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Differential Expression of Pax6 Following Bilateral Common Carotid Artery Occlusion

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Abstract. *Background/Aim: Chronic cerebral hypoperfusion causes neuronal damage involving cognitive impairment and development of dementia. Permanent bilateral common carotid artery occlusion (BCCAO) in rat models is used to study chronic cerebral hypoperfusion. Pax6 is used as an early neurogenesis marker which affects the maturation of neuronal cells. However, the expression of PAX 6 after BCCAO is not well understood. In this study, we investigated the expression of PAX6 in the neurogenic zones after BCCAO to evaluate the effects of Pax6 on chronic hypoperfusion. Materials and Methods: Chronic hypoperfusion was induced by BCCAO. Common carotid artery was laid parallel to the vagus nerve and separated from it. Both arteries were occluded using 4-0 silk sutures. Rats who underwent bi-common carotid artery occlusion formed in the BCCAO group, while unoperated rats served as the control group. Brain samples were obtained on days 3 and 14 after BCCAO and subjected to immunohisto-chemistry with NeuN and western blotting for Pax6 and HIF1α. Results: Compared to the control, the expression of Pax6 increased three days after surgery but did not differ on day 14, while that of NeuN showed the opposite trend. The expression of HIF1α increased three days after surgery. Conclusion: Bilateral common carotid artery occlusion induced early neurogenesis at three days after BCCAO but this result was not maintained at fourteen days after BCCAO.*

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Chronic cerebral hypoperfusion implies a significant reduction in cerebral blood flow (CBF) and insufficient perfusion in the cerebral region. It causes neuronal damage leading to cognitive impairment and development of dementia, such as Alzheimer's disease (AD) and vascular dementia (VaD) (1-3). Permanent bilateral common carotid artery occlusion (BCCAO) in rat models is widely used to study chronic cerebral hypoperfusion (4, 5). Cerebral blood flow (CBF) was rapidly reduced at three days after BCCAO and showed maintenance reduction at fourteen days after BCCAO (6). CBF reduction in BCCAO rats showed regional differences in the cerebral regions. For example, compared to normal-condition rats, CBF was reduced by 35-45% in the cortex and by 60% in the hippocampus (3).

We previously studied paired box-containing gene 6 (Pax6) in the cortex and hippocampus and found it to be related to neurogenesis during hypoxia (7). Pax6 is expressed in the midbrain and is involved in neuronal cell survival (8), and has also been observed in neuronal and glial cells in the adult brain, including the subgranular zone of the hippocampus (9). It is a crucial factor in neurogenesis as it reduces cell proliferation and induces cell maturation (10).

Notably, Pax6 is used as an early neurogenesis marker affecting the maturation of neuronal cells (11). However, expression of PAX 6 after BCCAO has not been addressed. In this study, we investigated the expression of PAX6 in the neurogenic zones at three days and fourteen days after BCCAO.

Materials and Methods

Experimental design. Seven- to eight-week old male Sprague–Dawley (SD) rats were obtained from a certified breeder (Damul Laboratory Animals, Daejeon, Republic of Korea). Rats were bred *ad libitum* and raised in a regulated environment at a temperature of 25˚C. All animal procedures were approved by the Chosun University Institutional Animal Care and Use Committee (approval number: CIACUC2019-A0008). The rats were divided in two groups: control and BCCAO. Rats who underwent bi-common carotid artery occlusion formed the BCCAO group (n=49), while unoperated rats were served as the control group (n=40).

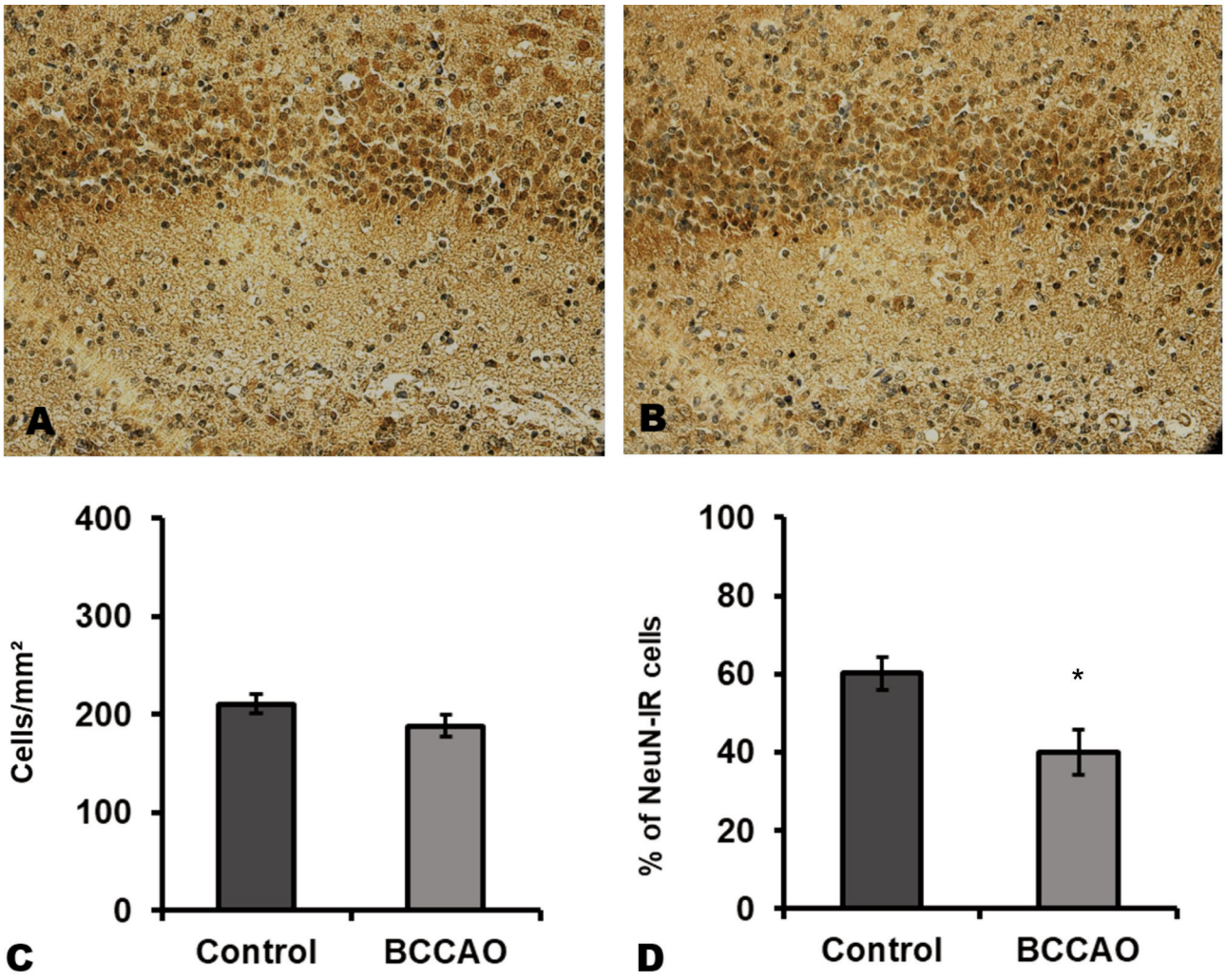

Figure 1. *Representative photomicrographs of NeuN-positive cells in dentate gyrus of hippocampus after three days BCCAO. Dark brown cells were NeuN positive cells. The density and proportion of NeuN-positive cells were lesser in the BCCAO group compared to the control group. This suggests that mature neurons were decreased in the BCCAO group (A) Control group. (B) BCCAO group (C) the density of NeuN-positive cells (D) the proportion of NeuN-positive cells (positive cells/total cells) Values are expressed as a Mean±SEM. *p<0.05. BCCAO: Bilateral common carotid artery occlusion.*

Animal surgery. Chronic cerebral hypoperfusion was induced by BCCAO as described in previous studies (12). Surgery anesthesia was induced using sevoflurane inhalation (1.0-2.0%, end-tidal concentration). The anterior neck portion was disinfected with a povidone–iodine solution and the bilateral common carotid arteries were found near the esophagus and trachea. They were laid parallel to the vagus nerve and separated from it. Both arteries were occluded using 4-0 silk sutures. After surgery, the rats recovered separately. Cages were maintained under aseptic conditions.

Immunohistochemistry. To measure the immunoreactivity of NeuN, a mature neuron marker, positive cells in hippocampus of control and BCCAO groups, brain samples were obtained at 3 and 14 days after surgery and fixed with 4% paraformaldehyde (PFA) solution. Samples were stored overnight at 4˚C. Paraffin sections were prepared using a previously described method, including washing, dehydration, and embedding (13). The cerebrums were cut at sagittal plane intervals of 7-8 μm. The cerebrums were mounted on gelatin-coated slides (Fisher Scientific, Hampton, NH, USA) and the sections deparaffinized with xylene and alcohol and washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4). Antigen retrieval was performed by cooking in a microwave oven in a jar filled with 0.01 M sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. The slides were stained with mouse anti-neuronal nuclear protein (NeuN, 1:100; Millipore, Burlington, MA, USA) primary antibodies overnight at 4˚C. On the second day, staining was performed using the avidin-biotin-peroxidase (ABC) detection system (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) to detect immunoreactivity. Thionin staining was used for counterstaining and the stained slides were mounted (Polysciences, Warrington, PA, USA). Immunoreactive cells (IR) were observed under a light microscope (BX41, Olympus) and quantified as previously described (14). Briefly,

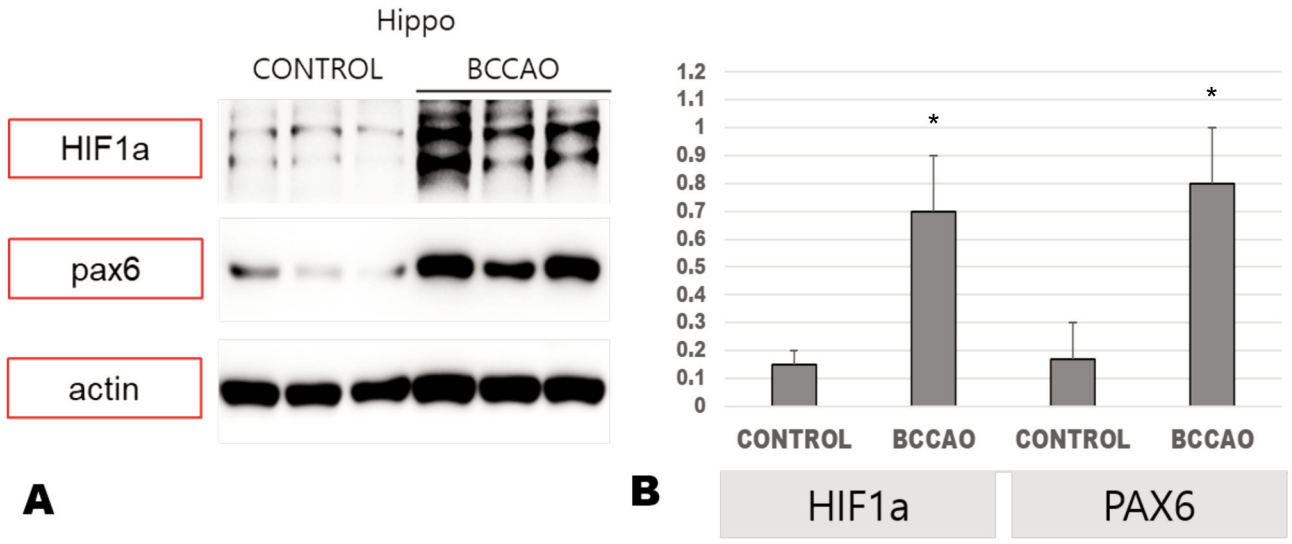

Figure 2. *Representative western blots (A) and band quantification (B) of HIF1α and PAX6 expression in hippocampus samples three days after BCCAO. HIF1α and PAX6 levels were significantly increased in the BCCAO group compared to those in the control groups. This suggests that early neurogenesis was increased in the BCCAO group under hypoxic condition. Results are expressed as a ratio compared to the expression of actin *p<0.05. BCCAO: Bilateral common carotid artery occlusion.*

NeuN-positive cells in each section of the slide were divided into five defined square regions in the cerebral hippocampus. The calculations were performed by two investigators manually.

Western blot analysis. Western blotting was performed at three and fourteen days after BCCAO, as previously described (15). Brains of rats in the ligated bi-carotid artery group and no surgery group were extracted using anesthesia (sevoflurane 1.0-2.0%, end-tidal concentration). Hippocampal tissues extracted from brains were lysed using 0.1% Triton X-100 buffer. Proteins in the tissue were quantified using the bicinchoninic acid protein assay method (BCA). Equal volumes of protein were subjected to gel electrophoresis using sodium dodecyl sulfate. Proteins in the gel were transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA) which was incubated with primary antibodies against β-actin (1:1,000; Santa Cruz, CA, USA), rabbit polyclonal Pax6 (1:500, Abcam, Cambridge, UK), and mouse anti-HIF1α (1:500, Abcam).

Statistical analysis. We used the Statistical Package for Social Sciences (Information Analysis Systems, SPSS) to compare the BCCAO and control groups. Mean values were analyzed using the Student's *t*-test.

Results

Three days after surgery. Immunohistochemistry revealed that the density of NeuN- positive cells was considerably reduced in the BCCAO group compared to the control at three days after surgery (Figure 1). The proportion of cells (positive cells/total cells) was also decreased (Figure 1). In the western blot performed in brain samples obtained at three days after surgery, the expression of HIF1α in the BCCAO group was greater than that in the control group (Figure 2). The mean Pax6 level was higher in the BCCAO group than that in the control group (Figure 2).

Fourteen days after surgery. The density and proportion of NeuN-positive cells did not differ from that of the control group (Figure 3). The expression of HIF1α was lower in the BCCAO, compared to control groups (Figure 4). There was no difference in the mean levels of Pax6 expression between the control and BCCAO groups (Figure 4).

Discussion

We investigated neuronal cell hypoxic status and expression levels of Pax6 at three days and fourteen days after permanent occlusion of BCCAO. We choose to perform measurements at 3 days after BCCAO because local cerebral blood flow reduction was presented in the autoradiographic measurement at least two days after BCCAO (16). Also, Tae-Kyeong *et al*. reported that NeuN-immunoreactivity (IR) neurons and GFAP-IR astrocytes decreased at least two days after BCCAO (17). We choose to perform measurements at 14 days after BCCAO because another study suggested that cerebral blood flow (CBF) was rapidly reduced at three days after BCCAO and presented a maintenance reduction up to fourteen days after BCCAO (6). Seven days after surgery, the survival rate of rats subjected to surgery was under 50%. Ligia *et al*. studied the transient ischemic injury effect using the reperfusion BCCAO model 21 days after surgery. Chronic hypoperfusion induced by BCCAO has been used in animal models of vascular

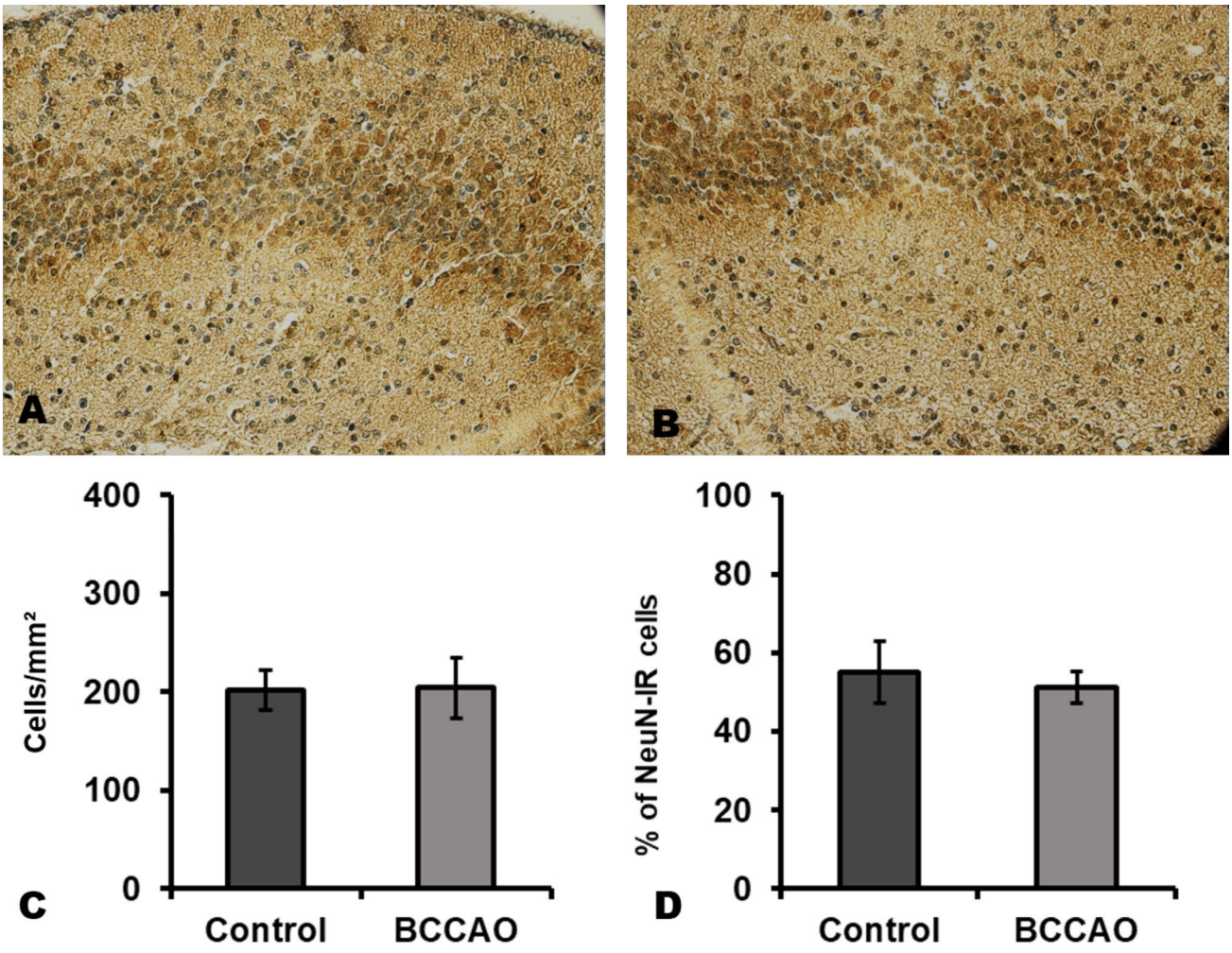

Figure 3. *Representative photomicrographs of NeuN-positive cells in the dentate gyrus of hippocampus fourteen days after BCCAO. No difference was observed in the density and proportion of NeuN-positive cells between the control and BCCAO groups. This showed that the number of mature neurons did not differ between two groups. (A) Control group (B) BCCAO group (C) density of NeuN-positive cells (D) proportion of NeuN-positive cells (positive cells/total cells). Values are expressed as a mean±SEM. BCCAO: Bilateral common carotid artery occlusion.*

dementia (18, 19). In this model, the dentate gyrus of the hippocampus was used for western blotting (20).

The density and proportion of NeuN-positive cells in the hippocampus differed in the BCCAO group at three days after surgery. Other studies have shown neuronal damage in the hippocampus of rats with BCCAO and have attempted to recover this damage (21, 22). BCCAO-induced hypoperfusion showed neuronal cell damage and apoptosis in the dentate gyrus of the hippocampus (23) which is related to learning and memory impairments (24). The number of NeuN-positive cells was reduced in the hippocampus seven days after BCCAO and was associated with the vascular endothelial growth factor (VEGF)-C/VEGFR-3 signaling (25), similar to our previous results (12). Fourteen days after surgery, there was no difference between the control and BCCAO groups. Otori *et al*. reported that the reduction of cerebral blood flow at two days after BCCAO did not continue till four weeks (16). Thus, a plan to create a BCCAO group for four weeks (28 days) is required.

The expression of HIF1α and Pax6 was higher in the BCCAO group compared to the control group at three days after surgery. HIF1α showed response to hypoxic conditions in many studies (26, 27). The expression of HIF1α was found increased under chronic prenatal hypoxia (7). Similar to our results, HIF1α expression was enhanced in the BCCAO model (28). Pax6 expression increased in hypoxic brain neuronal cells in previous studies (29, 30). In the BCCAO model, Pax6 was used as an early neurogenic marker, whose increased expression implies improved neurogenesis (31). Choi *et al*. reported that neurogenesis in the dentate gyrus of BCCAO

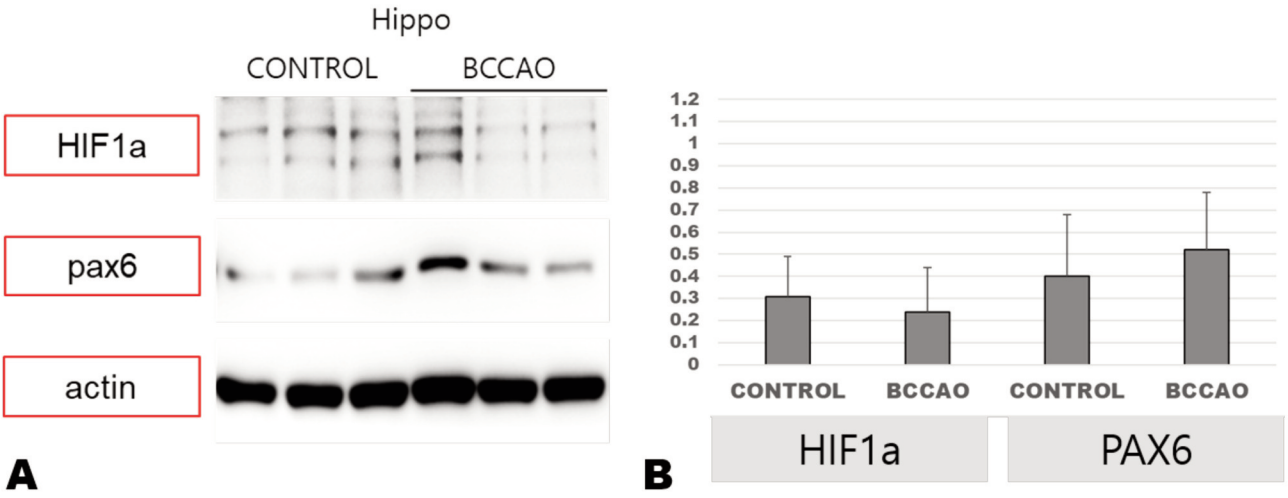

Figure 4. *Representative western blots (A) and quantification (B) of HIF1α and PAX6 expression in hippocampus region of brain tissues at 14 days after BCCAO. There was no difference in the mean levels of HIF1α and PAX6 expression between control and BCCAO group. This suggested that the hypoxic condition was not maintained to 14 days after BCCAO and early neurogenesis was not triggered. Results are expressed as a ratio compared to actin. BCCAO: Bilateral common carotid artery occlusion.*

group increased on day 1, but did not differ from the control group on day 28. The authors suggest that the difference in expression of Pax6 between days 3 and 14 after surgery indicated that early neurogenesis increased but did not persist 14 days after surgery.

Conclusion

The expression of Pax6 in western blot of the hippocampus of brain tissues was found increased three days after BCCAO, but presented no differences at fourteen days. HIF1α expression was similar with PAX6 expression. These results imply that chronic hypoperfusion induced early neurogenesis at three days after BCCAO but this effect was not maintained up to fourteen days after BCCAO.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

YHJ designed the experimental study. DJK and MJL participated in the BCCAO procedures. HBC, GHJ AND HKS performed the immunohistochemical analyses. HIH performed the western blot analyses. YYC analysed the obtained data. All Authors approved the final manuscript.

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