

# Differential Expression of Vascular Endothelial Growth Factor in the Cortex and Hippocampus upon Cerebral Hypoperfusion

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**Abstract.** *Background/Aim:* Vascular endothelial growth factor (VEGF) provides tolerance against ischemic brain injury, yet, the pattern of VEGF expression in the neurogenic zones following chronic cerebral hypoperfusion has not been studied. *Here we evaluated the immunoreactivity of VEGF in a rat model of chronic cerebral hypoperfusion. Materials and Methods:* Chronic hypoperfusion was induced by bilateral common carotid artery ligation in rats. Immunohistochemistry was performed against hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and VEGF on brain sections. *Results:* The density of HIF1 $\alpha$ -positive cells in the hypoxia group was increased in the cerebral cortex and hippocampus. Further, the density of VEGF-positive cells was significantly higher in the hypoxia group compared to the control group in the cerebral cortex whereas it was similar in the subventricular zone, and in the dentate gyrus in the hippocampus between the two groups. *Conclusion:* The pattern of VEGF expression varies in different brain regions following chronic cerebral hypoperfusion.

Abnormal cerebral circulation contributes to numerous neurological and psychiatric disorders. For example, disruption of the blood supply in a particular brain region can cause ischemic stroke and induce neuronal cell dysfunction, resulting in sudden neurological deficits (1).

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Moreover, chronic regional cerebral hypoperfusion may compromise the memory process. In particular, hypoperfusion in the temporal and parietal cortex has been related with Alzheimer's disease (AD) (2). Furthermore, global cerebral hypoperfusion can lead to a poor neurologic outcome in patients with heart issues, such as cardiac arrest or following complex cardiac surgery (3). Understanding the effect of cerebral hypoperfusion in cognitive dysfunction and dementia is important for the development of effective therapies. For this purpose, a number of animal models, such as the bilateral common carotid artery occlusion (BCCAO) model, have been introduced.

Angiogenesis describes the complex process of new blood vessel formation that implicates a number of vascular regulating factors (4). Chronic hypoperfusion triggers the repair brain capillary density *via* the hypoxia-inducible factor-1 (HIF-1) (5). In addition, the expression of angiopoietin-2 has been demonstrated to be up-regulated in sites of vascular remodeling following ischemic injury (6), whereas vascular endothelial growth factor (VEGF) induces angiogenesis by interacting with other angiogenesis-related factors (7). Interestingly, rats following bilateral common carotid artery occlusion (BCCAO) activate angiogenic processes in the CA1 region of the hippocampus by up-regulating VEGF expression (8). VEGF triggers angiogenesis and, in particular, VEGF-A a major regulator, member of the VEGF family, plays a critical role during brain development and disease by binding to VEGF receptor-2 (VEGFR-2) and stimulating angiogenesis (9, 10).

Ischemic stress has been shown to have a controversial effect on neuronal cells, either a harmful or a protective one (11). Many investigators have demonstrated the existence of protective mechanisms, referred to as ischemic preconditioning (12, 13). It has also been reported that certain transcription factors and growth factors, such as VEGF, provide tolerance against ischemic injury in the brain (14,

15). On the other hand, rodents with induced hypoxia present with neuronal loss and brain-derived neurotrophic factor (BCNF) down-regulation in the neurogenic zones, including the cortex, the subventricular zone and the dentate gyrus of the hippocampus (16-18). However, the pattern of VEGF immunoreactivity in the neurogenic zones following chronic cerebral hypoperfusion has not been investigated. Therefore, in the present study we sought to scrutinize the expression of VEGF in the neurogenic zones in a rat model of chronic cerebral hypoperfusion.

## Materials and Methods

**Animal surgery.** All animal procedures were performed according to the guidelines of the Chosun University Institutional Animal Care and Use Committee (approval no. CIACU2019-S0008). Sprague-Dawley rats were supplied by a certified breeder (Damul Laboratory animals, Daejeon, Republic of Korea). The rats were fed *ad libitum* and housed in a controlled environment. Six to seven-week-old male rats were anesthetized by inhalation of sevoflurane (1.0-2.0%, end-tidal concentration) and chronic hypoperfusion was induced by bilateral common carotid artery ligation, as described in previous studies (19, 20). Briefly, a midline incision was performed in the neck and both common carotid arteries were exposed and separated from the sheath and vagus nerve. Both arteries were permanently ligated using 4-0 silk sutures. Following the BCCAO surgery, the rats were housed separately and recovered in an aseptic room.

**Tissue preparation.** Three days following the BCCAO procedure, the rats were euthanized by cardiac perfusion fixation with 4% paraformaldehyde. The brain hemispheres were separated from the cerebellum and brain stem and were post-fixed in the same fixative. After 3 days, the specimens were washed for 16 hours with tap water and were subjected to dehydration by immersion in a series of ethanol solutions of incrementing concentrations. Subsequently, the brains were embedded in paraffin. Serial coronal sections of 6- $\mu$ m thickness were collected on gelatin-coated slides (Fisher Scientific, Hampton, NH, USA).

**Immunohistochemistry.** The slides were deparaffinized and washed with 0.1 M phosphate buffered saline (PBS, pH 7.4). An antigen-retrieval step was performed by heating the slides in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven and cooling them down for 1 hour. The deparaffinized slides were subjected to an endogenous peroxidase blocking process using 0.3% hydrogen peroxide solution for 20 min. They were then incubated with one of the following primary antibodies for 16 hours at 4°C: i) rabbit anti-HIF1 $\alpha$  (1:500, Abcam, Cambridge, UK) and ii) mouse monoclonal anti-VEGF (1:50, Santa Cruz Biotechnology, TX, USA). On the next day, the slides were washed in PBS several times and treated with the appropriate secondary antibodies which were the contents of VECTASTAIN® ABC HRP Kit (Vector Laboratories, Burlingame, CA, USA). The immunoreactivity was visualized using the avidin-biotin-peroxidase detection system (Vectastain ABC Elite Kit, Vector Laboratories) and the chromogen 3,3'-diaminobenzidine. Counterstaining was performed using thionin solution and the samples were mounted using the PolyMount mounting medium (Polysciences, Warrington, PA, USA).

**Quantification.** Immunopositive cells were observed using a light microscope (Olympus BX41, USA) connected to a digital CCD camera. Three investigators measured the densities of HIF1 $\alpha$ - and VEGF-positive cells (cells/mm<sup>2</sup>) using the Image-Pro Plus 7.0 image analysis software (Media Cybernetics Inc., Rockville, MD, USA). The density of positive cells was quantified within 5 randomly chosen fields, as described in a previous study (17, 21). BCCAO rats were defined as the hypoxia group (n=12) whereas not operated rats were defined as the control group (n=12).

**Statistical analysis.** The measurements collected from the hypoxic and control groups were compared using the Student's *t*-test in the Statistical Package for Social Sciences (Information Analysis Systems, SPSS, USA). The level of statistical significance was set at *p*<0.05.

## Results

**HIF1 $\alpha$  expression.** The density of HIF1 $\alpha$ -positive cells in the cerebral cortex increased in the hypoxia group as compared to the control group (Figure 1). Similarly, in the hippocampus the density of the HIF1 $\alpha$ -positive cells was higher in the hypoxia group compared to the control group (Figure 2).

**VEGF expression.** VEGF was strongly expressed in neurons of the cortex and the hippocampus whereas its expression was weaker in the subventricular zone. Notably, in the cerebral cortex, the density of VEGF-positive cells was significantly higher in the hypoxia group compared to the control group (Figure 3). However, the density of VEGF-positive cells was not significantly different in the subventricular zone between the hypoxia and control groups (Figure 4). In a similar manner, in the hippocampus and specifically in the granular (Figure 5) and subgranular (Figure 6) zones of the dentate gyrus, the densities of VEGF-positive cells did not differ significantly between the control and hypoxia groups.

## Discussion

Chronic hypoperfusion induced by BCCAO can lead to white matter and neuronal damage and may result to memory impairment (22, 23). In a previous study it was demonstrated that local cerebral blood flow can decrease to 33-58% of control levels in the cortex, only 2 days following BCCAO (24). To normalize the cerebral blood flow, compensatory mechanisms take part. In particular, angiogenic factors are activated to generate new microcapillaries (25), while vascular remodeling and arteriogenesis are also turned on (26).

Here, we evaluated the expression of HIF1 $\alpha$  and VEGF in 4 brain regions: i) the subventricular zone, ii) the cerebral cortex, iii) the subgranular and iv) the granular zone of the dentate gyrus in the hippocampus. The subventricular and subgranular zones are well-known niches of neurogenesis. Under hypoxic conditions, neurogenesis has also been observed in the cerebral cortex (27). The progenitor cells of

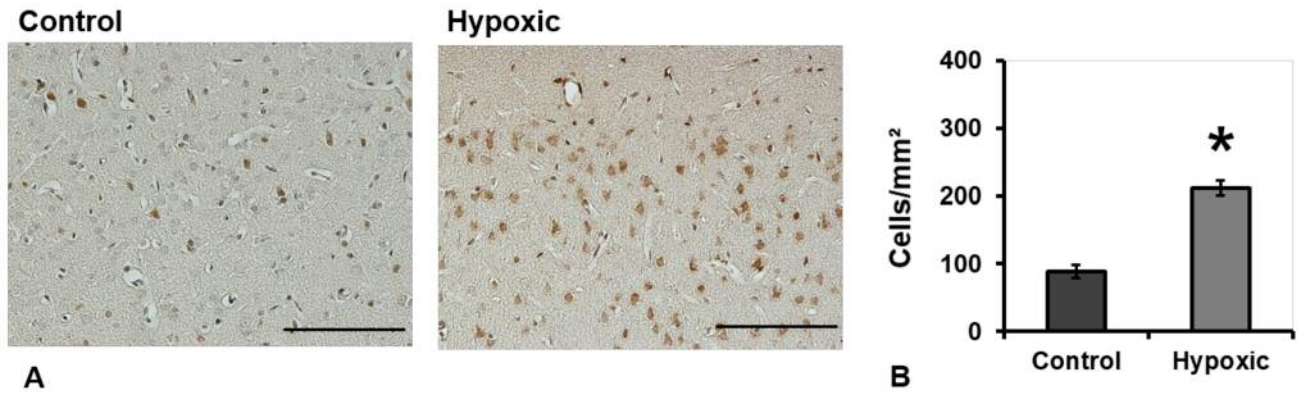


Figure 1. Representative photomicrographs (A) and density (B) of HIF1 $\alpha$  expression in the cerebral parietal cortex. Positive cells are stained dark brown. HIF1 $\alpha$ -positive cells in the hypoxia group were more compared to the control group. Scale bars=100  $\mu$ m; \* $p$ <0.005. HIF1 $\alpha$ : Hypoxia-inducible factor-1 $\alpha$ .

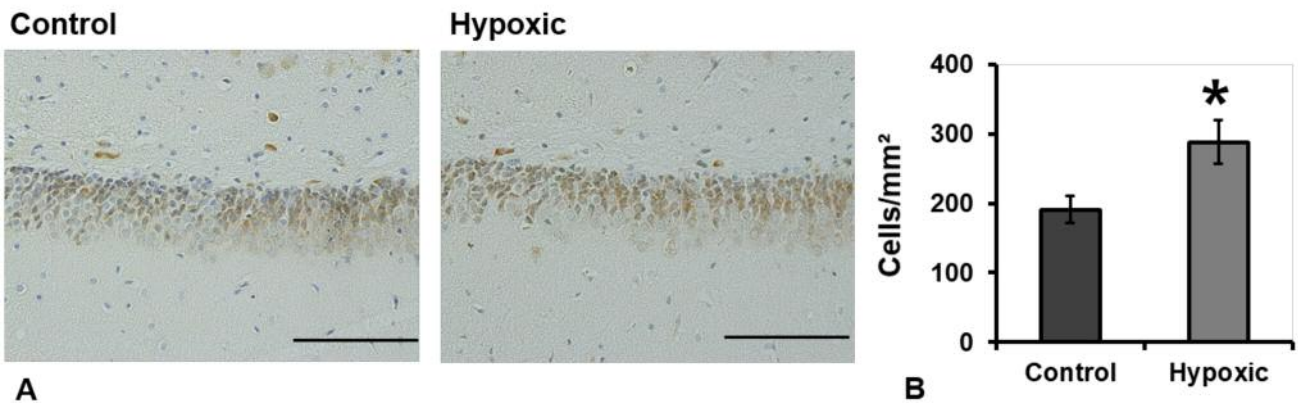


Figure 2. Representative photomicrographs (A) and density (B) of HIF1 $\alpha$  expression in the hippocampus. HIF1 $\alpha$ -positive cells in the hypoxia group were more in density than in the control group. Scale bars=100  $\mu$ m; \* $p$ <0.005. HIF1 $\alpha$ : Hypoxia-inducible factor-1 $\alpha$ .

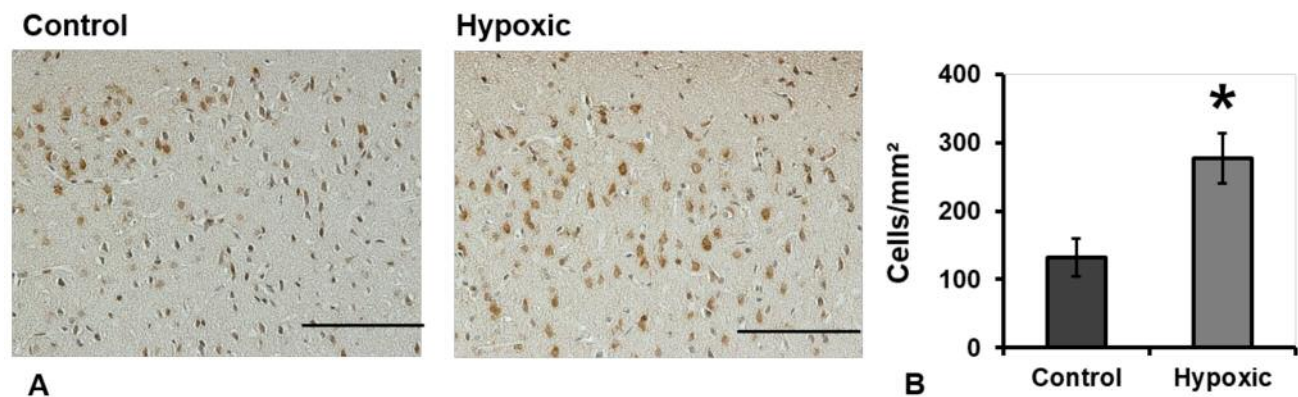


Figure 3. Representative photomicrographs (A) and density (B) of VEGF expression in the cerebral parietal cortex. Positive cells are stained brown. VEGF-positive cells in the hypoxia group were increased as compared with the control group. Scale bars=100  $\mu$ m; \* $p$ <0.005. VEGF: Vascular endothelial growth factor.

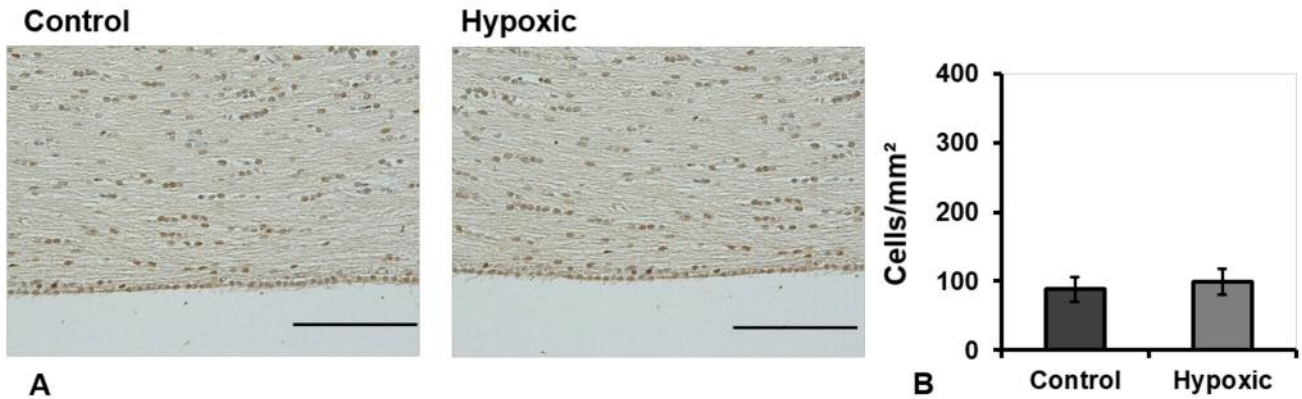


Figure 4. Representative photomicrographs (A) and density (B) of VEGF expression in the subventricular zone. The density of VEGF-positive cells did not differ between the hypoxia and control groups. Scale bars=100  $\mu$ m. VEGF: Vascular endothelial growth factor.

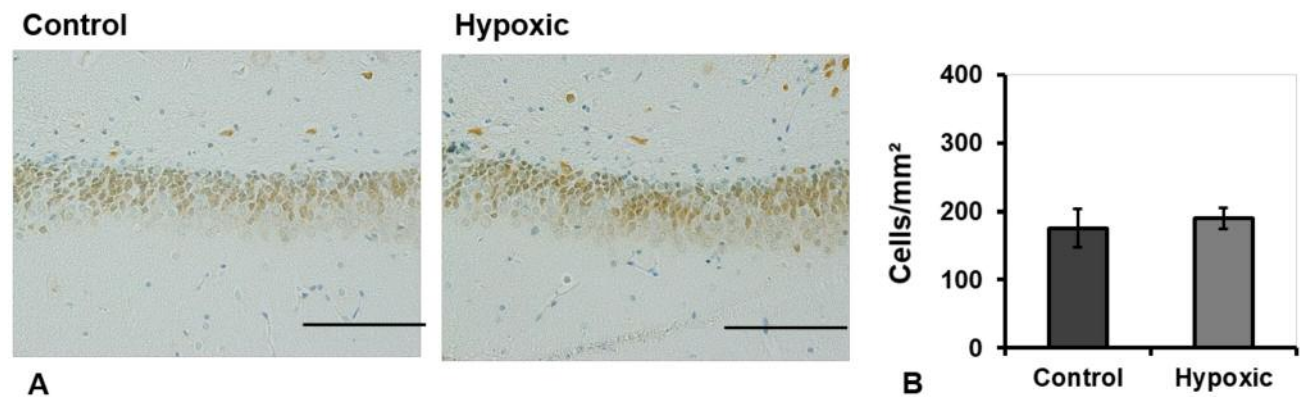


Figure 5. Representative photomicrographs (A) and density (B) of VEGF expression in the dentate gyrus. VEGF is expressed in neurons. VEGF-positive cells in the hypoxia group are similar in number with those in the control group. Scale bars=100  $\mu$ m. VEGF: Vascular endothelial growth factor.

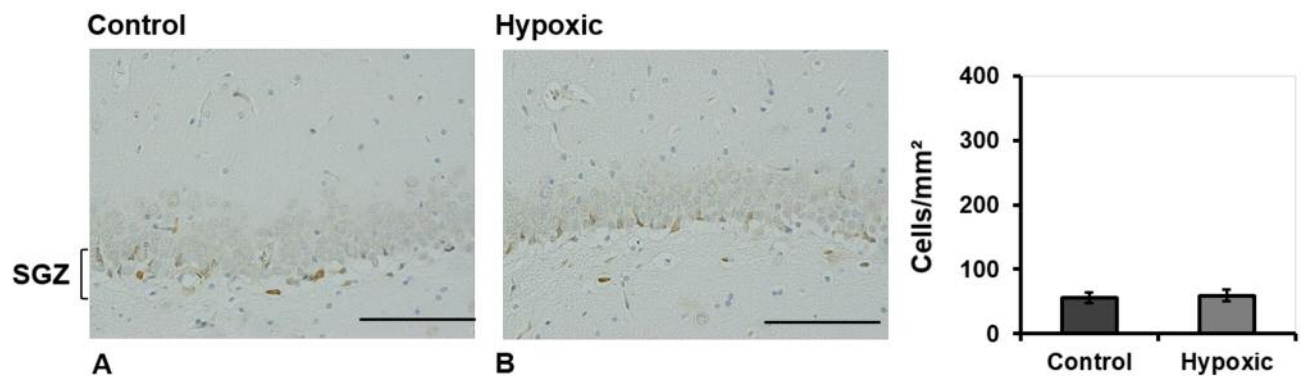


Figure 6. Representative photomicrographs (A) and density (B) of VEGF expression in the subgranular zone. VEGF-positive cells were rarely detected. The density of VEGF-positive cells has no difference between the hypoxia and control groups. Scale bars=100  $\mu$ m. VEGF: Vascular endothelial growth factor.

the subgranular zone proliferate and migrate into the granular layer of the dentate gyrus (28).

We found that the density of HIF1 $\alpha$ -positive cells increased in the cerebral cortex and hippocampus under hypoxia in the brain. HIFs are heterodimers having an oxygen-sensitive HIF-1/2 $\alpha$  subunit (29) and could bind with hypoxia-responsive enhancers (30). HIF1 $\alpha$  has been previously found to be strongly expressed under hypoxia-ischemia conditions (31). These findings support that the increase observed in the density of HIF1 $\alpha$ -positive cells is associated with the hypoxic state in the BCCAO group.

Furthermore, we observed an increased density of VEGF-positive cells in the cerebral cortex of the hypoxic group. It has been demonstrated that angiogenic factors promote neovascularization to adapt under conditions of decreased oxygen balance (32). Among these factors, VEGF is the most effective regulator of endothelial cell growth and differentiation (33), and thus the best regulator of vascular permeability (34). Marosi *et al.*, have shown that cerebral blood flow falls dramatically within 2-3 days following BCCAO, generating hypoxic-ischemic conditions (35), while elsewhere it has been reported that neuronal damage reached a high peak in the cortex 1-3 days following BCCAO (36). Considering a role of VEGF under hypoxic conditions, the up-regulation of its expression 3 days following BCCAO is likely a neuroprotective response.

Our study showed that the density of VEGF-positive cells in the dentate gyrus of the hippocampus was not different between the control and hypoxic groups. Similarly, Kim Min-Soo *et al.*, have shown that VEGF expression in the hippocampal CA1 region 1 week following BCCAO did not differ from that of not operated control rats, although at 4 weeks it was significantly increased (25). Moreover, in the adult hippocampus, VEGF upregulation leads to be sufficient neural stem cell microenvironment for neurogenesis (37). Our study captured differences between the cortex and the hippocampus which may possibly be attributed to the fact that the brain vascularization pattern has been described as “outside in” unlike other organs, such as the kidney and liver (38). Moreover, regions supplied by the cortical network are vulnerable to the reduction of cerebral blood flow (39).

Chronic cerebral hypoperfusion was induced by BCCAO. We confirmed hypoxia status with HIF1 $\alpha$  expression. we evaluated the expression of VEGF which has neuro-protective effect in neurogenic zones and the expression was different between cortex and hippocampus following BCCAO. Based on our results, we suggest that the VEGF expression pattern is different between neurogenesis zones in the cortex and hippocampus following chronic cerebral hypoperfusion induced by BCCAO.

## Conflicts of Interest

The Authors declare that they have no competing interests.

## Authors' Contributions

KMS designed the study. YHJ and DJK participated in the surgical procedures. GSJ, HKS and MSC analyzed the obtained data. YYC and STK performed the immunohistochemistry. All Authors read and approved the final manuscript.

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