ORIGINAL ARTICLE

The CAPE will Influence the Angiogenesis in Traumatic Brain Injury

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ABSTRACT

INTRODUCTION: Traumatic brain injury (TBI) is a frequent and highly heterogeneous neurological disorder which has the potential to cause major social and economic consequences. However, Caffeic Acid Phenethyl Ester (CAPE), obtained from propolis through extraction from honey bees, has long been known as a folk medicine. This study identifies the predictor factors for angiogenesis in a TBI rat model following the provision of CAPE. **MATERIALS AND METHODS**: This experimental control treatment and randomization study used fifteen male Sprague-Dawley rats with surgically induced brain injury. The rats were treated with CAPE. We measured vascular endothelial growth factor (VEGF) levels as an indicator and Myeloperoxidase (MPO) as a polymorphonuclear activity marker. The gauges for brain edema and oxidative stress were mRNA, AQP4, and F2 Isoprostane, respectively. The correlation test of TBI parameters in the form of MPO, mRNA AQP4, and F2 isoprostane against VEGF as an angiogenesis process indicator was performed to identify the factors associated with post-TBI angiogenesis. **RESULTS:** Mean values were obtained using a descriptive tests, while the correlation test results were VEGF (938274.352), AQP4 mRNA (10099.00), MPO (9284222.028), and F2-Isoprostane (307346.562). The findings suggested a strong correlation between all TBI parameters and VEGF as an angiogenesis indicator ($p<0.001$). In addition, polymorphonuclear activity (MPO) and the presence of brain edema, as indicated by mRNA AQP4 expression, were identified as the most significant influences. **CONCLUSION:** The post-TBI angiogenesis (VEGF) process, conducted by administering CAPE, was influenced by polymorphonuclear activity (MPO) and increased water content in the brain (mRNA AQP4).

Keywords
Traumatic brain injury; Angiogenesis; Caffeic Acid Phenethyl Ester; Predictors.

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INTRODUCTION

Angiogenesis is the growth of new blood vessels from existing ones. This process is initiated by the secretion of endothelial cell proteases, followed by cellular movement to the place of formation.[1,2] Therefore, proliferation and differentiation occur to form a new lumen. The secretion of growth factors by the endothelial cells attracts supporting cells, including the pericytes and smooth muscles, thus forming a basement membrane.[3] These components ensure function and stability. The final stage involves specific development according to the tissue or organ being supplied.[1–5]

Vascular endothelial growth factor (VEGF) is an indicator of blood vessel permeability and functions as a regulator for angiogenesis.[6] This triggers cerebral edema through the synthesis and release of NO, as a GMP-dependent cyclic activator.[5,6] In addition, VEGF phosphorylates occludin is one of the VEGF metabolites

capable of interfering with occludin function, instigating the opening of tight junctions and edema formation.[5–7]

Myeloperoxidase (MPO) is an enzyme secreted by neutrophils and active macrophages or microglia.[8,9] Higher amounts of the enzyme have been implicated in impaired endothelial function, as well as increased nitrite oxide formation and damage to active lipoproteins.[8,10] It is also known to trigger the development of ROS, which initiates ischemia processes.[10,11]

Aquaporin (AQP) is a protein compound that is essential for water transport across the plasma membrane. It is widely expressed from tissues, including the renal epithelium and erythrocytes, to various cells of the central nervous system. In addition, the main aquaporin molecules comprised of AQP1 and AQP4 play a role in regulating brain fluid and CSF movement. These also contribute to cytotoxic and vasogenic edema, which are implicated in the control of intracellular and extracellular fluid volume.[12,13] Specifically, AQP4 is a dysfunction center in glutamate metabolism, synaptogenesis, and memory consolidation. The expression is essential for cellular migration and angiogenesis in tumor growth.[12–15]

Caffeic Acid Phenethyl Ester (CAPE) therapy is obtained from propolis, which has an extensive history as a folk medicine.[16] The drug structure comprises a known powerful anti-oxidant, known as catechol. Furthermore, CAPE has been shown to decrease inflammatory initiation, brain lipid preoxidation, and free radical damage.[17] Previous reports have demonstrated the effect on xanthine/xanthine oxidase, nuclear factor-kB (NF kB), cyclooxygenase 2 (COX-2), 5-li-poxygenase (5-LOX), alongside the production of inflammatory cytokines and the release of cytochrome c from mitochondria.[16–18]

This study examines the relationship between the process of post-trauma angiogenesis in the brain, as characterized by the expression of VEGF with AQP4 mRNA and Myeloperoxidase (MPO) levels in the blood after the provision of CAPE.

MATERIALS AND METHODS

Animal

Fifteen male Sprague-Dawley rats weighing 200–300g were used in this study. All animal procedures received approval from the Health Research Ethical Committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, number: 771/UN4.6.4.5.31/ PP36/2019.

Examination of the mRNA AQP4 gene expression and the levels of MPO, VEGF, and F2-IsoProstane

The mRNA expression of the AQP4 gene was examined by extracting RNA in alignment with the guanidium thiocyanate method.[19–22] 100 µl of fresh blood was mixed with L6 buffer containing guanidium thiocyanate. A quantitative real-time PCR was performed with HPRT as a housekeeping gene (internal control). In addition, the AQP4 gene was the Sequence Nucleotide used, with the primary sequence of sense 5-CCA-CTG-GA T-ATA-TTG-GGT-TGG-A-3; antisense 5-CCA-CGT-CAG-GAC-AGA-AGA-CAT-A-3. However, the primer sequences included HPRT sense 5-GGA-CCT-CTC-GAA-GTG-TTG-GAT-3 and HPRT antisense 5-CCA-ACA-ACA-AAC-TTG-TCT-GGA-A-3. The PCR cycle was initiated at 94^{o}C for 5 minutes, which was sustained for another 20 seconds, followed by a decline and maintenance at 54^{o}C for 30 seconds. This rotation was repeated 25 times. The procedure was carried out according to the Tomomi Yajima protocol, and the oligonucleotide primers used were obtained from Macrogen Laboratory, Korea, with NO. DG 190726.

A total of 12.5 µl from 2 x SYBR Green QRT-PCR master mix was added to 10 µl of the initial primer (concentration optimized), Nuclease - PCR free - H2 level x µl of final primer (concentration optimized) as well as 0.375 µl of the reference dye solution collected from step 1 (optional), and 1.0 µl of the RT/Rnase enzyme block mixture with 25 µl of total reaction volume. This combination was mixed slowly (not rotated) to avoid bubble formation. Then, the mixture was distributed to test tubes containing 10 µl of experimental RNA. The reaction was briefly centrifuged and placed in the

instrument before running the PCR program using a Real -Time PCR machine (CFX Connect system, Biorad Laboratories, Real-Time PCR 96 well 0.1 ml, USA).[23–26]

Meanwhile, the other 3 parameters were evaluated using the sandwich ELISA method, Myeloperoxidase (MPO) Catalog No. Ls-F24875, VEGF Catalog No. Ls-F978 was purchased from Life Span BioSciences, Inc., while the 8-iso-PGF2 (8-isoprostane) Catalog No. MBS7606827 was obtained from MyBioSource Inc.

CAPE provision

CAPE powder was diluted with saline solution and administered through intraperitoneal injection (IP) 30 minutes after trauma. This required a dose of 10mg/kg,[8] repeated daily for 7 days.

Preparation for surgery

Five virus-free male Sprague-Dawley rats were used in the study. These rats, weighing 280–300 g, were adapted and reared for approximately 2 months, with unrestricted access to food and drink. The trauma model was performed according to the marmourou journal model for developing countries with modification to Nasution et al..[26,27]

Statistical analysis

Basic data were presented in terms of mean ± SD. The angiogenesis predictor factors in the experimental animals after administering CAPE were evaluated by performing a correlation test on the initial analysis, using the Pearson correlation, resulting in normally distributed data. Furthermore, multivariate linear regression analysis was conducted for all examined parameters in instances where p-value was statistically significant at <0.05. All data were processed and analyzed using Excel 2013 and SPSS version 23 software (Armonk, NY: IBM Corp.).

RESULTS

All rats survived after trauma up to a predetermined experimental time point.

Table I: Average TBI parameters after CAPE provision

Parameter	Mean	Standard Deviation
VEGF	938274.352	96049.339
mRNA AQP4	10099.00	531.490
MPO	9284222.028	953098.093
F2 isoprostane	307346.562	31119.798

The average weight of Sprague-Dawley rats in this experiment was 290.07 grams. Table I shows each TBI parameter tested by descriptive analysis to determine the average following the administration of CAPE.

Table II: Correlation test results between VEGF and parameters mRNA AQP4, MPO, and F2 isoprostane

Variable		R value			
		mRNA	sAQP4	sMPO	F2iso
VEGF	Pearson Correlation	0.957	0.910	0.957	0.917
	Sig. (2-tailed)	<0.001*	<0.001*	<0.001*	<0.001*

Note: * p <0.05 is significant

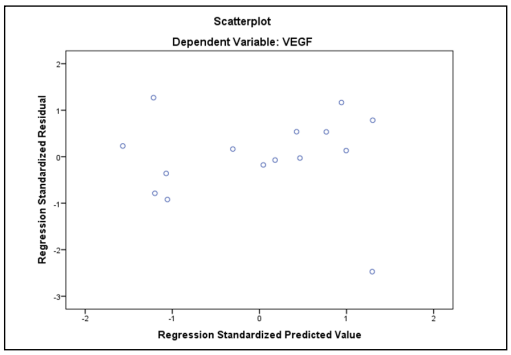

Figure 1. VEGF variable scatterplot graph with independent variable parameters after TBI.

Table II shows the Pearson correlation analysis. A strong correlation was observed between all parameters and VEGF (range r=0.910–0.957), with a significance value of (p<0.05). Figure 1 is a scatter plot graph demonstrating the homogeneity of the residual variant. The non-specific pattern prompts the need to perform multivariate analysis.

The correlation results are followed by multivariate linear regression analysis to determine the most influential parameters of angiogenesis. Statistical analysis showed a 95% contribution of AQP4 and MPO mRNA post-trauma in the brain after the administration of CAPE (Table III).

Table III: VEGF multivariate analysis with MPO, F2 isoprostane, and AQP4 parameters

Model		Unstandardized Coefficients		Standardized Coefficients	T	p-value
		B	Std. Error	Beta		
1	(Constant)	-504601.221	161188.755		-3.130	.011
	mRNA	99.960	33.668	.575	2.969	.014
	sAQP4	-.798	.603	-.252	-1.324	.215
	MPO	.041	.015	.395	2.770	.020
	F2iso	.952	.431	.298	2.211	.051
2	(Constant)	-361525.329	123653.703		-2.924	.014
	mRNA	69.528	25.436	.400	2.733	.019
	MPO	.042	.015	.405	2.755	.019
	F2iso	.684	.393	.214	1.741	.110
3	(Constant)	-422825.615	128174.539		-3.299	.006
	mRNA	86.192	25.483	.496	3.382	.005
	MPO	.053	.015	.504	3.441	.005

[a] VEGF dependent variable
[b] Predictor in the model: (Constant) F2isoprostane, mRNA AQP4 and MPO
[c] Predictor in the model: (Constant) mRNA AQP4 and MPO

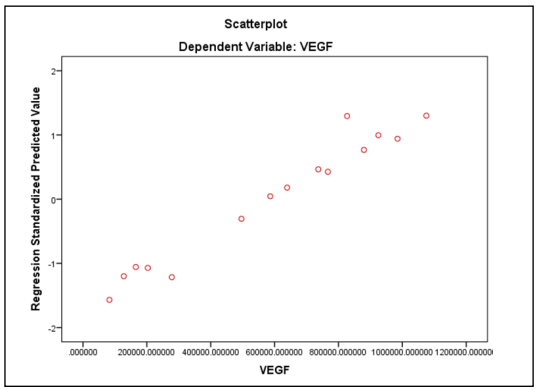

Figure 2. VEGF variable scatterplot graph with independent variable of MPO, AQP4 mRNA

The scatterplot graph in Figure 2 illustrates a correlation between VEGF as a dependent variable and the independent linear variable.

DISCUSSION

The effect of vascular endothelial growth factor (VEGF) against angiogenesis occurs normally or pathologically.[28] This molecule binds to endothelial cells through an interaction with high-affinity tyrosine kinase receptor flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR2), produced predominantly in SDO endothelial cells.[29,30] In addition, VEGF has strong vascular permeability activity (several thousand times superior to histamine) and a direct effect on the tight junction endothelial SDO.[29–31] This molecule, alongside platelet-derived growth factor (PDGF), is a potent angiogenic factor with activity as a proinflammatory cytokine, estimated to improve endothelial cell permeability and upregulate molecular adhesion.[32] In addition, VEGF binds to specific transmembrane stimuli, thus signalling a pathway for the proliferation and migration of endothelial cells. This further ensures the maintenance of the immature types and increases vascular permeability.[32,33]

VEGF is mitogenic, angiogenic, and a potential mediator of vascular permeability. It also serves as a potent activator of angiogenesis and neurogenesis. Previous studies have demonstrated the role of CAPE in reducing vascular permeability in the blood of rats subjected to brain injury, while angiogenesis continues by evaluating the clinical condition of survivors up to the termination of the study.[34,35]

A strong correlation was identified between VEGF and Myeloperoxidase as well as AQP4 mRNA. This is associated with the presence of Myeloperoxidase (MPO) as an enzyme secreted by neutrophils and active macrophages or microglia. Hence, ROS formation is triggered, followed by the incidence of ischemia and the consequent disruption of the sodium pump in the astrocytes. This phenomenon further instigates swelling and cerebral edema.[3] Interleukin-1 induces endothelium in the brain vascular system, thus releasing the VEGF.[36] This product is known to induce astrocytes and release aquaporin (AQP) -4, which stimulate the vascular wall to facilitate water discharge into the interstitial brain tissue, resulting in cerebral edema.[37] These findings are congruent with the statistical tests, where AQP4 and MPO mRNA demonstrated a 95% contribution towards angiogenesis in the brain, following the incidence of an injury.

CONCLUSION

This study established a relationship between the angiogenesis process in the brain post-trauma, marked by the VEGF expression, and AQP4 mRNA as well as blood Myeloperoxidase (MPO) levels, following the administration of CAPE. These three factors were shown to have a strong correlation because statistical tests using multivariate linear regression analysis indicated a 95% contribution to the angiogenesis process of blood vessels in the brain.

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