlation is positive. **Conclusions:** Green tea with EGCG active compound increases CD206 expression as an M2 marker in the *Rattus norvegicus* with MCAO model.

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## Full Text

Stroke is the second leading cause of death and the third leading cause of disability worldwide in adults.[1] Ischemic stroke is the most common type of stroke, accounting for approximately 87% of all stroke cases.[2] The incidence of stroke increases sharply, with 5–12 per 1,000 population.[3] The prevalence of stroke increases twofold in low- and middle-income countries.[4] Stroke also has a sizeable economic impact, because of stroke burden.[5]

The first-line defense against brain injury is microglia. During an acute stroke, microglia and peripheral macrophages rapidly go to the injury site to initiate the release of effector molecules and recruit other immune cells.[6] Microglia also have the capability to change their morphology. Activated microglia release various cytokines and contribute to increased cell damage, or in later cases, these are also involved in cell repair. During an ischemic stroke, microglia polarizes into the M1 or M2 phenotypes depending on their signaling pattern.[7] Activation of microglia into M1 phenotypes occurs after infection or injury. M1 phenotypes are characterized by the presence of pro-inflammatory cytokines and high levels of free radicals. Contrary to M1 phenotypes, M2 phenotypes are activated by anti-inflammatory cytokines such as interleukin-4 (IL-4) and IL-13. These are also activated by T-helper 2 (Th2), which further upregulate scavenger receptors on M2 cell surfaces such as the mannose receptor (MRC1/CD206), and M2 phenotypes secrete anti-inflammatory cytokines such as resolution molecules.[8],[9]

The cluster of differentiation 206 (CD206; mannose receptor) is part of the C lectin type. It is a transmembrane glycoprotein and acts as a pattern recognition receptor (PRR).[10],[11] CD206 is expressed in response to increased macrophage levels, particularly M2 macrophages, and M2 microglia are also activated in this case. CD206 is considered a reliable marker for M2 activation in mice and humans because it is expressed mainly in M2 phenotypes. CD206 in M2 increases significantly, when M2 is given IL-4, IL-13, IL-10, or glucocorticoids, while CD206 decreases if there are interferon-gamma (IFNγ) and lipopolysaccharide (LPS).[12],[13] M2 microglia can inhibit inflammation, increase tissue repair and healing, and play a role in neurogenesis and functional repair.[7],[14] Recent studies have shown that M2 microglia assist neurogenesis in a post-stroke model; thus, so it is suitable for functional recovery.[15]

Thrombolysis using iv-rTPA is the only drug approved by the Food and Drug Administration (FDA), but its efficacy and safety are limited because the time limit for giving iv-rTPA is narrow; thus, only a few acute stroke patients can receive it.[16] Green tea, with its abundant bioactive polyphenols, including epigallocatechin-3-gallate (EGCG), is the second most common drink in the world, and its consumption is associated with health benefits.[17],[18] EGCG has been shown to prevent pro-inflammatory mediator production and strongly inhibits leukocyte elastase. EGCG also inhibits the activation of inflammation inducers such as matrix metalloproteinase-2 (MMP-2) and MMP-9. Therefore EGCG acts as an anti-inflammatory agent. It also affects phenotype transition from M1 to M2.[8],[19],[20] Moreover, EGCG can inhibit IFNγ-inducing expression and LPS regulation while increasing IL-10 and IL-13.[14],[21],[22],[23] Therefore, EGCG should boost CD206 stimulator and decrease CD206 inhibitor. According to this information, it is important to know the effect of green tea with its active compound EGCG on increasing CD206 expression as an M2 marker.

## Subjects and Methods

### Animal

We used a 4-month-old healthy Wistar male mice (Rattus norvegicus) weighing 175–225 g (from Gadjah Mada University breeding center, Indonesia) after getting proper acclimatization in the animal house conditions (12-h lighting cycle) for 1 week with free access to water and standard rodent chow. We performed all experimental procedures according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Universitas Airlangga, Indonesia. We divided the animals randomly into six groups. The first group is the negative control group (Sham), the healthy mice. The second group is a positive control group (P0) and included mice that were given water. The third group was treated with 10-mg/kg BW EGCG (P1). The fourth group (P2) was treated with 20-mg/kg BW EGCG. The fifth group was treated with 30-mg/kg BW EGCG (P3). The sixth group (P4) was treated with 30-mg/kg BW standardized green tea extract.

### Middle cerebral artery occlusion model

We performed middle cerebral artery occlusion (MCAO) on animals before giving the treatment. First, we anesthetized Rattus norvegicus with 80-mg/kg BW ketamine and 10-mg/kg BW xylazine intraperitoneally. Then, we made an incision in the right neck until the common carotid artery was exposed. After finding the internal carotid artery, the artery was clamped using a small bulldog brace for 180 min. We looked at mouse consciousness and whether or not a stroke model emerged.[24] We performed this model because the technique is easier and the ischemic model in Rattus norvegicus can be achieved.

Intervention

We gave the treatment to the mice with EGCG (Xi'an Rongsheng Biotechnology CO., LTD, Keji 3rd Road, Xi'an, China) or green tea extract (PT. Dharma Putra Airlangga, Tegalsari, Surabaya, Indonesia) diluted in aquades with a concentration of 1 mg/ml for seven consecutive days once daily every morning before having a meal. All groups received equivalent volumes using rat sonde. Then, we sacrificed mice after anesthetizing them with 0.1 mg/100 gr BW propofol. Subsequently, we performed an incision on the coronal section of infarcted hemispheric brain tissue from each mouse with a thickness of 1.5 cm before and behind the bregma for immunohistochemical examination. The tissue was preserved in a paraffin block.[24]

Immunohistochemistry

The paraffin block was placed on a slide, dipped in xylene, and then in ethanol 100%, 95%, and 70% for rehydration. Then, we used 3% peroxide solution to eliminate peroxidase activity. Next, we diluted anti-CD206 mAb (Lsbio ABIN1861753). The slide was then given conjugate enzyme antibodies and dissolved in TBS with 1% BSA before being incubated at room temperature. Chromogen was given for 10 min and rinsed. We provided counterstain if needed. After the slides were dried, we read the expression using a light microscope with 400 × magnification.

We assessed IHC in a semiquantitative manner based on D.C. Allred, MD guideline scoring. The assessment of proportion score was as follows: 0 = no positive cells, 1 = 0%–1% positive cells, 2 = 1%–10%, 3 = 10%–33%, 4 = between 33%–66%, and 5 = 66%–100% positive cells. There is an assessment of the intensity score based on the average staining intensity, with 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. Allred's score is the total score obtained when the proportion score is added up by the intensity score, with a value that can be 0 or between 2 and 8. Scores of 0 and 2 are interpreted as negative.[25]

Statistical analysis

All the data were analyzed using a descriptive test and then tested for homogeneity using the Levine test, and for normality using the Kolmogorov–Smirnoff test. The differences in CD206 expression between the two groups were assessed using an independent sample t test. Then, we used the Pearson correlation test to examine the correlation between two variables, which are green tea with its active compound EGCG and CD206 expression enhancement.

## Results

Independent sample t tests were used to differentiate CD206 expression between every two groups shown in [Table 1]. We discovered that there is a significant difference in CD206 expression between the Sham and P0, with $P < 0.05$. There are also significant differences between the sham group and the other two groups (P1 and P2) with $P < 0.05$. Furthermore, when we compared the P0 group to each treatment group, we found that CD206 expression between P0–P2, P0–P3, P0–P4 are significantly different.{Table 1}

In addition, we discovered that both EGCG and standardized green tea extract can increase CD206 expression, but standardized green tea extract outperforms EGCG, as shown in [Figure 1] and [Figure 2]a,[Figure 2]b,[Figure 2]c. The following section of the analysis, as shown in [Table 2], was concerned with determining the correlation between two variables using the Pearson correlation test. As a result, there is a significant correlation between green tea with its active compound EGCG and CD206 expression enhancement. The correlation is positive.{Figure 1}{Figure 2}{Table 2}

## Discussion

Ischemic stroke occurs when the supply of oxygen to the brain is blocked, often caused by a blood clot.[26] The middle cerebral artery (MCA) is the most frequent area (approximately 80%) to experience occlusion; thus, this artery has been used as a stroke model in experimental animals.[27] In normal brain conditions, microglia are considered as “resting microglia,” but recent findings have shown that microglia are the brain's most sensitive sensors. These continuously scan and monitor the parenchyma environment. Once the presence of a brain lesion or dysfunction is detected, the microglial cells are “activated,” displaying inflammatory and phagocytic features.[28] When an ischemic stroke occurs, through different signals, microglia can be polarized into the M1 phenotype or M2 phenotype.[7]

The effect of green tea with EGCG active compounds on CD206 M2 marker expression

In some studies, tea has been ranked second as the most consumed beverage after water.[29] Among the many tea types, green tea has the largest number of polyphenols and the least amount of caffeine.[30] Green tea contains abundant EGCG. EGCG can cross the blood–brain barrier and reach the brain parenchyma, which has attracted many researchers.[31],[32]

Based on independent sample t test, we found that sham and P0 significantly differ in CD206 expression. This was also observed for Sham-P1 and Sham-P2. Meanwhile, the analysis results for Sham-P3 and Sham-P4 did not show significant differences in CD206 expression. Microglia is always “active” even though there is no pathological condition. In normal and healthy conditions, microglia do not distinguish precisely the way between inflammatory and anti-inflammatory phenotypes. In contrast, the microglia shift slightly toward the anti-inflammatory phenotype, which is beneficial for brain homeostasis. Even without stimulation, microglia are an essential source of neuro-supportive cytokines such as insulin-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF). In other words, M2 also acts as an anti-inflammatory in normal brain conditions.[33] There is a significant difference in CD206 expression between sham and P0. In this study, the sham group consisted of healthy mice, which did not experience a brain infarct, dissimilar to the P0 group. Therefore, the amount of CD206 in sham is higher than in P0. Meanwhile, when CD206 expression was compared between Sham and P3, it showed that the expression is not significantly different. It also happened when CD206 expression of sham compared with P4. In other words, the CD206 level of Sham, P3, and P4 are all high. We compared the P0 group (MCAO mice were given water) with the treatment groups. CD206 expression differs significantly between P0 and P2 (MCAO mice were given EGCG 20 mg/kg BW), P0 and P3 (MCAO mice were given EGCG 30 mg/kg BW), and P0 and P4 (MCAO mice were given standardized green tea extract 30 mg/kg BW). This result shows that the addition of Camellia sinensis can increase CD206 expression compared with giving water only.

Our results show that CD206 is abundant in ischemic brains treated with 30-mg/kg BW EGCG and 30-mg/kg BW standardized green tea extract. EGCG can inhibit nitrite oxide, which causes oxidative stress.[8],[19] EGCG has also shown its ability to prevent pro-inflammatory mediators' production and strongly inhibit leukocyte elastase; thus, the activation of inflammation inducers (MMP-2 and MMP-9) cannot be mediated. This process inhibits M1 polarization and increases polarization changes in M2 phenotypes.[8],[19],[20] CD206 acts as a marker of M2 microglia; thus, its expression enhancement signifies increased polarization of M2.[34] EGCG enables increased CD206 expression by enhancing IL-10 and IL-13. EGCG also inhibits IFNγ-induced expression and LPS regulation.[14],[21],[22],[23] IL-4, IL-13, and IL-10 are stimulators of CD206 expression, while IFNγ and LPS are its inhibitors.[12],[13] M2 is capable of assisting in tissue repair, remodeling, and wound healing.[8] It is synergistic with the anti-inflammatory effect of M2 for repairing the post-stroke brain. M2 can inhibit inflammation as well as participate in neurogenesis and functional repair.[7],[14]

Green tea has four leading polyphenol derivatives (known as catechins) due to their structure, namely epigallocatechin-3-gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EG), and epicatechin (EC).[24],[35] Interestingly, EGCG is very abundant in brewed green tea, with 60%–65% of 240–320-mg catechins.[18],[29],[36] According to the comparison of CD206 expression mean total score, we found a significant enhancement of marker expression. However, standardized green tea extract (P4) has a better effect on it than EGCG itself. It may have occurred because standardized green tea extract's compounds work synergistically rather than EGCG individually, which has a better effect and potential for improving cognition post-MCAO.

After we performed the Pearson correlation test, we got the result that there is a significant correlation between green tea with its active compound EGCG and CD206 expression enhancement with positive correlation characteristics. The positive correlation shows that increasing the dose of EGCG or standardized green tea extract enhances the expression of CD206. CD206 itself has a role in resolving inflammation by clearing inflammatory molecules from the blood, as evidenced by the lack of CD206 improving inflammatory protein serum levels.[37] In addition, our study's results are in line with research conducted by Zhang et al.[14] Therefore, data analysis

results show that green tea with EGCG active compound increases CD206 expression as an M2 marker in the Rattus norvegicus with MCAO model. Our research has the advantage that biased factors affecting the research results can be controlled because it is a true experimental design. Moreover, this study can be applied to humans because both stroke and MCAO pathology occur in the brain. Our study's limitation is the semi-quantitative characteristic of the immunohistochemistry method, and that experimental animals do not accurately have the same biological mechanisms as humans.

## Conclusion

Green tea with EGCG active compound increases CD206 expression as an M2 marker in the Rattus norvegicus with MCAO model.

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Conflicts of interest

There are no conflicts of interest.

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