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Research Paper

Potential Mechanisms Involved in the Anticonvulsant Effect of Recombinant Human VEGF on Maximal Electroshock-induced Seizure

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ABSTRACT

Background: Vascular endothelial growth factor (VEGF) signaling pathway plays an important role in the pathogenesis of seizure. The oxidant/antioxidant factors and miRNA expression in the brain are differentially regulated in seizure.

Objectives: We aim to investigate the potential mechanism of action for the recombinant human VEGF (rhVEGF) in mice with maximal electroshock (MES)-induced seizure.

Materials & Methods: A total of 40 male mice (weight: 20-25 g) were treated intraperitoneally with normal saline, or rhVEGF (50, 100, and 150 µg/kg, daily for 4 consecutive days). One hour after the last injection, seizures were induced in each animal by MES. The latency for the onset of the first clonus and the duration of hind limb extension (HLE) were recorded. The levels of nitric oxide (NO), total antioxidant capacity (TAC), and micoRNA-142-5p expression were determined in the hippocampus of mice. Blood-brain barrier (BBB) permeability was also estimated by Evans blue dye extravasation method.

Results: The administration of rhVEGF at all doses significantly reduced the HLE duration. However, latency for the seizure onset increased after administration of 50 and 150 µg/kg rhVEGF and decreased after administration of 100 µg/kg rhVEGF. In the brain, the NO level decreased, while TAC level and microRNA-142-5p expression increased by rhVEGF treatment in mice with MES-induced seizure. Pretreatment with rhVEGF at doses of 100 and 150 µg/kg reversed the increase in BBB leakage induced by MES-induced seizures.

Conclusion: The rhVEGF administration can prevent MES-induced seizures by regulating NO, TAC, and miR-142-5 expression levels in the hippocampus and reducing BBB leakage in mice.

Keywords: Vascular endothelial growth factor, Generalized tonic-clonic seizure, Blood-brain Barrier, microRNAs, mouse, Oxidative stress

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Highlights

• Recombinant human vascular endothelial growth factor (VEGF) has dose-dependent proconvulsant and anticonvulsant effects on maximal electroshock-induced seizures.

• Recombinant human VEGF increases otal antioxidant capacity (TAC) and microRNA-142-5p expression in the brains of mice with seizures.

• Recombinant human VEGF prevents the seizure-induced increase in blood-brain barrier permeability and brain NO level.

Introduction

Generalized tonic-clonic seizure (GTCS) is one of the most prevalent neurological diseases in Iran, with a prevalence of 16.6 per 1000 people [1]. Hippocampal and extra-hippocampal pathologies contribute to GTCS [2, 3]. Vascular endothelial growth factor (VEGF) is a neuroprotective agent, and its administration may promote neuronal survival, neurogenesis, and cerebral angiogenesis [4, 5]. Its inhibitory effect on the firing rate of the action potentials of hippocampal neurons is mediated by modulation of the voltage-gated sodium channel [6]. The beneficial effects of VEGF on neurodegenerative and neuropathic conditions have been reported in people with neurological disorders such as trauma, seizures, and ischemia [7]. In an animal study, a decrease in the VEGF level caused a rapid and sustained change in the brain activity of rodents [8]. Also, VEGF can protect neuronal damage caused by seizures [9]. Moreover, VEGF has positive effects on neuroinflammation, brain vascular permeability, neurotransmitter release, synaptic function, reactive oxygen species (ROS)-induced cellular damage, and N-methyl-D-aspartate (NMDA) receptor function [10, 11]. In a study, recombinant human VEGF (rhVEGF) was used as an anti-cancer agent and showed a stimulatory effect on axonal growth and enhanced Schwann cell proliferation and survival [9, 12, 13]. Its administration can lead to a decline in glutamate excitotoxicity in the cultured hippocampal neurons [14]. Administration of rhVEGF may exert an anti-inflammatory action and improve neural survival in neuropathological conditions [15]. Therefore, rhVEGF seems to be an appropriate alternative drug for the prevention and treatment of seizures. Since rhVEGF can pass the blood-brain barrier (BBB), it is distributed throughout the brain [7]. Moreover, it affects neuronal activity in the central nervous system [16, 17]. Therefore, further investigation of its potential mechanism of action can be beneficial.

Several studies have demonstrated an increase in hippocampus nitric oxide (NO) level and BBB permeability in neurodegenerative diseases, including epilepsy [18-22]. Reduction in brain antioxidant capacity due to neuronal hyperexcitability plays an essential role in epileptogenesis and influences anti-epileptic drug therapy [23]. Furthermore, microRNA 142-5p is involved in epileptic behaviors [24, 25]. However, the manipulation of miR-142–5p attenuates pilocarpine-induced seizures in mice [26]. On the other hand, administration of rhVEGF reduces BBB permeability following focal cerebral embolic ischemia and accelerates neurological recovery in rats [27]. Therefore, targeting the above-mentioned mediators in the brain can exert both anticonvulsant and proconvulsant effects.

In this study, we hypothesize that administering rhVEGF can prevent GTCS by modulating several mechanisms. We aim to investigate whether rhVEGF has anticonvulsant effect or a proconvulsant effect. We also evaluate the effects of NO, total antioxidant capacity (TAC), and miR-142-5p expression in the hippocampus and alteration of the BBB permeability as rhVEGF's possible mechanisms of action, on maximal electroshock (MES)-induced seizure in mice.

Materials and Methods

In this study, rhVEGF was purchased from Zeist Pajoohan Mahbob Company (Tehran, Iran). The cDNA of mice was prepared according to the method reported in a previous study [28]. The L-NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as a non-specific inhibitor of NO synthase. Template DNA from clinical specimens was prepared using the high pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Twenty-four male Swiss albino mice weighing 25-30 g were used in the experimental work. Animals were kept in the Experimental Research Laboratory of Shahed University. Habituation occurred under laboratory conditions at 25˚C in seven days, with 12:12 h light-dark cycles. Moreover, the mice received a standard commercial diet and ad libitum water. Mice put in groups of seven in standard polypropylene cages and were given food and ad libitum water and maintained in a temperature-controlled (25±2˚C) room with a 12:12 day/night cycle. The mice were randomly divided into five groups: Control group without receiving MES (for molecular test), group 1 received 0.1 M phosphate-buffered saline (PBS) injection for 4 consecutive days followed by MES (for seizure tests), group 2 received intraperitoneal (i.p.) saline injection for four consecutive days followed by MES, and groups 3 to 5 received i.p. injection of rhVEGF at 50, 100 and 150 µg/kg/day, respectively, for four consecutive days followed by MES. The doses were determined based on a previous study [29].

An alternating current (0.2 seconds, 50 mA at 60 Hz) generated by an stimulator (Borj Sanat, Iran) was used to induce MES seizures. The current was applied by ear-clip electrodes moistened with normal saline [30]. The latency for the onset of the first clonus and duration of tonic hind limb extension (HLE) were recorded. Immediately after the MES, the mice were quickly decapitated, and their brains were removed from the skull and placed in an ice-cold normal saline solution. Brain tissue samples were homogenized in PBS (4˚C, pH=7.4). Then, samples were centrifuged at 4000×g for 10 min at 4˚C, and the supernatant was used for various biochemical assays. The TAC level in the homogenate was measured using the Naxifer™ TAC assay kit [31] purchased from Navand Salamat Company (Urmia, Iran). The NO level in the homogenate was measured by the Griess method.

Total RNA was extracted from brain tissues (1 cm^3) using a miRNA assay kit (AnaCell, Ana Cell Tec., Iran) according to the manufacturer's protocol. Reverse transcription of miR142-5p and cDNA was performed using miRNA cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and cDNA synthesis kit, respectively. Expression levels of miR-142-5p and a housekeeping gene control (*18S rRNA*) detected with a TaqMan miRNA assay kit (AnaCell extraction kit), were measured using a thermocycler (Applied Biosystems; Thermo Fisher Scientific Inc., USA), according to the manufacturer's protocol. Thermocycling conditions were as follows: 95˚C for 30 sec, followed by 35 cycles of 95˚C for 10 sec and 60˚C for 25 sec. Primers were as follows: miR-142-5p, forward 5'-AACTCCAGC TGGTCCTTAG-3', reverse 5'-TCTTGAACCCT-CATCCTG T-3'; and housekeeping gene forward 5'-GCTTCGGCAGCACATATACTA AAAT-3', reverse 5'-CGCTTCACGAATTTGCGT-3' [32].

A separate group of mice was tested for BBB permeability by using Evans blue dye extravasation method [33]. Mice were decapitated 4 h after MES-induced seizure according to previously published protocol with some modification, and their brains were removed, weighed, and homogenized in 1.0 mL of trichloroacetic acid (50% in pure water), and centrifuged at 10,000 rpm for 20 min. The absorbance of supernatants was read by a microplate reader (SpectraMax M5, Molecular Devices, USA) at 630 nm. The amount of Evans blue dye was quantified according to external standard curve of Evans blue dye concentration (25–2,000 ng/mL) in 50% TCA/ethanol (1:3), and expressed in µg/g of brain tissue.

Statistical comparisons were made using GraphPad Prism software, version 5. To determine the differences in seizure threshold, latency of seizures, and brain TAC and NO levels, one-way ANOVA followed by Tukey's post hoc test were used. The data were presented as Mean±SEM.

Results

As shown in Table 1, all animals exposed to MES had GTCS. The results showed that the treatment by rhVEGF (50 and 150 µg/kg, i.p.) for four days before MES application had no significant effect on seizure onset. However, treatment with rhVEGF at 100 µg/kg i.p was associated with significantly lower latency for the onset of first clonus. The HLE duration was significantly reduced following treatment with all doses of rhVEGF compared to the MES group ($P<0.01$).

Compared to the vehicle-treated control group, the NO level significantly increased in the hippocampus of mice after MES-induced seizures ($P<0.01$). Conversely, treatment with all doses of rhVEGF for four days significantly prevented from MES-induced increase of NO level in the hippocampus ($P<0.01$, Figure 1).

As shown in Figure 2, the TAC of the hippocampus in the MES-induced seizure group was lower (25.26± 0.3 µmol) compared to the control group (30.32±0.27 µmol). Compared to the MES group, rhVEGF treatment significantly increased the TAC in the brain in a dose-independent manner. The data indicate that treatment with 100 µg/kg dose of rhVEGF increased the TAC more than the 50 and 150 µg/kg doses of rhVEGF.

Table 1. The mean latency for the onset of first clonus and the HLE duration in different study groups (n=10 in each group)

Groups	Mean±SEM	
	Latency for the Onset of Seizure (s)	Duration of HLE (s)
Vehicle (PBS)	2.77±0.28	2.89±0.39
rhVEGF (50 µg/kg)	2.24±0.36	1.76±0.05** ##
rhVEGF (100 µg/kg)	1.9±0.38**	1.99±0.04** ##
rhVEGF (150 µg/kg)	2.49±0.07	1.65±0.01** ##

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**Significant compared to vehicle+MES group (P≤0.01), ##Significant compared to the rhVEGF+MES group (P≤0.01).

Note: Data was analyzed by one-way ANOVA followed by Dunnett's test.

To investigate the central anti-inflammatory mechanism of rhVEGF in the MES model, the miR-142-5p in the hippocampus, relative to a housekeeping gene expression, was measured by real-time quantitative PCR following MES-induced seizure in mice. The results showed a high expression level of miR-142-5p in the brain of MES-induced seizure group (Figure 3). Compared to the MES group, miR-142-5p expression increased significantly in the hippocampus of mice in all rhVEGF-treated groups (P<0.05). These results indicate that rhVEGF enhanced miRNA-142-5p expression in the hippocampus of MES-treated mice.

To investigate the neuroinflammation mechanism of rhVEGF in the MES model, we examined the BBB permeability. The reduction of the leakage showed the improvement of neuroinflammation, indicating the enhancement of BBB leakage following epileptiform activity in the MES group compared to the control group. Treatment with 100 and 150 µg/kg doses of rhVEGF reduced the seizure-induced increase in the BBB permeability (Figure 4).

Discussion

Our results suggested that rhVEGF may have both proconvulsant and anticonvulsant effects. Decreased myoclonic response was an convulsive response, while reduced HLE duration showed anticonvulsant effect. These results raise questions regarding rhVEGF's mechanisms of action. A possible explanation for the opposite effects of the same dose of rhVEGF is its dual effect on both presynaptic and postsynaptic mechanisms in the hippocampus [34]. In support of this hypothesis, a study showed that VEGF dependent mechanisms are likely to be the underlying mechanism for control of balance between glutamate and

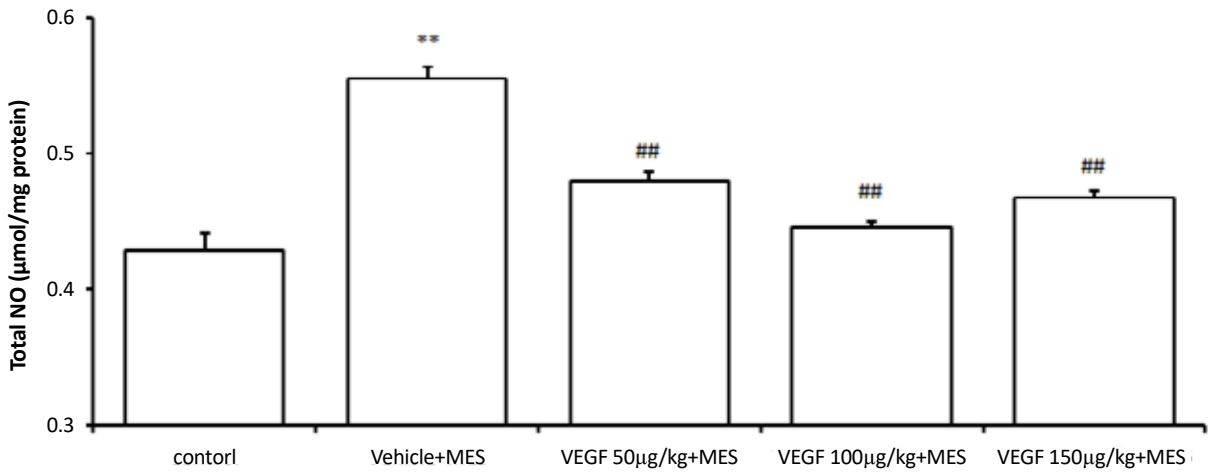

Figure 1. The total NO content (µmol/mg protein) in the hippocampus of mice in different study groups

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**Significant compared to the control group (P<0.01), ## Significant compared to the vehicle+MES group (P<0.01).

Note: Data were reported as Mean±SEM and analyzed using one-way ANOVA followed by Tukey's post hoc test.

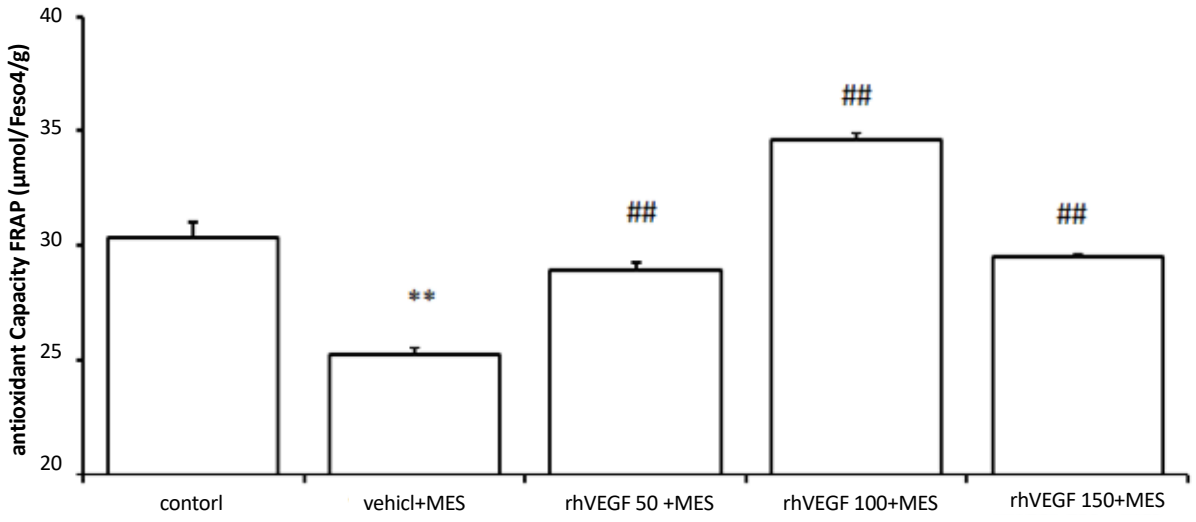

Figure 2. The TAC of hippocampus in the study groups estimated by ferric-reducing antioxidant power (FRAP)

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**Significant compared to the control group (P<0.01), ##Significant compared to the vehicle+MES group (P<0.01).

Note: Data are reported as Mean±SEM, data were analyzed using student's t-test.

γ-aminobutyric acid (GABA) in the brain [35]. Thus, the VEGF in the brain addresses a key challenge in neurobiology of determining how it induces or suppresses effects on neuronal activity to produce proconvulsant or anticonvulsant effects. In addition, molecular studies have shown that VEGF inhibits astrocytic calcium influx, which is a promising therapeutic approach for preventing seizures [36, 37]. On the other hand, it has been found that cytokines stimulate the production of VEGF, and this compound, in turn, increases seizure potential [9]. Investigation of the rhVEGF mechanisms identified the modulation of NO level, the increase of TAC and microRNA expression in the hippocam-

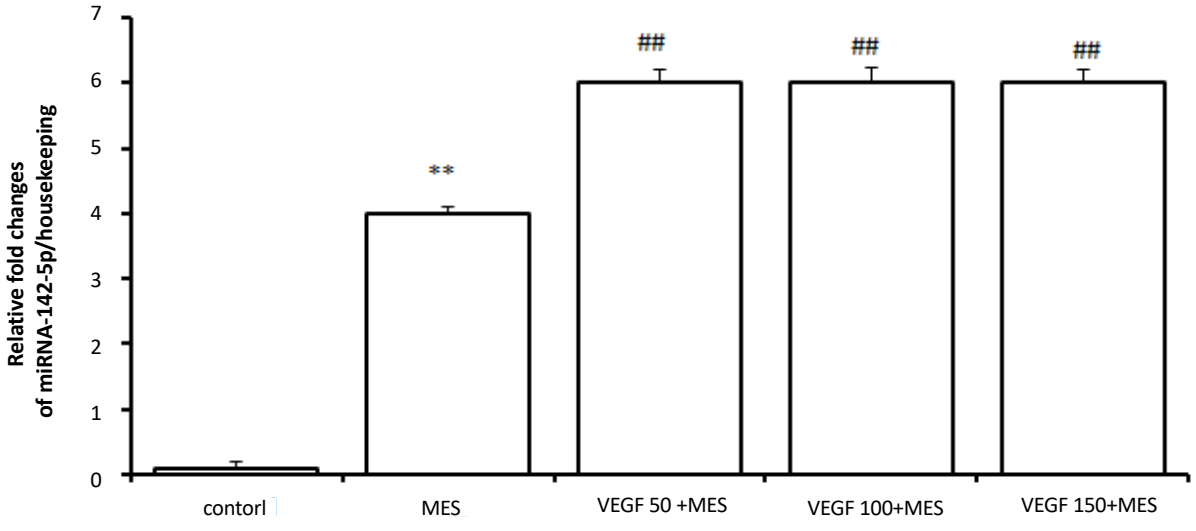

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Figure 3. Relative fold change of miRNA-142-5p expression in the hippocampus of mice in different study groups

**Significant compared to the control group (P<0.01), ##Significant compared to the vehicle+MES group (P<0.01) (student's t-test).

Note: Data are expressed as Mean±SEM, and analyzed using ANOVA followed by Tukey's post hoc test.

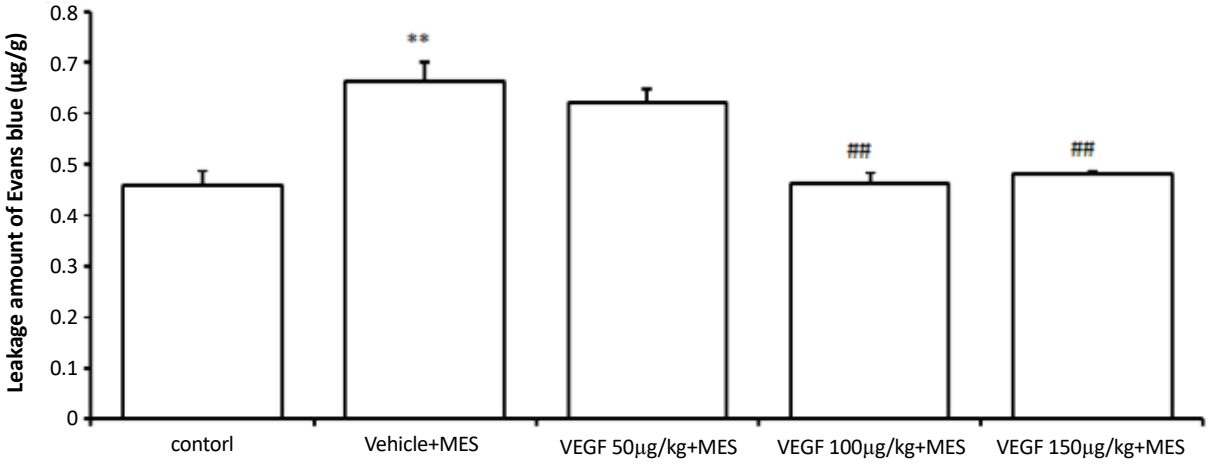

Figure 4. Leakage amount of Evans blue dye in the study groups

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**Significant compared to the control group (P<0.01), ##Significant compared to the vehicle+MES group.

Note: Measured in collected blood samples by spectrophotometer. Data are reported as Mean±SEM, data were analyzed using student's t-test.

pus and the regulation of BBB permeability as the factors that can cause the prevention of seizure development by rhVEGF. In line with this finding, a study suggested that miR-142-5p up-regulation is a natural neuroprotective mechanism against several neurodegenerative disorders [38]. Therefore, miRNA-142-5p may play a role in rhVEGF's neuroprotective effect against seizure-induced oxidative stress factors in the brain. As previously reported, VEGF modulates neuronal circuits and suppresses the epileptiform activity in the hippocampus of rats [35]. It has a regulatory effect on hippocampal neurogenesis and a reverse effect on epileptic seizure in murine [38]. Furthermore, the VEGF upregulation response following seizures has a protective role in hippocampal neurons [39]. Thau-Zuchman et al. also showed neuroprotective activity for rhVEGF against traumatic brain injury [40]. Moreover, the results of a study showed an increase in VEGF and its receptors in the temporal cortex of patients with drug-resistant temporal lobe epilepsy, such that it acted as a proconvulsant [41]. Additionally, VEGF is related to the increase in the postsynaptic responses mediated by the NMDA receptors in hippocampal neurons [11]. On the other hand, a study showed that a reduction in the expression of VEGF had an anticonvulsant effect on pentylentetrazole-induced acute seizures in mice [42]. Blocking the VEGF signaling pathway can reduce the amplitude and width of the action potential in dissociated hippocampal neurons [43]. Furthermore, VEGF was found to increase the lactate and can increase the excitability of neurons [44]. These studies suggest that rhVEGF may have a proconvulsant effect.

There are reports regarding the levels of brain NO and oxidative stress markers in epilepsy. Increased NO level and reduced antioxidant capacity can produce anti-epilepsy and may not be merely a consequence of seizures. Electroconvulsive seizures can stimulate the VEGF pathway [45]. According to Bussolati et al., the VEGF-stimulated NO release is inhibited by the blockade of VEGFR-1 [46]. Furthermore, it was shown that NO plays an important role in VEGF-induced changes in neuronal activity of the brain in mice [47]. Similar to another study [48], we showed the protective effects of rhVEGF on the regulation of BBB permeability. Moreover, several studies showed miRNA-142-5p role in neuronal hyperexcitability in epileptic seizures, oxidative stress, BBB dysfunction, and increased NO level in the brain [49-53]. Other subtype of VEGF, such as VEGF-B, has shown potent antioxidant property [54]. In addition, inhabitation of miRNA-142-5p expression was shown to has an antioxidant effect by reducing the ROS generation in the epileptic rats [26]. Therefore, the augmenting effect of rhVEGF on miRNA-142-5p expression may be part of its neuroprotective role [55, 56].

Conclusion

The rhVEGF has both proconvulsant and anticonvulsant effects against MES-induced seizures by modulating NO, TAC, and miRNA-142-5p expression and reducing BBB leakage. Our results provide empirical evidence that rhVEGF has a dose-dependent biphasic effect on MES-induced seizures.

Ethical Considerations

Compliance with ethical guidelines

All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Shahed University (Code: IR.SHAHED.REC.1401.058).

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Authors contributions

Conceptualization, methodology, investigation, and funding acquisition: All authors; Supervision, resources and writing: Majid Hassanpourezatti.

Conflict of interest

The authors declared no conflict of interest.

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