ORIGINAL ARTICLE

Snakehead fish extract as an enhancer of vascular endothelial growth factor and nitric oxide levels in cerebral angiogenesis: an insight of stroke therapy

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ABSTRACT

Aim To assess the effect of snakehead fish extract administration in angiogenesis focusing on the level of vascular endothelial growth factor (VEGF), nitric oxide (NO) and VEGF receptor 2 (R2) expression is ischemic stroke models.

Methods An experimental study was conducted on 5 groups of ischemic stroke rats models: Group K- without carotid artery ligation, Group K+ with artery ligation, Group P1 with artery ligation and administration of 200 mg/day extract, Group P2 with artery ligation and 400 mg/day extract, and Group P3 with artery ligation and 800 mg/day extract. The VEGF expression and NO levels were assessed on day 3.

Results Snakehead fish extract significantly increased VEGF levels along with increasing doses, in which the highest VEGF level was observed in P3 group (361.7±40.2; $p<0.001$). The NO level also increased along with an increasing dose of snakehead fish extract, in which the highest NO level was found in P3 group (59.43±0.88 μmol/gr; $p<0.001$). The VEGFR2 expression also increased significantly after snakehead extract administration along with increasing doses ($p<0.001$) in which administration of 800 mg extract yielded the highest VEGFR2 expression compared with lower doses (17.7 vs. 15.6; $p<0.001$)

Conclusion Snakehead fish extract administration increased angiogenesis process marked by an increased level of VEGF, NO and VEGFR2 expression in ischemic stroke rat models.

Key words: arginine, nitric oxide, cerebral ischemia

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INTRODUCTION

Various studies have raised the possibility of snakehead fish extract as a potential treatment in ischemic stroke (1,2). Snakehead fish extract is rich in arginine. Arginine acts as a coenzyme in the formation of nitric oxide (NO), an important mediator for vasodilation, from endothelial nitric oxide synthase (eNOS) (3-5). Several studies have also found that snakehead fish extract may increase vascular endothelial growth factor (VEGF), in which binding of VEGF to its receptor, VEGF-Receptor-2 (VEGFR2) is involved in an increase of proliferation of endothelial cells, and are expected to enhance angiogenesis in patients with ischemic stroke (6,7). However, studies focusing on the definite role of snakehead fish extract in cerebral angiogenesis are still very limited.

This study aimed to assess the effect of snakehead fish extraction administration in angiogenesis focusing on the level of VEGF, NO and VEGF receptor 2 (R2) expression is ischemic stroke models.

METHODS

Study design

This experimental study was conducted in Biomolecular Laboratory, Brawijaya University Malang-Indonesia from January – April 2019. The study was conducted on 5 groups of ischemic stroke rats models to assess the effect of snakehead fish extract administration in angiogenesis focusing on the level of vascular endothelial growth factor (VEGF), nitric oxide (NO) and VEGF receptor 2 (R2) expression is ischemic stroke models.

The experimental animals used were 25 Sprague - Dawley strain rats (*Rattus norvegicus* sp.) with body weight of 305 - 425 grams. Twenty five rats were divided randomly into 5 treatment groups, each group consisted of 5 rats: group K (-) was control group without carotid artery ligation, and without extract administration, group K (+) was control group with artery ligation, but without extract administration; groups P1, P2 and P3 all underwent artery ligation and were then treated with 200 mg, 400 mg and 800 mg/day, respectively, snakehead extract for three days.

All animal procedures were based on the Helsinki Declaration and approved by the Animal Research Ethics Commission, Universitas Sumatera Utara.

Methods

Stroke induction was done by the unilateral carotid artery occlusion method at the internal and external carotid arteries of rats on the left side (8,9). Distribution of microglia cells in occlusion area of the internal and external carotid arteries was assessed in all groups. Neurological deficits in rats were assessed within 24 hours after an ischemic event (10,11).

The dosage of snakehead fish extract (VipAlbumin, Royal Medicalink Pharmatab, Makassar, Indonesia) given was calculated based on animal equivalent dose (AED) formula (11). Snakehead fish extract 100% were given intraorally by inserting feeding tube daily. The focus of this research is the formation of vascular (histological) structures of brain tissue in rats ischemic stroke models after the treatment with Snakehead fish extract. The NO, VEGF and VEGFR2 levels were assessed on the third day after stroke, according to previous studies (9).

The VEGF and NO level measurement was done from rat plasma on the third day after stroke, e.g. after occlusion of the internal and external carotid arteries. Histological examination of brain tissue was done by routine staining technique of hematoxylin – eosin. The brain tissue of rat's ischemic stroke models in each group was processed for immunohistochemistry examination. This showed that there was an activation of the immunological process. Measurement of VEGFR2 expression was carried out on immune labelled tissue (Ab182981 anti-VEGFR2 antibody Flk-1 A-3: sc-6251, and ELISA kit LS-F542, LifeSpan-Bio Life Bioscience Inc, Seattle, USA), then viewed using a fluorescence microscope (Nikon E-100, with attachment SonyA7 Camera, Tokyo, Japan). Cells with increased area of vascular formation for each antibody were counted in six randomly selected fields from one optical field on microscope located along the border of the ischemic lesion. Two pieces of brain tissue specimens of each animal were counted in random order, and the average value was determined (7).

The VEGFR2 examination and calculation was observed by looking at the brown colour in the cytoplasm of cells, then calculated according to a formula by Soini et al. (13) and Pizem et al. (14). To guarantee representation and reduce the

errors, it was needed to observe approximately 20 fields with 1000x magnification, each containing approximately 1500 cells (12-14).

Statistical analysis

All data were analysed by investigators for which the experimental group was blinded. An analysis of increased levels of VEGF, NO and VEGFR2 expression was done by ANOVA test and post hoc comparison, in which $p<0.05$ was stated as significant (15).

RESULTS

The results showed that in the occlusion area of the internal and external carotid arteries, there was an increase of the number of microglia cells in the K (+) group. This showed that there was activation of the immunological process in the K (+) group (Figure 1).

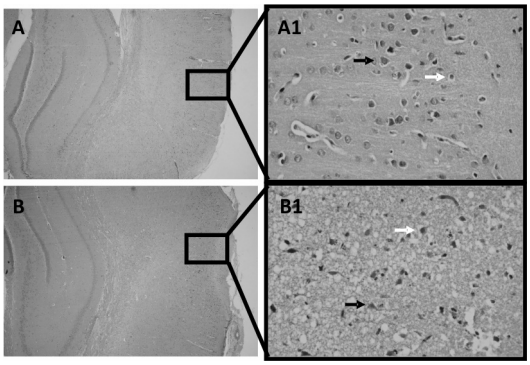

Figure 1. Histological picture of brain tissue due to occlusion of the internal and external carotid arteries (hematoxilen-eosin staining). A) Normal tissue - control, A1) 400x magnification normal tissue, B) Occlusion of the artery, B1) 400x magnification in the tissue with occlusion artery. White arrows indicated microglia cells and black arrows indicated neuron cells (Nikon E-100 microscope with magnification of 100x and 400x; photomicrographs were carried out with a Sony ICLE-A7 camera)

A significant increase was found in plasma VEGF level in rat groups treated with snakehead fish extract (P1, P2 and P3) compared to K (+) group (control group —without extract administration). The increase of VEGF occurred along with an increasing dose (Table 1). The level of VEGF in group P1 (200 mg extract administration), group P2 (400 mg) and group P3 (800 mg) was 182.0 ($p=0.28$), 267.4 ($p<0.001$) and 361.7 pg/mL ($p<0.001$).

The administration of snakehead fish extract produced also an increase in NO level, along with the dose increase (Table 1). The level of NO in group P3 was the highest (59.4 μM) compared with P2 (53.6 μM; $p<0.001$), P1 (51.9 μM; $p=0.001$) and K (+) group (control group without extract administration) group (40.2 μMq; $p<0.001$).

Table 1. Level of vascular endothelial growth factor (VEGF), nitric oxide (NO) and VEGF receptor 2 (R2) expression in each treatment group

Group*	VEGF (pg/mL)		NO (μM)		VEGFR-2 (No of cells/field)	
	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p
K (-)	202.1 ± 54.7		25.4 ± 2.54	Ref	7.0 ± 2.44	Ref
K (+)	128.0 ± 34.0	0.07	40.2 ± 3.01	<0.001	5.9 ± 2.3	0.96
P1	182.0 ± 28.7	0.28	51.9 ± 5.3	0.001	12.0 ± 2.47	0.01
P2	267.4 ± 47.3	<0.001	53.6 ± 4.75	<0.001	15.6 ± 2.79	<0.001
P3	361.7 ± 40.2	<0.001	59.4 ± 0.87	<0.001	17.7 ± 2.95	<0.001

*K (-),control group without carotid artery ligation, and without extract administration, K (+), control group with artery ligation, but without extract administration; P1, P2 and P3, treated with 200 mg, 400 mg and 800 mg/day, respectively, snakehead extract for three days

A significant increase of VEGFR2 expression (no of cells) in the groups P1 (12.0; $p=0.01$), P2 (15.6; $p<0.001$) and P3 (17.7; $p<0.001$) was noticed comparing to K+ group (control group without extract administration) (Table 1). The increase of VEGFR2 expression occurred along with the dose extract indicating that the vascularization process began after the administration of snakehead fish extract for 3 days of exposure (Figure 2).

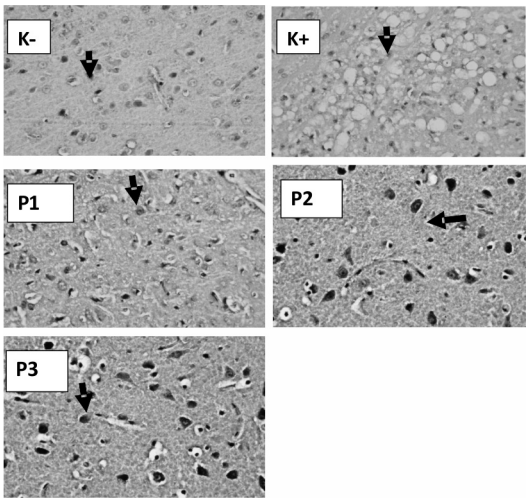

Figure 2. Histological representation of vascularization in the groups. K (-) control group without carotid artery ligation, and without extract administration, K (+), control group with artery ligation, but without extract administration, P1, treated with 200 mg of snakehead fish extract, P2, treated with 400 mg of snakehead fish extract, P3 treated with 800 mg of snakehead fish extract. Black arrows indicated vascular formation by VEGFR2 expression (Nikon E-100 microscope with magnification of 100x and 400x; photomicrographs were carried out with a Sony ICLE-A7 camera)

DISCUSSION

Angiogenesis is a process of vascular compensation or protection, which is also a target of stroke therapy (16,17). An immunohistochemistry analysis indicates angiogenesis begins to be active on day 2 to 7 after stroke attack (9).

Snakehead fish extract contained many important amino acids, particularly arginine. Arginine, which will be oxidized to citrulline, is a cofactor in the conversion of eNOS to NO. The NO has a protective effect by reducing platelet aggregation, and increases vasodilation, blood flow and regulates vascular tone (18-22). It in turn promotes blood flow and tissue oxygenation (23). A study by Zhang et al. showed that administered exogenous NO/donor NO significantly increased vascularization and proliferation of cerebral endothelial cells (24).

This study found VEGF level increased significantly with increasing doses of snakehead fish extract. Interestingly, VEGF levels in K (-) group (without carotid artery ligation) were higher compared with K (+) group (control group with artery ligation, but without extract administration). The decrease of VEGF level in the K (+) group probably occurred because it was still in the acute phase (3 days). Longer observation period is needed to obtain increased plasma VEGF levels.

The VEGF is very important for endothelial cell function associated with angiogenesis. The signal transduction initiated by VEGF/VEGFR2, leading to proliferation and formation of new blood vessels has multiple protective effects in regulating angiogenesis and neurogenesis (25-27).

The VEGF-VEGFR system is an important target for pro-angiogenic therapy (28). It is involved in the growth of new blood vessels, endothelial cell mitogenesis, vasodilatation (via the NO dependent pathway) and vascular permeability (29,30).

In hypoxic state, increased VEGF and VEGFR2 expression stimulates angiogenesis (30), proved by the presence of vascular formation area in brain tissue. This study found that after the administration of 200 mg dose of snakehead fish extract, there was a significant increase in VEGFR2 expression compared to control group, and the increase of VEGFR2 expression occurred along with dose extract increase.

After all, it can be concluded that administration of snakehead fish extract for 3 days after exposure to ischemic stroke accelerated the ongoing vascularization process.

This is by far the first study with histologically proven effect of administered snakehead fish extract to increase cerebral angiogenesis. However, it is important to understand that VEGF, NO and VEGFR2 expression are highly determined by the hypoxic condition. This study did not assess the levels of inducible-NOS (iNOS) that are known to play a role in stimulating NO expenditure. Knowing the iNOS and NO ratio will help to make clear that NO rise comes from arginine and not from iNOS.

The administration of snakehead fish extract can increase the level of VEGF, NO and VEGRF2 expression in cerebral angiogenesis process of ischemic stroke rat models.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

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