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Catechins decrease neurological severity score through apoptosis and neurotropic factor pathway in rat traumatic brain injury

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ABSTRACT

BACKGROUND
Catechins inhibit apoptosis through anti-oxidative and anti-inflammatory pathways, also increases brain-derived neurotrophic factor (BDNF). Only a few research that explained the role of catechins in traumatic brain injury (TBI). The objective of the study was to evaluate the effect of catechins administration on neurological severity score (NSS) through apoptosis and neurotrophic pathway in TBI rat model.

METHODS
A post test only controlled group design was conducted using *Rattus norvegicus* weight-drop models of TBI. It was devided into negative control, positive control, TBI+catechins 513 mg/kgBW, TBI+catechins 926 mg/kgBW, and TBI+catechins 1113 mg/kgBW groups . Catechins was administered daily for three days and seven days post-trauma. NSS was examined in the first hour, third day, and seventh day. Expression of TNFα, Bax, Bcl-2, caspase 8 caspase 3, BDNF, total expression of NFκB p65, and apoptosis cells in brain tissue was measured by immunohistochemistry method. One way Anova and Kruskal Wallis were used to analyse the data.

RESULTS
Catechins decreased the expression of TNFα, caspase 8 caspase 3, total expression NFκB p65, apoptosis cells and NSS significantly ($p<0.050$) on the third day, either on the seventh day. Catechins increased the expression of BDNF significantly ($p<0.050$) on the third day, either on the seventh day. Catechins increased the expression of Bcl-2/Bax significantly ($p<0.050$) on the third day, neither on the seventh day ($p\geq0.050$).

CONCLUSIONS
Administration of catechins decrease NSS by inhibiting inflammation and apoptosis, as well as triggering the neurotrophic factors in rat TBI.

Keywords: Catechins, TBI, apoptosis, inflammation

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INTRODUCTION

TBI is divided into primary and secondary injuries. Primary injury occurs during trauma while secondary injury does not occur immediately. TBI therapy targeted at secondary injury since there is a time interval for inhibiting it, whereas no time interval in primary injury.[1] Inflammation and apoptosis occurred on secondary injury and is the cause of clinical deterioration in TBI. Apoptosis is responsible for two-thirds of cell death in TBI.[2,3] Intervention on inflammatory and apoptotic pathways are the targets of therapy in TBI.[4]

The initiation of apoptosis is due to an increase in calcium influx.[5] Apoptosis, can occur through intrinsic and extrinsic pathways. In intrinsic pathways, apoptosis is regulated by the B cell lymphoma 2 (Bcl-2) family, such as Bcl-2 and Bcl-2-associated X (Bax) protein. In TBI, a decrease in Bcl-2 and a increase in Bax occurs.[6] Bax activation causes cytochrome c release which activates caspase 9, leading the activation of caspase 3.[3,6,7] Bax activation is inhibited by Bcl-2. It is known that the Bcl-2/Bax ratio influences the permeability of the mitochondrial membrane. An increased in Bcl-2/Bax ratio decreases the number of apoptotic cell.[3] Meanwhile, extrinsic pathways of apoptosis progresses due to inflammation. An increase of NFκB induces an increase in TNFα, thus activating the death receptor,[2] Activation of death receptor activates caspase 8 which further activates caspase 3. Caspase 8 also affects intrinsic pathways by activating Bax through activation of BH3-interacting domain death (Bid).[3]

Besides the activation of apoptotic pathways due to TBI, brain naturally has brain-derived neurotrophic factor (BDNF) which can protect neurons from death. Decreased BDNF is known to contribute on neurodegenerative disease, whereas increase of BDNF can protect the brain from ischemia, trauma, and toxic.[8]

Green tea (*Camellia sinensis*) catechins is known to have anti-inflammatory and anti-oxidative feature, which may inhibit apoptosis in a head injury.[9,10] Epigallocatechin gallate (EGCG) is the most predominant flavonoid compound in green tea catechins. Epigallocatechin, epicatechin gallate, epicatechin, and catechin was also found in green tea catechins.[11] In brain, each flavonoid of catechins is known to have different effects. Epicatechin has an anti-oxidative effect for traumatic neurons and can inhibit TNFα release. But, it can not reduce the expression of inducible nitric oxide synthase (iNOS), lower production of nitric oxide (NO), and inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[12,13] Catechin and EGCG has an anti neuroinflammation effect.[11] EGCG can reduce brain edema, also support cell life by take care of the protein kinase c pathway and extracellular signal-related kinase ½ pathway.[2,14] The administration of EGCG in 1 hour after spinal injury can reduce inflammation through decreased expression of TNFα, interleukin-1β (IL-1β), nitrotyrosine, cyclooxygenase-2 (COX-2), and poly (ADP-ribose) polymerase (PARP).[15]

Many previous studies have given catechins ad libitum in the drinking water so that the amount of catechins consumed would be different. According to the research by Itoh et al., there was decrease in apoptosis and cognitive improvement occurred in the TBI rats which administered by EGCG ad libitum for 7 days.[16] Based on the research of Suzuki et al., there was a decrease of nitrogen oxide and clinical improvement in ischemic rats by giving catechins 0.5 % ad libitum. Based on the research of Suzuki et al., rats had clinical improvement by consuming catechins of 513, 926, and 1113 mg/kgBW/day.[17]According to that research, in this study, catechins were administered by oral with the same doses as above for TBI rat in 3 days and 7 days. Although there have been many studies using EGCG, epicatechin, or catechin in TBI, no research has examined the total use of catechins in TBI. We hypothesized that administration of catechins might inhibit

inflammation and apoptosis and increase BDNF. By inhibition of apoptosis through both intrinsic and extrinsic pathways and the activation of BDNF, it was expected that the severity of the injury may be reduced, marked by decreased NSS in model rat.[18] This study aimed to assess the effect of catechins on expression of NFκB, TNFα, caspase 8, caspase 3, apoptotic cells, Bcl-2/Bax ratio, BDNF, and NSS in wistar rats of the TBI model.

METHOD

Design of the study

This research used experimental design of post test only controlled group in Laboratory of Physiology, Anatomic Pathology, and Biochemistry Faculty of Medicine Brawijaya University Malang. The study was conducted from July to September 2016.

Experimental animals

Rattus norvegicus wistar strain has served as an animal model for research, which obtained from Pharmacology Laboratory of Airlangga University Surabaya. The inclusion criteria was male rats, 6-8 weeks old, weight 100-150 grams, NSS 0. The drop out criteria was dead rat at the time of the research.

Forty rats were randomly distributed to 5 intervention groups: group A (negative control, group without TBI), group B (positive control, TBI model group without catechins), group C (group of TBI model with catechins 513 mg/kgBw/day), group D (TBI model group with catechins 926 mg/kgBW/day), and group E (group of TBI models with catechins 1113 mg/kgBW/day).[17] Catechins were administered orally every day as much as once daily 1 hour after trauma.

After obtained catechins for 3 days, 4 rats from each group (total of 20 rats) performed dissection while 20 other rats performed dissection after got catechins daily for 7 days. Dissection on the third day aims to observe at apoptosis peak. Dissection on the seventh day was performed because secondary injury through calcium influx and increase NO took place until 7 days.[1] Furthermore, functional improvement in mild TBI was observable on the seventh day.[19]

TBI Model

The TBI method was performed according to research which was conducted by Riawan et al.[20] Rats were anesthetized with Ketamine 44 mg/kgBW intra muscular then scalp was incised. The 45 gram iron cylinder (0.4 cm diameter) was dropped at an angle of 90º from a height of 100 cm once, resulting in a 0.45 joule impact energy on the rat vertex. After the load collision scalp was sewn back by chromic catgut 3-0 thread. Rats were placed in cages and observed until they regain consciousness.

Catechins Extraction

GMB-4 strain of green tea was obtained from Gambung Tea and Quinine Research Center. Catechins was isolated from green tea GMB-4 strains in powder form. The isolation procedure was performed by Ciptati[21] in Department of Chemistry, Faculty of Science, Institute of Technology Bandung.

Preparation of immunohistochemistry slides

The rat brain was placed in the bottle which has 10% buffered formalin, then made into paraffin block which were cut into 2-3 μm thick slide section taken from the vertex region. The immunohistochemical technique was performed on the slides using primary antibodies (NFκB p65 bs-0465R BiossUSA, TNFα bs-2081R Bioss USA, TUNEL ba-2220 Enogen, Bax bs-0127M BiossUSA, Bcl-2bs-4563R BiossUSA, caspase 8 bs-0052R BiossUSA, caspase 3 bs-0081R Bioss USA, BDNF bs-4989R Bioss USA), each of which was dissolved in phosphate buffered solution (PBS) at a ratio of 1:100 and incubated for 1.5 hours. Subsequently the slides were twice washed with PBS, each time for 5 minutes. The secondary antibody was dripped onto the slides and the slides incubated for 10 minutes. The slides were then washed again with

PBS (2x5 minutes). Then Trekavidin-Hrp label was dripped onto the slides and the slides incubated for 10 minutes. The slides were then washed with PBS (2x5 minutes). Subsequently diamino-benzidine (DAB) was added and the slides incubated for 2-4 minutes. Especially for slides that were used NFκB p65 antibody, it incubated for 60 minutes.Then the slides were rinsed in running water for 5 minutes. After counterstaining with Mayer's hematoxylin, the slides were covered with a coverglass.

Preparation of deoxynucleotidyl transferase terminal dUTP nick end labeling (TUNEL)

The TUNEL assay was performed to observe apoptotic neurons in the brain tissues. The slides were washed with PBS at pH 7.4 and incubated with 20μg/mL proteinase-K for 15 minutes at 37^0C. The slides were washed and then incubated with H_2O_2 3% for 15 minutes and again washed for 5 minutes. The slides were incubated with TUNEL fragmented DNA labelling solution for 60 minutes at 37^0C. The slides were washed again and incubated with peroxidase solution for 40 minutes at 37^0C. After that, the slides were washed again. The substrate for peroxidase (DAB) was dripped onto the slides for 20 minutes at room temperature, the slides washed, then counterstained with Mayer's hematoxylin for 10 minutes, rinsed in tap water and washed with distilled water (dH_2O), dried and covered with a coverglass.

Neurological Severity Score (NSS)

An initial NSS examination prior to experiment was performed to ensure that there were no defect on rats that used in this study. Subsequently, the NSS was measured after anesthesia effects disappeared (approximately 1 hour after the weight-drop injury) and after the completion of experiment before the subjects were sacrificed (third and seventh day according to rat day group). NSS 1 hour after trauma was performed to assess the severity of the injury.[22] The NSS measurements were performed according to the research of Wang et al.[22] The lowest value is 0 (normal) and the highest value is 10.[23] This weight-drop method generated mild brain injury.

Evaluation method

The total count of neurons which were expressed NFκB, TNFα, Bax, Bcl-2, caspase 8 caspase 3, BDNF and apoptotic cells were done by "hot-spot method." The procedure was performed with a 400x magnitude of binocular microscope (Olympus type BX51). Positive cells were counted in 10 fields of view and rearranged. Positive neurons expressed TNFα, Bax, Bcl-2, caspase 8 caspase 3, and BDNF were shown with a brown-colored cytoplasm, whereas TUNEL positive neurons exhibit a brown colored nucleus. Neurons with positive NFκB p65 expression were stained with brown in the nucleus and the cytoplasm. Furthermore, positive Bcl-2 and Bax expressions were presented in the Bcl-2/Bax ratio.

Statistical analysis

The results were analyzed by statistical analysis program, IBM SPSS, version 22.0 for Windows. The confidence level used was 95% (α=0.05). One way Anova for parametric data and Kruskal Wallis for non parametric data were used to analyse expression of NFκB p65, TNFα, Bax, Bcl-2, caspase 8, caspase 3, TUNEL and BDNF on the third and seventh days. Significant data were tested by post-hoc Tukey or Mann Whitney.

Ethical clearance

The study protocol was approved by the Ethics Commission, Faculty of Medicine, Brawijaya University, Malang (No.276/EC/KEPK/07/2016).

RESULTS

Mean NSS and total expression NFκB p65, expression of TNFα, caspase 8, caspase 3, apoptotic cells count, Bcl-2/Bax ratio and BDNF on the third and seventh days were presented in Table 1. NFκB p65, TNFα, caspase 8, caspase

3, and apoptotic cells were highest in the positive control and lowest in the negative control. There was decreased in expression of the average TNFα expression, caspase 8, caspase 3, total expression of NFκB p65 and apoptotic cells after catechins administration. In contrast, the smallest expression of Bcl-2/Bax ratio and BDNF were in the negative control and highest in positive controls. Upon catechins administration, there was also increased of Bcl-2/Bax ratio and BDNF expression (Figures 1 and 2).

Effect of catechins dose on expression of total NFκB p65, TNFα, caspase 8, caspase 3, number of apoptosis cells, NSS, Bcl-2/Bax ratio and BDNF on days 3 of intervention

There were significant differences in mean expression of total expression NFκB p65 (p=0.000), TNFα (p=0.002), Bcl-2/Bax ratio (p=0.005), caspase 8 (p=0.000), caspase 3 (p=0.000) BDNF (p=0.000), and number of apoptotic cells (p=0.004) among groups on the third day (p<0.05) (Table 1). The results of the Tukey multiple comparisons analysis showed a significant decrease in NFκB (p=0.000; p=0.000; p=0.000), caspase 8 (p=0.000; p=0.000; p=0.000), and caspase 3 (p=0.000; p=0.000; p=0.000) in the groups receiving catechins at doses of 513, 926, and 1113 mg/kgBW, respectively, as compared with the positive control group (p<0.05). A significant decrease in TNFα in comparison with the positive control group (p<0.05) was only found in the group receiving catechins at doses of 926 mg/kg BB (p=0.021) and 1113 mg/kgBW/days (p=0.020), respectively. There was also a significant decrease in BDNF in the group receiving catechins at doses of 513 mg/kg BB (p=0.002), 926 mg/kg BB (p=0.001), and 1113 mg/kgBW (p=0.000), respectively, as compared with the positive control group (Figure 1).

The results of the Mann Whitney test showed that a significant decrease in number of apoptotic cells as compared with the positive controls (p<0.05) occurred only in the group receiving catechins at 1113 mg/kgBW/days (p=0.021). There was a significant increase (p<0.05) in the Bcl-2/Bax ratio in the group receiving catechins at 513 mg/kgBW (p=0.021), 926 mg/kgBW (p=0.021) and 1113 mg/kgBW) (p=0.021), respectively (Table 1).

Effect of catechins dose on expression of total NFκB p65, TNFα, caspase 8, caspase 3, number of apoptotic cells, NSS, Bcl-2/Bax ratio and BDNF on the days 7 of intervention

There was a significant difference in mean expression of NFκB p65 (p=0.000), TNFα (p=0.000), Bcl-2/Bax ratio (p=0.013), caspase 8 (p=0.000), caspase 3 (p=0.000), BDNF (p=0.000), and mean apoptotic cell count (p=0.000) between groups (p<0.05) (Table 1). The results of the Tukey multiple comparisons analysis showed a significant decrease in NFκB p65 (p=0.000; p=0.000; p=0.000), TNFα (p=0.000; p=0.000; p=0.000), caspase 8 (p=0.000;p= 0.000; p=0.000), caspase 3 (p=0.000; p=0.000; p=0.000) and apoptotic cell count (p=0.000; p=0.000; p=0.000) in the groups receiving catechins at doses of 513, 926, and 1113 mg/kgBW, respectively, as compared with the positive controls (p<0.05). There was a significant increase in BDNF between the groups receiving catechins at 513 mg/kgBW (p= 0.000), 926 mg/kg BB (p=0.000) and 1113 mg/kgBW (p=0.000), respectively, as compared with the positive controls (p<0.05). The results of the Kruskal Wallis test showed no significant decrease in the Bcl-2/Bax ratio (p>0.05) in the groups receiving catechins at 513 mg/kg BB (p=0.083), 926 mg/kg BB (p=0.083) and 1113 mg/kgBW (p=0.083), respectively, as compared with the positive controls (Figure 2).

Effect of catechin dose on NSS on days 3 and 7 of intervention

On day 3, there was significant difference in NSS between the intervention groups (p=0.000) (Table 1). The results of the Tukey multiple comparisons analysis showed a significant decrease in NSS in the groups receiving catechins at 513 mg /kgBW (p=0.002)

Table 1. Mean expression of NFκB, TNFα, Bcl-2, Bax, Bcl-2/Bax ratio, caspase 3, caspase 8, TUNEL, and BDNF and mean NSS score in the intervention groups

	Control - (n=4)	Control + (n=4)	TBI + Catechins 513 mg/kgBW (n=4)	TBI + Catechins 926 mg/kgBW (n=4)	TBI + Catechins 1113 mg/kgBW (n=4)	p value
Catechins for 3 days						
NFκB on day 3*	8.70 ± 2.05 [a]	41.70 ± 3.18 [e]	33.15 ± 3.59[d]	28.20 ± 3.50[c]	17.25 ± 3.01[b]	0.000
TNFα on day 3 †	2.80 ± 0.85[a]	26.48 ± 0.88[d]	25.00 ± 3.31[cd]	21.78 ± 0.69[c]	14.48 ± 1.67[b]	0.000
Bcl-2 on day 3 ‡	16.00 ± 4.25 [a]	8.00 ± 2.24[a]	11.23 ± 3.50[a]	12.23 ± 3.80[a]	12.08 ± 3.13[a]	0.066
Bax on day 3§	4.93 ± 1.05[a]	26.10 ± 0.85[d]	15.3 ± 0.85[b]	16.33 ± 0.86[c]	14.9 ± 0.12[b]	0.002
Bcl-2/Bax ratio on day 3	3.40 ± 1.46[c]	0.30 ± 0.09[a]	0.73 ± 0.21[b]	0.75 ± 0.22[b]	0.81 ± 0.20[b]	0.005
Caspase 3 on day 3	3.03 ± 0.34[a]	27.88 ± 0.35[c]	14.13 ± 0.46[b]	13.93 ± 0.17[b]	13.73 ± 0.46[b]	0.000
Caspase 8 on day 3	10.27 ± 5.37[a]	46.40 ± 5.56[e]	39.80 ± 4.26[d]	29.55 ± 4.97[c]	17.50 ± 3.78[b]	0.000
BDNF on day 3**	9.83 ± 0.88[c]	2.43 ± 0.46[a]	4.30 ± 0.50[b]	4.43 ± 0.50[b]	5.40 ± 0.39[b]	0.000
TUNEL on day 3	2.20 ± 0.43[a]	28.18 ± 4.61[c]	23.78 ± 0.59[c]	25.60 ± 2.53[c]	17.30 ± 0.96[b]	0.004
NSS on day 3¶	0.00 ± 0.00	4.25 ± 1.25[c]	1.75 ± 0.5[b]	3.00 ± 0.81[bc]	2.50 ± 0.57[b]	0.000
Catechins for 7 days						
NFκB on day 7	6.45 ± 1.98[a]	19.47 ± 3.17[c]	17.22 ± 3.27[d]	12.45 ± 2.85[c]	11.02 ± 3.24[b]	0.000
TNFα on day 7	2.10 ± 0.14[a]	24.95 ± 2.68[d]	16.02 ± 1.00[c]	13.83 ± 0.85[c]	9.43 ± 1.53[b]	0.000
Bcl-2 on day 7	15.50 ± 4.74[a]	12.20 ± 3.18[a]	14.13 ± 4.29[a]	12.88 ± 4.62[a]	13.00 ± 4.18[a]	0.677
Bax on day 7	5.17 ± 0.33[a]	16.9 ± 1.10[c]	13.75 ± 0.31[d]	12.93 ± 0.10[c]	12.03 ± 0.10[b]	0.001
Bcl-2/Bax ratio on day 7	3.04 ± 1.11[b]	0.71 ± 0.15[a]	1.03 ± 0.33[a]	0.99 ± 0.36[a]	1.08 ± 0.34[a]	0.013
Caspase 3 on day 7	3.03 ± 0.34a	27.88 ± 0.35[c]	14.13 ± 0.46[b]	13.93 ± 0.17[b]	13.73 ± 0.46[b]	0.000
Caspase 8 on day 7	4.87 ± 4.43[a]	29.75 ± 5.80[d]	24.97 ± 4.18[c]	12.07 ± 4.12[b]	6.40 ± 4.07[a]	0.000
BDNF on day 7	10.28 ± 0.40[c]	2.65 ± 0.30[a]	4.73 ± 0.10[b]	4.75 ± 0.33[b]	5.25 ± 0.21[b]	0.000
TUNEL on day 7	2.20 ± 0.64[a]	25.70 ± 3.14[d]	19.20 ± 1.61[c]	16.57 ± 0.45[c]	9.10 ± 0.86[b]	0.000
NSS on day 7	0.00 ± 0.00	5.00 ± 0.82[c]	1.75 ± 0.50[b]	2.25 ± 0.50[bc]	1.25 ± 0.57[b]	0.002

Values are mean ±SD; *NFkB=nuclear factor kappa B; †TNF-α=tumor necrosis factor alpha, ‡BCL-2=B cell lympoma gene 2; §Bax=Bcl-2-associated protein X; **BDNF=brain derived nuclear factor, ¶NSS= neurological severity score; mg/kgBW=milligram/kilogram body weight. [a,b,c,d,e] =markers indicating differences between intervention groups; p=p-values for one-way Anova significant results ($p<0.05$)

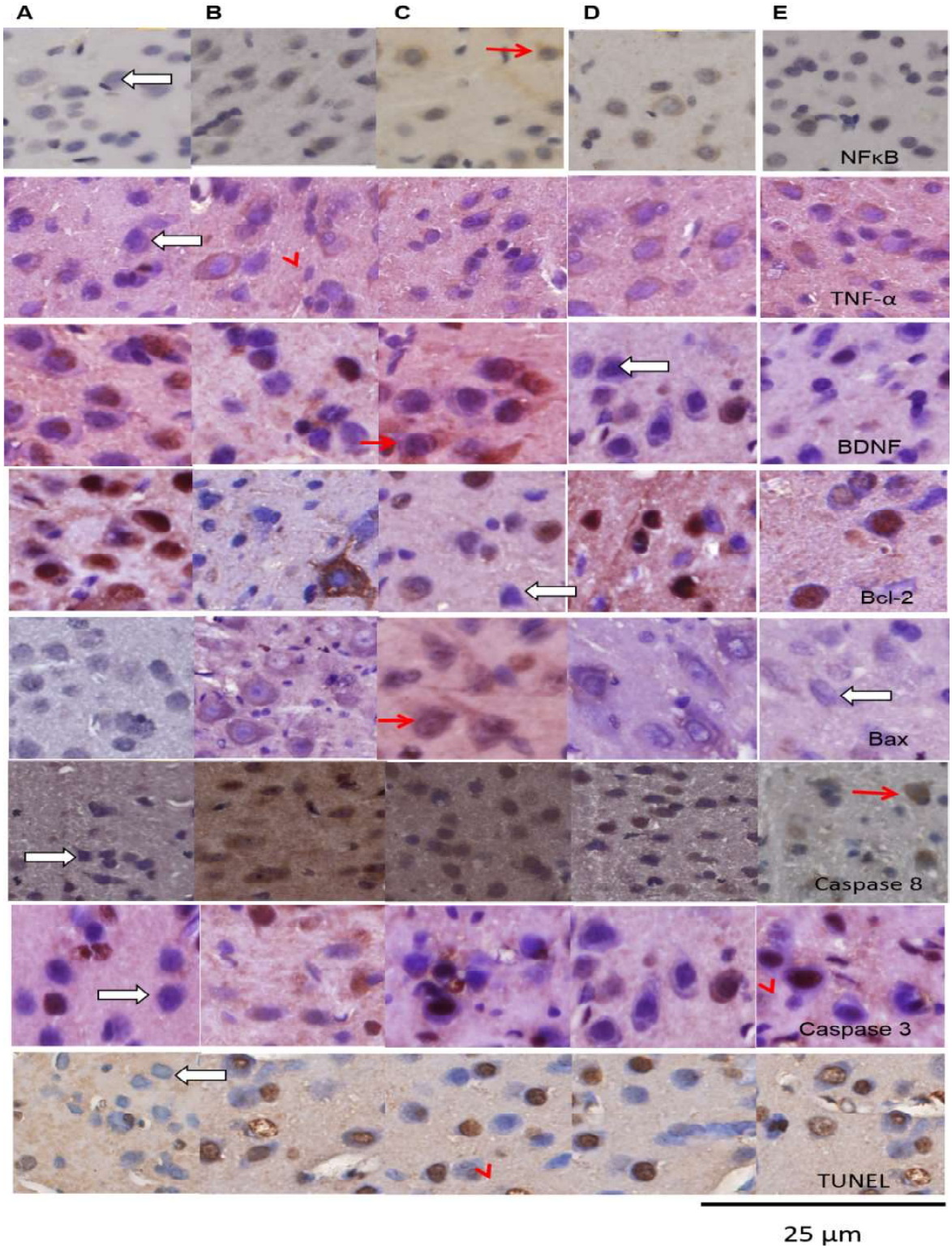

Figure 1. Expression of NFκB, TNFα, Bcl-2, Bax, caspase 3, caspase 8, TUNEL, and BDNF on day 3 using immunohistochemical technique
(A) Negative controls, (B) Positive controls, (C) TBI+catechins 513 mg/kgBW/day, (D) TBI+catechins catechins 926 mg/kgBW/day, (E) TBI+catechins 1113 mg/kgBW/day. Red arrows point to neurons with positive expression (TNFα, Bcl-2, Bax, caspase 3, caspase 8, and BDNF, stained brown in the cytoplasm; TUNEL apoptosis stained brown in cell nucleus ; NFκB stained brown in nucleus and cytoplasm). White arrows point to neurons with negative expression

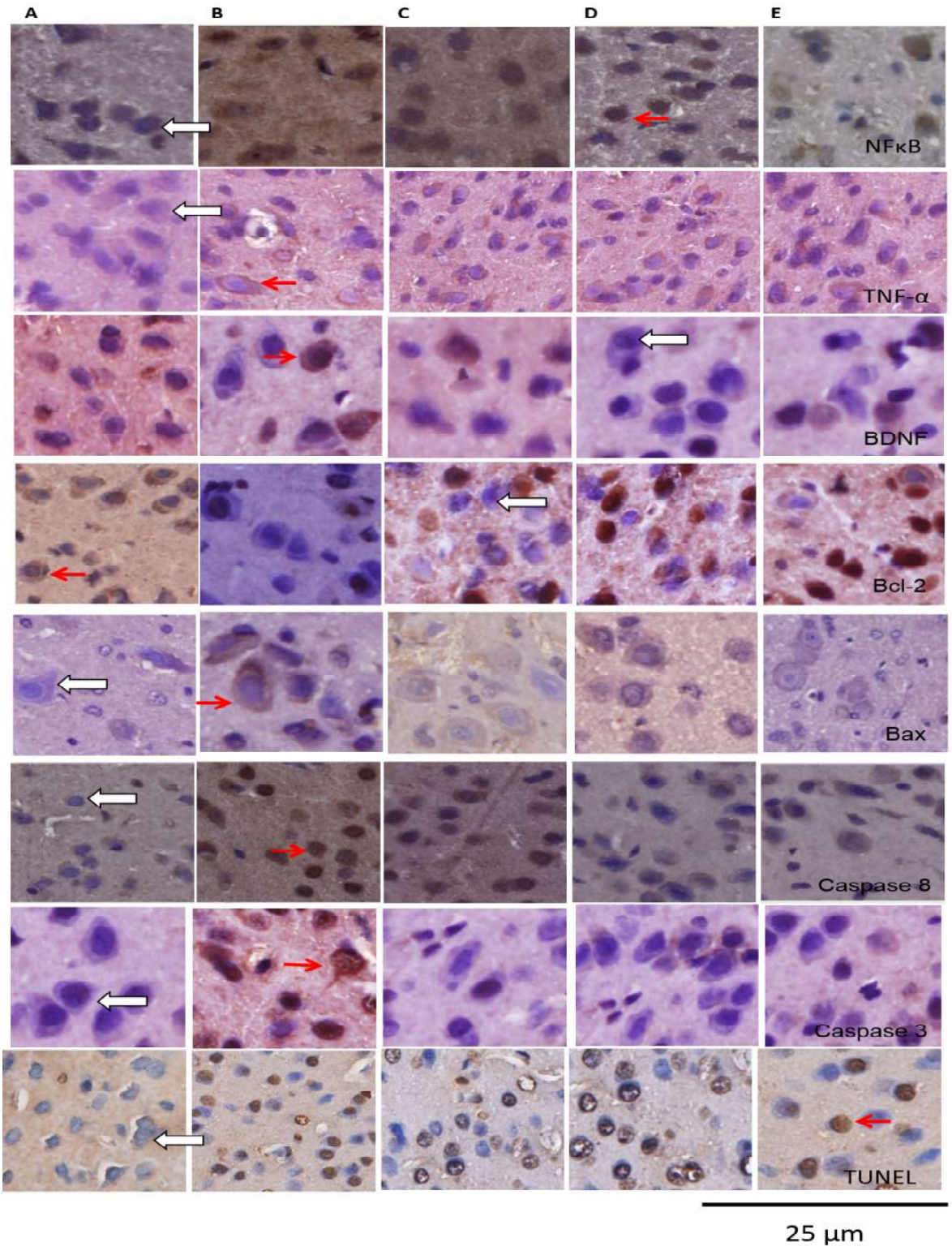

Figure 2. Expression NFκB, TNFα, Bcl-2, Bax, Caspase 3, Caspase 8, TUNEL, and BDNF on day 7 using immunohistochemical technique

(A) Negative control, (B) Positive control, (C) TBI+catechins 513 mg/kgBW/day, (D) TBI+catechins catechins 926 mg/kgBW/day, (E) TBI+catechins 1113 mg/kgBW/day. Red arrows point to neurons with positive expression (TNFα, Bcl-2, Bax, caspase 3, caspase 8, and BDNF, stained brown in the cytoplasm; TUNEL apoptosis stained brown in cell nucleus ; NFκB stained brown in nucleus and cytoplasm). White arrows point to neurons with negative expression

and 1113 mg/kgBW (p=0.034) as compared with the positive controls. On day 7, there also was a significant difference in NSS between the intervention groups (p=0.002) (Table 1). The results of the Mann Whitney test showed a significant decrease in NSS in the groups receiving catechins at 513 mg/kgBW (p=0.017), 926 mg/kgBW (p=0.017) and 1113 mg/kgBW (p=0.017), respectively, as compared with the positive controls.

DISCUSSION

Expression of total NFκB p65 and TNFα of the third day was higher in the TBI group than the negative control. On the seventh day, there was still an increase in TNFα and total NFκB p65 expression even though it was not as high as on the third day. There was a significant decreased total expression of NFκB p65 in all three groups which was received catechins on the third day compared with positive control. There was significantly decrease of TNFα against positive control only in the group which was treated with catechins 926 and 1113 mg/kgBW. On the seventh day, administration of catechins at all studied doses, significantly decreased total NFκB p65 and TNFα expression compared with positive controls. The highest decreased in NFκB p65 and TNFα occurred at doses 1113 mg/kgBW, both on the third and seventh days. Decreased expression of NFκB and TNFα in these studies showed anti-inflammatory activity of catechins on the model of head injury.[4] NFκB was a modulator of inflammation which induced TNFα through an increase in TNF receptor 1 and 2.[24] Decreased of TNFα after catechins administration in accordance with the results of previous studies which was shown the decreased of TNFα in spinal cord injury after EGCG administration, which is the largest component of catechins.[15] In the previous study, it was found decreased expression of NFκB p65 nucleus in injured spinal cord after administration of catechins.[25] In this study, there were decreased in the total expression of NFκB p65, which was not specific in the nucleus only. Activation of NFκB occured because of the free radicals which is produced as a result of TBI.[16] The administration of epicatechin (one of the flavonoids contained in catechins) also can inhibit an increase in NFκB and TNFα on neurotoxicity due to doxorubicin.[13] There was no significant difference of TNFα between giving catechins dose of 513 mg/kgBW and positive control in this study shows the effect of catechins can be different at different doses.[14] Therefore, it was necessary to test the most optimal dose to decrease inflammation in the next study. Based on this study, administration of third day catechins on TBI was shown to decrease inflammation through decreased total expression of NFκB p65 and TNFα. The effects of decreased inflammation can still be found when administration of catechins is continued for up to 7 days.

In this study, TUNEL positive apoptotic cell counts was higher in the TBI group than negative control group. The number of apoptotic cells in rat brain on the third day injury was higher than on the seventh day. On the third day, a significant decrease in the number of apoptotic neurons occurred only in groups of rat which were received catechins 1113 mg/kgBW. On the seventh day, there was a significant decrease in the number of apoptotic neurons in all three doses of catechins observed with the highest decreased at doses of 1113 mg/kgBW. It showed that the effect of decreasing by the catechins depends on the dose administered.[14] These results were supported by previous studies where administration of epicatechin for 3 and 7 days in mice with TBI decreased the number of apoptotic cells, as determined by TUNEL assay.[26] These results also support previous study that the peak of apoptosis in TBI occured on the third day, while apoptosis on the seventh day has begun to decline.[16]

The expression of caspase 8 and caspase 3 in rat TBI model were higher as compared with negative control upon the observations of the third and seventh days. On the seventh day, caspase 8 and caspase 3 expression were lower than on the third day but still higher than negative

control. Significant reduction of caspase 8 and caspase 3 than the positive control by administration on the third dose of catechins observed, both in the group which was given catechins for 3 days until 7 days. The most decreased in caspase 8 occurred at a dose of 1113 mg/kgBW. A significant decreased in caspase 3 versus positive control did not differ on the three doses observed. These results were consistent with previous studies which administration of EGCG shown can decrease caspase 8 and caspase 3 in neurotoxic brain due to bupivacaine.[27] Decreased in caspase 3 by administration of EGCG also shown in the rat model of spinal cord injury.[15] According to the theory, decreased of caspase 8 occured due to decreased of TNFα. TNFα forms complex with Fas-Associated Death Domain (FADD), thus releasing TNFR-1. TNFR-1 activates caspase 8, which then activates caspase 3. Furthermore, caspase 3 translocate to the nucleus to initiate apoptosis.[3] After TBI, caspase 3 begin to decline after 72 hours.[16] Activation of caspase 8 occurs in the extrinsic apoptotic pathway due to inflammation, while caspase 3 activation may be affected by intrinsic and extrinsic pathways. Decreased caspase also influenced apoptotic intrinsic pathway arranged by family of Bcl-2, such as Bcl-2/Bax ratio.[3] Decreased of caspase 8 by administration of catechins for three days indicated the role of catechins on inhibition of protein markers of apoptosis in the extrinsic pathway. The inhibitory effect of extrinsic apoptotic pathways could still be seen if catechins was continued for up to 7 days. The differences in the effect of catechins dose on the studied dose of caspase 3 and caspase 8 were due to the effect of catechins may differ at different doses.

In this study, there was decreased in the Bcl-2/Bax ratios of TBI rats group compared to negative control on the third and seventh days. With catechins administration, there was significant increase in the Bcl-2/Bax ratio compared with positive control on the third day. There was no significant increased in the Bcl-2/Bax ratio of TBI rat which administered catechins compared to positive control on the seventh day. Decreased in apoptotic of this study might be influenced by an increase in the Bcl-2/Bax ratio on the third day. These results support the previous study that an increase in the Bcl-2/Bax ratio may decrease apoptosis.[6] From another study it was found that the decrease of the Bcl-2/Bax ratio mainly occurred in the first 72 hours after TBI.[16] In this study, increased in the Bcl-2/Bax ratio was more influenced by a decrease of Bax expression in the group which was received catechins. There was no significant change in Bcl-2 expression with catechins (Table 1). Rat models of TBI with Bax deletions have decreased caspase 3 expression and number of apoptotic neurons compared with normal rat in TBI.[3,28] These results differ from previous studies in rat models of spinal cord injury. In addition to the decrease in Bax, there was also an increase in Bcl-2 with the administration of green tea extract in rat models of spinal cord injury.[15] The difference in these results may be due to the dose of catechins and the different location of neuron that was observed.

In this study, there was decreased in BDNF of TBI rats compared to negative control. Administration of catechins could significantly increase BDNF, either on the third day or on the seventh day after injury. There was no significant difference in BDNF expression in all three doses of catechins observed. This is in accordance with previous studies in rat models of spinal cord trauma. Administration of green tea extract may increase BDNF and decrease apoptosis in mice model of spinal cord trauma.[8] Based on previous theories, it is recognized that BDNF can support cell resistance through inhibition of proapoptotic proteins and modulation of anti-apoptotic protein. Increased BDNF may also decrease inflammation through decreased TNFα, decrease excess intracellular calcium, and inhibit apoptosis by increasing Bcl-2 protein.[8]

In this TBI model, mild brain injury (NSS<5) according to the first hour of NSS examination (data not shown). On the third day,

there was no difference in NSS in control group positive with mice received catechins 926 mg/kgBW. However, there was a significant decrease of NSS in administration of catechins dose 513 and 1113 mg/kgBW compared to positive control. On the seventh day, a significant decrease in NSS occurred in all three groups which were administered catechins versus positive controls. The group that received catechins, had a seventh day NSS lower than on the third day. Decreased of NSS on the third day was in accordance with the previous study. Based on other studies of TBI, decreased in NSS was noticeable on day 3.[22] In accordance with other studies, NSS can still decline on the seventh day.[19] In addition to being influenced by inflammation and apoptosis, clinical improvement was also influenced by different genetic susceptibilities in each individual.[5] In this study catechins was shown to decrease NSS of the TBI rat on the third day, and will be continued on the seventh day.

Based on this study, there was a evidence that weight-drop TBI model resulting apoptosis through intrinsic pathways and extrinsic pathways. In theory, the increased expression of Bcl-2/Bax ratio and caspase 8 led to increased caspase 3 expression and result in apoptosis. Administered of catechins which contained EGCG, epicatechin, catechin and epigallocatechins was proven synergy in improving clinical output of rat.

A limitation of the present study was that the investigators did not prepare sections of the brain itself, so that there is the possibility that the selected tissue sampling site was not what had been expected. In addition, the staining of Bcl-2 and Bax which was presented as a ratio, was not done by double-staining, which may have introduced bias because the counting was not performed in the same fields of view. Limitations of this study also in the calculation of total NFκB p65 expression that was not specific to the nucleus, so we can not evaluate the effect of catechins toward active NFκB. Before catechins can be recommended for treatment of patients, a more detailed study needs to be conducted on their bioavailability in plasma and the brain, on the dose-response effect, the safety of the medication, its tolerability, efficacy, and the possible interactions with other medications according to the specific disease.

CONCLUSION

Administration of catechins in head injury was proven to have anti-inflammatory effects, to decrease apoptosis, increase neurotropic factors, and improve clinical outcomes. The apoptotic process in the intrinsic pathway was proven to be inhibited, particularly during third day, but the inhibition of inflammation still continued until seventh day. The clinical impact of catechins is that they have potential for the treatment of TBI in the foreseeable future.

CONFLICT OF INTEREST

There is no conflict of interest in this study. The funder did not intervene in the conduct of the study or the publication of this article.

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CONTRIBUTORS

RR, ANA, AN, SDU, and MAH contributed to drafting the manuscript, conception and design

of the study. ANA, AN, SDU, and MAH contributed to collection, analysis and interpretation of the data. RR, MR, SBA, HP, and MD were responsible for revising the manuscript critically for important intellectual content and the final content. All authors read and approved the final manuscript.

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