Source details

Journal of Basic and Clinical Physiology and Pharmacology

Formerly known as: Reviews in Clinical and Basic Pharmacology

Scopus coverage years: from 1985 to 1988, from 1990 to 2021

Publisher: Walter de Gruyter

ISSN: 0792-6855 E-ISSN: 2191-0286

Subject area: Pharmacology, Toxicology and Pharmaceutics: Pharmacology

Pharmacology, Toxicology and Pharmaceutics: Drug Discovery

Biochemistry, Genetics and Molecular Biology: Physiology

Source type: Journal

CiteScore 2020
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- Physiology

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- Medicine (miscellaneous)

Pharmacology, Toxicology and Pharmaceutics
- Drug Discovery
- Pharmacology

PUBLISHER

Walter de Gruyter GmbH

H-INDEX

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PUBLICATION TYPE

Journals

ISSN

07926855, 21910286

COVERAGE

1985-1988, 1990-2020

INFORMATION

Homepage

How to publish in this journal

m.horowitz@mail.huji.ac.il

SCOPE

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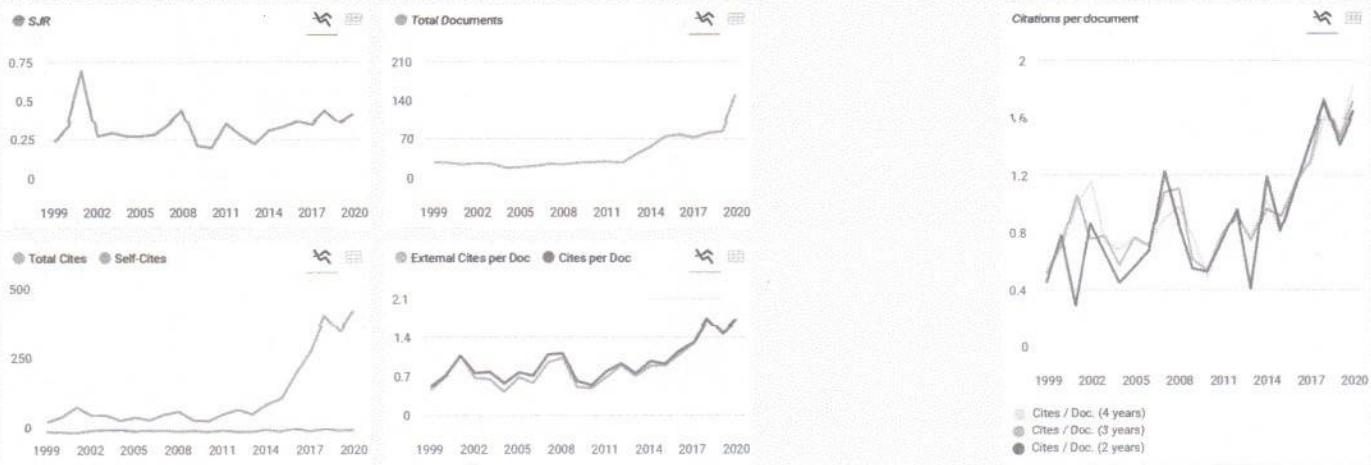

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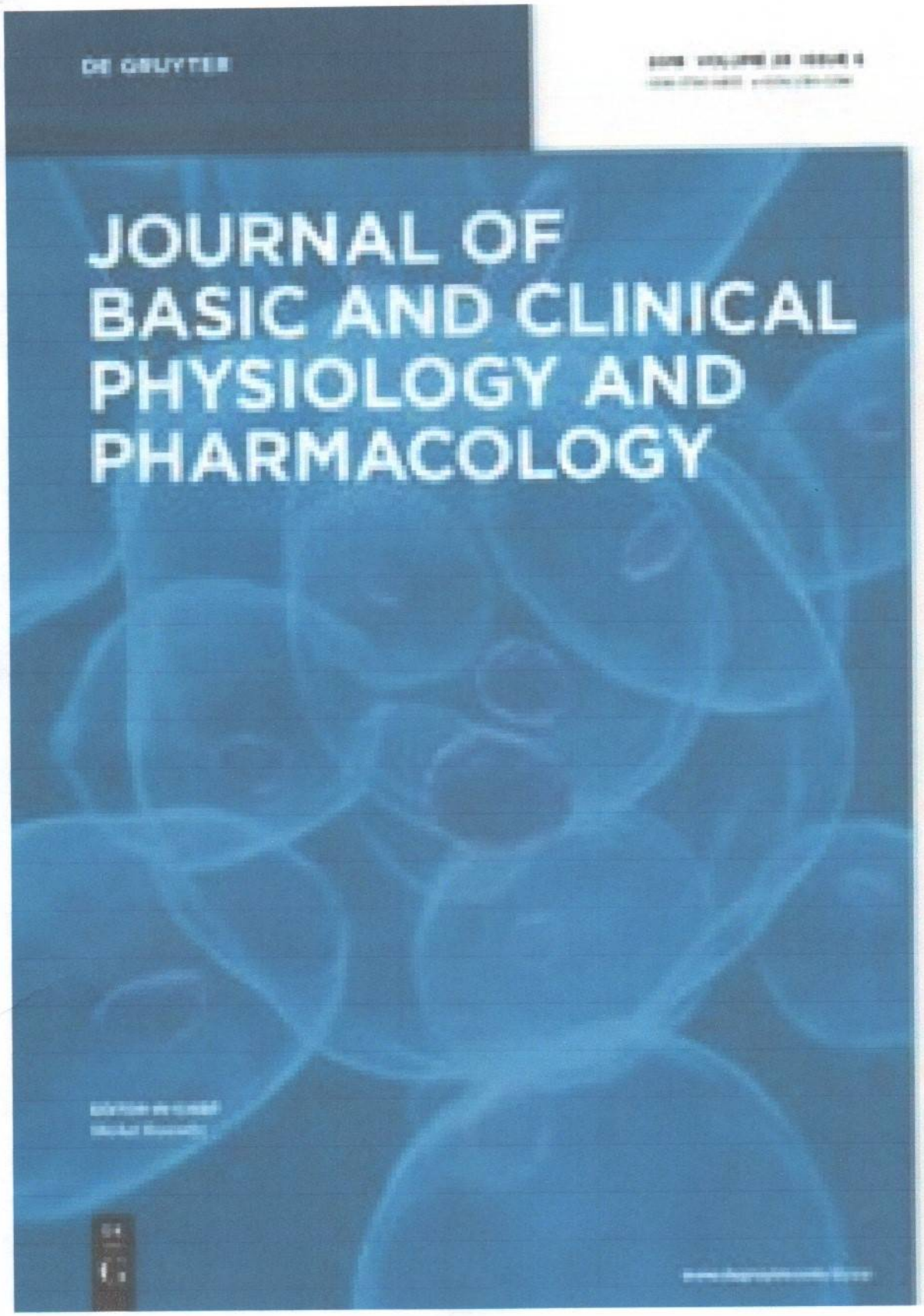
DE GRUYTER
JOURNAL OF
BASIC AND CLINICAL
PHYSIOLOGY AND
PHARMACOLOGY

Published since December 1, 1986

Journal of Basic and Clinical Physiology and Pharmacology

ISSN: 2191-0286
Editor-in-chief: Ugo Oliviero
Managing Editor: Alberto Marra

Editorial

Online ISSN: 2191-0286
Type: Journal
Language: English

Publisher: De Gruyter
First published: December 1, 1986
Publication Frequency: 6 Issues per Year
Audience: researchers and health professionals in the field of clinical physiology and pharmacology

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Published by De Gruyter

Volume 32 Issue 4 - INTERNATIONAL CONFERENCE OF PHARMACY AND HEALTH SCIENCES: The 3rd JOINT CONFERENCE UNAIR - USM; Guest Editors: Suciati & Andang Miatmoko

July 2021

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Case Report

Abdulloh Machin*, Imam Susilo and Djoko A. Purwanto

Green tea and its active compound epigallocathechin-3-gallate (EGCG) inhibit neuronal apoptosis in a middle cerebral artery occlusion (MCAO) model

https://doi.org/10.1515/jbcpp-2020-0454
Received November 28, 2020; accepted February 20, 2021

Abstract

Objectives: To determine the effect of green tea with the active ingredient epigallocathechin-3-gallate (EGCG) on the inhibition of apoptosis in the middle cerebral artery occlusion (MCAO) model.

Methods: Four month old male *Rattus norvegicus* rats with a body weight of 200–275 g was used for the MCAO model and divided into five groups, and the treatment was carried out for 7 days. Before being sacrificed, the subject had 1 cc of blood drawn for high mobility group box 1 (HMGB-1) examination using enzyme-linked immunosorbent assay (ELISA), and after being sacrificed, the brain tissue specimen was taken to examine caspase-3 and B-cell lymphoma 3 (BCL-3) using immunohistochemistry methods.

Results: There was no significant difference in HMGB-1 results for the treatment group compared to the control group (P1: 384.20 ± 231.72 [p = 0.553]; P2: 379.11 ± 268.4 [p = 0.526]; P3: 284, 87 ± 276.19 [p = 0.140]; P4: 435.32 ± 279.95 [p = 0.912]). There is a significant increase in BCL-2 expression between the treatment group compared to the control group (P1: 2.58 ± 0.51 [p = 0.04]; P2: 3.36 ± 0.50 [p<0.001]; P3: 4.00 ± 0.42 [p<0.001]; P4: 3.60 ± 0.52 [p<0.001]). There was a significant difference in caspase-3 expression compared to the control group in the P3 group (P1: 4.33 ± 0.49 [p = 0.652]; P2: 4.09 ± 0.30 [p = 0.136]; P3: 3.58 ± 0.51 [p = 0.01]; P4: 3.89 ± 0.42 [p = 0.063]). There is no correlation between HMGB-1 and caspase-3 (r = –0.063; p = 0.613) or BCL-2 (r = –0.106; p = 0.396). There is significant negative correlation between caspase-3 and BCL-2 (r = –0.459; p = 0.000).

Conclusions: Green tea with the active ingredient EGCG can inhibit neuronal cell death through the apoptotic pathway and not through the activation of HMGB-1.

Keywords: BCL-2; Caspase-3; EGCG; green tea; HMGB-1; MCAO.

Introduction

There is a decrease in blood flow during ischemic stroke that causes a decrease in adenosine triphosphate (ATP) production and causes lactic acidosis and homeostatic ion imbalance [1–3]. Homeostatic ion imbalance will cause calcium influx that will trigger phospholipase and protease and will damage cell membrane and protein [4, 5]. An increase in intracellular calcium will also trigger reactive oxygen species on mitochondria that will cause damage [6, 7].

High mobility group box 1 (HMGB-1) is a nuclear factor protein and a stress marker in the cells [8–10]. Some studies show that HMGB-1 has capability as a proinflammatory mediator that triggers inflammation during ischemia or sepsis [11–13]. HMGB-1 also increases during a pathologic condition and hours after ischemic injuries such as myocardial infarction or ischemic neuronal injury and cell death [13]. Some studies show that the HMGB-1 level increases after ischemic stroke, myocardial infarction, and hemorrhagic shock; thus, it plays an important role in the pathogenesis of ischemic stroke [8, 9, 14].

Ischemic injury can cause apoptotic cell death, and apoptotic cell death also occurs in stroke. The key mediator of apoptotic cell death is caspase-3 [15, 16]. Caspase-3 is the most well-characterized effector caspase and will trigger apoptosis when it is activated by other caspases [17]. Caspase-3 as an effector caspase plays a crucial role during neuronal development and under pathological conditions in the brain tissue such as cerebral ischemia, intracerebral hemorrhage, and brain injury. Caspase-3 is the most abundant cysteine

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aspartate expressed in adult rodent brains; thus, a change in its expression is easier to monitor [2]. Asahi et al. reported in his study that during 1 h after ischemic stroke there is upregulation of mRNA caspase-3 in the brain ischemic tissue that will increase caspase-3 expression. Namura et al. also detected caspase-3 and its product in mouse brain tissues during early reperfusion or 2 h after middle cerebral artery occlusion (MCAO) [16, 18, 19].

B-cell lymphoma 2 (BCL-2) was first identified in B-cell lymphoma, and it functions as a key regulator in the intrinsic apoptosis pathway in the mitochondria. BCL-2 also have function in the antiapoptotic pathway with B-cell lymphoma extra large (BCL-XL), myeloid cell leukemia 1 (MCL-1), and BCL-2-like protein 2 (BCL-2L2) with contour BCL-2 homology (BH) domain. BCL-2 is widely expressed during development in the brain including neuroepithelial cells and cerebral ventricle [20]. Study on primary neuronal cell culture and animal model shows that BCL-2 overexpression can protect neuronal damage and infarct size again N-methyl-D-aspartate (NMDA) induced excitotoxicity [21, 22]. Overexpression of BCL-2 also blocks apoptosis-inducing factor (AIF) and will improve cortical neuron survival after cerebral ischemia [22]. BCL-2 also acts as a key regulator of mitochondrial permeability and maintains mitochondria homeostasis. It is released in response to molecular-induced apoptosis and will prevent cell from apoptotic cell death. BCL-2 and BCL-XL protein are located in the mitochondria and endoplasmic reticulum. BCL-2 in the mitochondria maintains mitochondrial integrity and inhibits the release of proapoptotic molecule [20, 22, 23].

Green tea is the second most common drink in the world and has some beneficial effect because it has some polyphenol known as catechin and also has antioxidant property. Epigallocatechin-3-gallate (EGCG) is one of the green tea catechins and it makes up 63% of total catechin. EGCG is a potent antioxidant and a free-radical scavenger [24, 25]. Green tea can prevent neurodegenerative disease; a meta-analysis shows that people who drink more than three cups of tea a day have 21% less chance of having stroke than people who drink less than one cup a day [26]. Studies on animal models show that treatment using EGCG in an ischemic reperfusion brain tissue model decreases ischemic lesion [25, 27–29]. EGCG has free-radical scavenger function and can protect neuronal cell from oxidative damage induced by pro-oxidant agent [30]. Some animal studies show that EGCG increases mitochondrial function and decreases oxidative stress [31, 32]. Treatment with 30 mg/kg BW of EGCG can prevent isoproterenol-induced mitochondrial damage in the animal model [32].

Based on information above, green tea with its active compound EGCG may play an important role in cerebral neuroprotection because it has antioxidant and free-radical scavenger effect. It is important to conduct research to determine the effect of green tea treatment with its active compound EGCG on the level of HMGB-1 and expression of neuronal apoptosis marker in an MCAO model.

Materials and methods

We performed an MCAO model using male *Rattus norvegicus* rats obtained from Gajah Mada University's breeding center that have been published on Eurasia J Biosci 14, 1813–1820 (2020). We evaluated the HMGB-1 level using enzyme-linked immunosorbent assay (ELISA) methods (LS Bio). On the 7th day, we obtained blood for serum ELISA examination.

Immunohistochemistry examination

The brain tissue used is from a male *R. norvegicus* MCAO model and was obtained from the animal laboratory in the Faculty of Pharmacy, Universitas Airlangga. The tissue taken is the 1.5 cm area of the brain tissue in front of and behind the bregma and was then stored in paraffin blocks. The paraffin block was then cut as thick as 5 μm, and the slice was placed on the slide and heated at 65°C for 2 h so that the tissue can stick to the microscopic slide. The microscopic slides were dipped in xylene three times for 5 min each to remove paraffin. The tissue was rehydrated by dipping in 100% ethanol, then 95% ethanol, and then in 70% ethanol; then the microscopic slides were removed from 70% alcohol and then soaked in Tris buffer for 1 h. Peroxide activity is then removed by dipping in a 3% peroxide solution for 3 min. The microscopic slides were cleaned from Tris buffer for 3 min and given a conjugate enzyme after administration of the body and dissolved in Tris buffer saline with 1% bovine saline albumin and incubated at room temperature for 1 h, then give chromogen for 10 min at room temperature and rinse with running water for 5 min. The microscopic slide was read according to the guidelines of DC Allred, MD, et al. [33] by means of scoring consisting of proportion scores, with a score of 0 indicating no cells showed marker expression in all fields of view, a score of 1 when an expression of 0–1% was obtained in the field of view examined, a score of 2 when an expression of >1–10% was obtained in the field of view, a score of 3 when an expression of >10–33.3% was obtained, a score of 4 when an expression of >33.3–66.6% was obtained, and a score of 5 when a marker expression of >66.6–100% was obtained. Then, the intensity score is calculated, that is, a score of 0 if no restraint is obtained, a score of 1 if weakening is obtained, a score of 2 if moderate warming is obtained, and a score of 3 if strong warming is obtained. The final score is the proportion score added to the intensity score.

Statistical analysis

All variables were tested descriptively, and then, normality was tested using the Kolmogorov–Smirnov test. Differences in expression

between the two groups were analyzed using the Kruskal–Wallis test, followed by the Mann–Whitney test. Furthermore, all variables were tested for correlation using the Pearson or Spearman test. Then, all variables were analyzed using path analysis.

Results

We performed Immunohistochemical assessment for BCL-2 and caspase-3. We also performed ELISA for HMGB-1. There is a significant difference between all groups ($p > 0.01$), so we performed Kruskal–Wallis and Mann–Whitney tests to test the difference between groups (see Table 1).

Our data show that there is no significant difference between the control and intervention group with repect to the HMGB-1 level. According to our finding, neither EGCG nor green tea extract influenced the HMGB-1 level because of excessive release of HMGB-1 during acute ischemic stroke.

According to Table 2, the higher dose EGCG (30 mg/kg BW) and green tea extract (30 mg/kg BW) can suppress caspase-3 expression. This result shows that EGCG can inhibit apoptosis in the *R. norvegicus* MCAO model. There is a decrease in caspase-3 expression in the group treated with the lower dose of EGCG, but the decrese was not statistically significant. This result shows that inhibition of caspase-3 expression is dose dependent and significant inhibition of EGCG started at the dose of 30 mg/kg BW. Figure 1 shows expression of caspase-3 in neuronal cells after treatment with 30 mg/kg BW of EGCG.

According to Table 3, either EGCG or extract green tea can increase BCL-2 expression in the MCAO model. The intensity of expression of BCL-2 is dependent on dosage. Green tea extract also increases BCL-2 expression; the expression in the group treated with green tea extract is similar to that in the group treated with 30 mg/kg BW of EGCG. This result shows that either EGCG or green tea extract can increase antiapoptotic activity and that this activity depends on the dose of EGCG. Green tea extract also can decrease apoptotic activity, and this activity is similar to that of its active compound EGCG. Figure 2 shows expression of BCL-2 in neuronal cells after treatment using 30 mg/kg BW of EGCG.

According to Table 4, there is negative correlation between HMGB-1 and caspase-3, but is not statistically significant ($r = -0.063$; $p = 0.613$), and there is no statistically significant and negative correlation between HMGB-1 and caspase-3 ($r = -0.106$; $p = 0.396$). There is also significant negative correlation between BCL-2 and caspase-3 ($p = -0.459$; $p = 0.000$). These data show that the HMGB-1 level did not influence the expression of either caspase-3 or BCL-2. Correlation between caspase-3 and BCL-2 shows that EGCG can decrease caspase-3 expression and increase BCL-2 expression or in another

Table 1: Mean difference of HMGB-1 between the groups shows that there is no significant difference between intervention groups compared to the stroke control group.

Group	Mean ± SD	p-Value
P0 (n = 10)	1458.42 ± 329.11	–
P1 (n = 12)	384.20 ± 231.72	0.553
P2 (n = 11)	379.11 ± 268.47	0.526
P3 (n = 12)	284.87 ± 276.19	0.140
P4(n = 10)	435.32 ± 279.95	0.912

SD = standard deviation; HMGB-1 = high mobility group box 1; EGCG = epigallocathechin-3-gallate; P0 = control group; P1 = group treated with 10 mg/kg BW of EGCG; P2 = group treated with 20 mg/kg BW of EGCG; P3 = group treated with 30 mg/kg BW of EGCG; P4 = group treated with 30 mg/kg BW of green tea extract.

Table 2: Mean difference of caspase-3 shows that there is the significant difference in caspase-3 expression in the group treated with 30 mg/kg BW of EGCG.

Group	Mean ± SD	p-Value
P0 (n = 10)	4.40 ± 0.70	–
P1 (n = 12)	4.33 ± 0.49	0.652
P2 (n = 11)	4.09 ± 0.30	0.136
P3 (n = 12)	3.58 ± 0.51	0.010
P4(n = 10)	3.89 ± 0.42	0.063

SD = standard deviation; EGCG = epigallocathechin-3-gallate; P0 = control group; P1 = group treated with 10 mg/kg BW of EGCG; P2 = group treated with 20 mg/kg BW of EGCG; P3 = group treated with 30 mg/kg BW of EGCG; P4 = group treated with 30 mg/kg BW of green tea extract.This result shows that only the higher dose of EGCG can suppress caspase-3 expression.

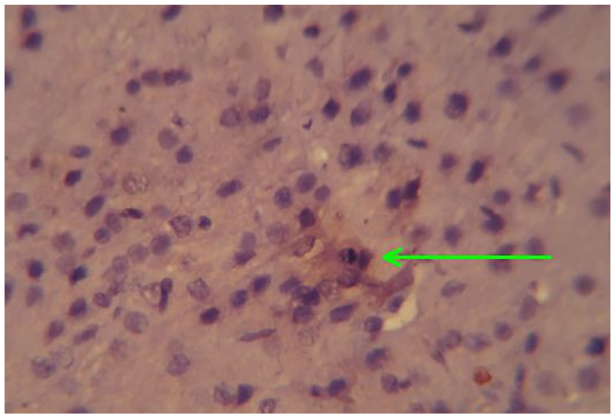

Figure 1: Caspase-3 expression in neuronal cell, shown in green arrow. There is increase in expression of caspase-3 expression (brown) in neuronal cells in the MCAO model. MCAO = middle cerebral artery occlusion.

Table 3: Mean difference of BCL-2 expression between the groups shows that there is increase in BCL-2 expression in intervention groups compared to the control group.

Group	Mean ± SD	p-Value
P0 (n = 10)	1.80 ± 1.03	–
P1 (n = 12)	2.58 ± 0.51	0.040
P2 (n = 11)	3.36 ± 0.50	0.001
P3 (n = 12)	4.00 ± 0.42	0.001
P4(n = 10)	3.60 ± 0.52	0.001

SD = standard deviation; BCL-2 = B-cell lymphoma 2; EGCG = epigallocathechin-3-gallate; P0 = control group; P1 = group treated with 10 mg/kg BW of EGCG; P2 = group treated with 20 mg/kg BW of EGCG; P3 = group treated with 30 mg/kg BW of EGCG; P4 = group treated with 30 mg/kg BW of green tea extract.There is significant difference between the control group and the sham group.

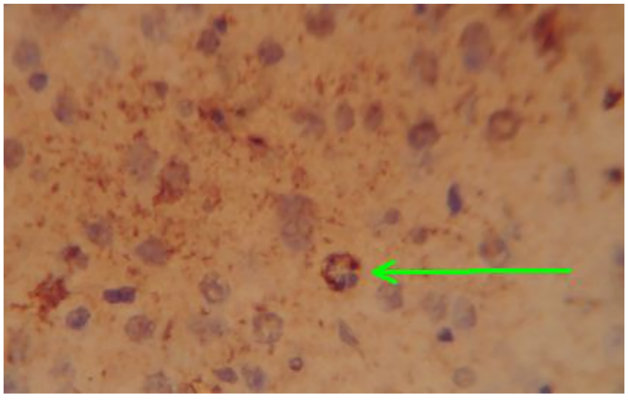

Figure 2: Expression of BCL-2 in neuronal cells is shown in green arrow. There is increase in BCL-2 expression in neuronal cells (brown). BCL-2 = B-cell lymphoma 2.

Table 4: Correlation between variables.

Variable I	Variable 2	r	p-Value
HMGB-1	Caspase-3	−0.063	0.613
Caspase-3	BCL-2	−0.459	0.000
BCL-2	HMGB-1	−0.106	0.396

BCL-2 = B-cell lymphoma 2; HMGB-1 = high mobility group box 1.There is significant negative correlation between expression of caspase-3 and BCL-2, but there is no significant correlation between HMGB-1 and caspase-3 expression or between BCL-2 and HMGB-1 expression.

word EGCG can protect neuronal cells from apoptosis through decreasing proapoptotic protein expression and increasing antiapoptotic protein expression.

Discussion

Our study shows no difference in the HMGB-1 level between the intervention group and control group, and in another way, it shows that neither green tea extract nor EGCG, as its active compound, influenced the HMGB-1 level. Our finding is different from that of other studies that show that EGCG can decrease HMGB-1 expression [34, 35].

HMGB-1 is secreted actively and passively from cells, active release of HMGB-1 have function to maintain cell and tissue homeostasis and passive release is triggered with insult that threatens the cells. HMGB-1 is a marker of cell damage, and it is released in cells during stress as danger cell signals [8, 36–39]. In response to many stresses or injuries in cells, HMGB-1 is released passively as danger-associated molecular patterns that will trigger inflammation; thus, during acute ischemic stroke, HMGB-1 is released from neuronal cells or other cells in cerebral tissue, and this will accelerate cell injury in cerebral tissue [10, 40]. During acute ischemic stroke, there is increase infree radicals as a result of oxidative stress, and this will increase HMGB-1 levels [41–43]. EGCG as an active compound of green tea has strong antioxidant property and also acts as a free radical scavenger that can decrease the HMGB-1 level [25, 34, 35, 43, 44]. Our study shows that EGCG has no effect on the HMGB-1 level because during acute stroke, there is excessive cell damage not only from necrosis mechanism but also from other forms of cell death; this excessive cell death will let neither green tea extract nor its active compound EGCG to block the release of HMGB-1 from neuronal cells [45, 46]. Our study also found that neuroprotective mechanism of EGCG is not through HMGB-1 pathways [46].

Caspase-3 is a key regulator of apoptotic cell death because it involves in the final common pathway of apoptosis. Our study shows that the high dose of EGCG can inhibit caspase-3 expression, and this implies that the high dose of EGCG can prevent neuronal apoptotic cell death in the MCAO model throughout its inhibition [2, 47]. This result is similar to that of the study by Nan et al. [48] which shows that treatment using EGCG will diminish expression of caspase-3. Some studies also show that EGCG can inhibit apoptosis through its antioxidant activity and as a free radical scavenger [25, 32]. During cerebral ischemia, there is increase in excitatory amino acids, free radicals, NO, and inflammation that will trigger neuronal death including apoptosis. There is increase in mitochondrial function during acute ischemia, which will activate cytochrome c, caspase, and apoptotic bodies. EGCG that has free radical scavenger effect thus will decrease apoptotic cell death [48]. EGCG also stabilized mitochondria through its antioxidant and free radical scavenger properties [49]. Our intervention group treated with standardized green tea extract shows no significant difference compared with the control group; this may be because it needs a higher dose of

EGCG to inhibit apoptosis in the MCAO model because in the EGCG-treated group, significant caspase-3 inhibition is shown only in the group treated with the highest dose of EGCG (30 mg/kg BW).

BCL-2 is a main antiapoptosis protein; it regulates apoptosis through the mitochondrial complex that maintains the integrity of the outer mitochondrial membrane [20, 22]. Expression of BCL-2 is increased in our intervention study, which means that either green tea extract or its active compound EGCG can inhibit apoptosis through increasing BCL-2 expression [48, 50].

There is a different mechanism of EGCG in the context of stroke and malignancy with regard to the effect on apoptosis. Effect of EGCG in malignancy can induce apoptosis; however, in stroke, EGCG can prevent neuronal apoptosis, and this is different because of different expression of *silent information regulator* (SIRT3). EGCG will significantly reduce SIRT3 expression in malignancy, but not in normal cells; thus, EGCG can prevent apoptotic neuronal death [51].

There is no correlation between HMGB-1 and caspase-3 in our study. Zhang et al. [52] studied colorectal cancer and found that increase in HMGB-1 levels will increase activation of caspase-3. Another study also found that HMGB-1 release promotes apoptosis in cancer cells [53]. The role of HMGB-1 in stroke is different between the acute phase and recovery phase. HMGB-1 in the acute phase promotes necrosis and triggers inflammation, but in the recovery phase, HMGB-1 mediates plasticity and recovery [54]. Oxidation of EGCG will produce dimer EGCG (theasinensin) and cause aggregation of HMGB-1 and promote autophagia; in our study, EGCG treatment showed no effect because excessive release of HMGB-1 prevented EGCG from blocking HMGB-1 [46]. In our study, there were no significant correlation between HMGB-1 and caspase-3, this result shows that caspase-3 expression is not through the HMGB-1 pathway [46].

Our study found that antiapoptotic property of EGCG through inhibition of caspase-3 expression and increasing BCL-2 expression. This action may be because of antioxidant property and free radical scavenger effect of EGCG [25, 27, 55]. Neuroprotective effect of EGCG has been demonstrated in many stroke model studies [25, 27, 29, 55]. One of the mechanisms of neuroprotective EGCG is through antiapoptotic effect and properties that can stabilize the mitochondrial membrane [24, 32, 48, 49]. Mitochondria has been long known to play a role in apoptosis through oxidative stress that triggers cytochrome c which can stimulate apoptosis [32].

Our study is a true experimental study using either EGCG as the active compound or green tea extract, giving new insights into neuroprotective mechanism of EGCG in the stroke model that should continue in clinical trials. Our study is a true experimental trial that we can control other confounding factors that will affect this study.

The limitation of our study is that it is an animal model study that should be proven in clinical trials to know the usefulness in stroke patients. Our study using immunohistochemistry method, resulting in semiquantitative data. For more objective result and assessment we used Allred score.

Conclusions

Green tea treatment with its active compound EGCG inhibits the expression of the neuronal apoptosis marker in the MCAO model and shows no effect on the level of HMGB-1.

Acknowledgments: Gratitude is due to the Head of the Department of Pathology, Faculty of Medicine, Universitas Airlangga, and Head of the Department of Pharmaceutical chemistry, Faculty of Pharmacy, Universitas Airlangga.
Research funding: Research fund was obtained from Research and Community Service Management Information Systems Ministry of Research, Technology, and Higher Education of the Republic of Indonesia.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: The authors state no conflict of interest.
Ethical approval: Ethical approval was obtained from The Research Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga, number: 2.KE.161.09.2018.

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