

IGF-1 Protects Neurons in the Cortex and Subventricular Zone in a Periventricular Leucomalacia Model

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Abstract. *Background/Aim:* Chronic cerebral hypoperfusion affects early and mature neurons in the subventricular zone (SVZ) and cerebral cortex. Herein, we investigated the effects of insulin-like growth factor-1 (IGF-1), a neurogenesis-promoting agent, on neurons in these regions in periventricular leucomalacia (PVL) model rats. *Materials and Methods:* Following right carotid artery ligation, the rats were placed in a hypoxia chamber and injected with recombinant IGF-1 (0.1 and 1 µg/µl). Their brain sections were immunohistochemically analysed using anti-*nestin* and anti-*NeuN* antibodies. *Results:* The numbers of early-neuronal cells in the SVZ and mature neurons in the cerebral cortex were higher and lower, respectively, in the PVL group than in the control group. The number of *NeuN*-positive cells was significantly higher in the IGF-treated group than in the PVL group. *Conclusion:* PVL increased the number of early neuronal cells in the SVZ, reducing the survival of mature neurons in the cerebral cortex; IGF-1 reversed these effects.

Clinical syndrome in the white matter damage is associated with significant neurological outcomes (1). Immature brain is caused by reasons such as ischemic injury and infections (2, 3). Periventricular leucomalacia (PVL) occurs predominantly in premature infants. Predominant neurodevelopmental sequelae in patients with PVL are cerebral palsy, mental retardation, and behavioural deficits (4, 5). PVL is primarily characterised by white matter damage, but gray matter damage

can also occur (6, 7). PVL leads to neuronal cell apoptosis and demyelination, causing axonal loss (8). PVL occurs in association with motor and sensory deficits (9, 10). However, thus far, there are no available treatments for PVL (11, 12).

Insulin-like growth factor-1 (IGF-1) is a single-chain peptide; it is abundantly expressed in the central nervous system (CNS) (13). In animal models, members of the IGF family play an important role in brain development and functional stability (14). IGF-1, as a potent tropic factor, has neuroprotective effects against cerebral ischemia (15). Intracranial injections of IGF-1 after hypoxia reduce the infarct volume, enhance cell survival, and improve cognitive performance (16, 17). These effects are caused by IGF-1 signalling, which could stimulate the neuronal cell proliferation, survival, and differentiation (18). Several studies have revealed that IGF-1 improves neurogenesis and angiogenesis in brain tissues or cells (19, 20).

Previous studies have shown that chronic cerebral hypoperfusion affects the number of early and mature neurons in the neurogenic zone, including the subventricular zone (SVZ) and cerebral cortex (21, 22). As mentioned above, IGF affects cell survival. In this study, we investigated the effects of IGF-1 on neurons in the neurogenic zones in a PVL rat model.

Materials and Methods

Animal surgery. The use of certified Sprague–Dawley (SD) rats (Damul Laboratory Animals, Daejeon, Republic of Korea) for this study was approved by the Chosun University Institutional Animal Care and Use Committee (approval no. CIACUC2019-A0031). We simulated a PVL condition based on the methods described in a previous study (23). Seven-day-old SD rats (n=50) were anaesthetised with the inhalation of sevoflurane (1.0%-2.0%, end-tidal concentration). After shaving the fur, a midline incision was made below the anterior cervical region under aseptic conditions. The right carotid artery was exposed, and the ligature was performed using silk sutures (4/0). After the procedure, the neck was disinfected using povidone–iodine solution. After 10 min, the rats that underwent this

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surgical procedure were placed in a hypoxia chamber (8% O₂ and 92% N₂ conditions at 37°C) for 2 h. Rats that underwent surgery and were placed in the hypoxic chamber were assigned to the PVL group, whereas the unoperated rats were assigned to the control group (n=50).

Two hours after the rats were subjected to hypoxic incubation, they underwent intracranial IGF-1 injection using stereotaxic injector (QSI/53311). The IGF-1 injection was administered as previously described (24). Briefly, recombinant IGF-1 (Genentech, San Francisco, CA, USA) at a concentration of 0.1 µg/µl and 1 µg/µl was dissolved in sterile phosphate-buffered saline (PBS; 0.15 M; pH 7.4). The rats were anaesthetised with sevoflurane (1.0%-2.0%, end-tidal concentration), and the solutions were injected using a stereotaxic injector. Hamilton syringes (Hamilton Co., Reno, NV, USA) for injection were located at 1.0 mm posterior and 1.0 mm lateral to the bregma and at a 3.0 mm depth from the skull surface. The duration of injection was 5 min.

Tissue preparation. Seven days after hypoxia treatment and IGF-1 injection, the rat brains were harvested and fixed in 4% paraformaldehyde solution. The cerebrum was separated from the brain stems and fixed in 4% PFA at 4°C. After one day, the cerebrum samples were washed with water, dehydrated using graded ethanol solutions, and embedded in paraffin. A series of 6-7-µm-thick sagittal sections were cut and mounted on gelatine-coated slides (Fisher Scientific USA).

Immunohistochemistry. The slides were deparaffinised and rehydrated. The slides were washed with 0.9% NaCl in 0.1 M phosphate buffer (PBS, pH 7.4). Microwave-based antigen retrieval process was performed with 0.01 M sodium citrate buffer (pH 6.0). After cooling, the slides were treated with 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase. After rinsing in PBS, the slides were blocked with normal horse serum in 0.5% bovine serum albumin (BSA) solution for 30 min at room temperature. The slides were incubated overnight with the primary antibodies, mouse anti-hexaribonucleotide-binding protein-3 (NeuN; 1:100, Millipore, Burlington, MA, USA) and anti-nestin antibodies (1:200, Millipore), at 4°C. On the following day, the slides were rinsed in PBS several times and incubated with secondary antibodies matched to the primary antibodies in 0.5% BSA solution for 90 min at room temperature. The slides were incubated with avidin-biotin-peroxidase (Vector Laboratories, Burlingame, CA, USA) and immunoreactivity was visualised using the chromogenic substrate 3, 3'-diamino-benzidine (DAB). Thionin counterstaining was performed, and the slides were dehydrated and covered with DPX.

Statistical analysis. A light microscope (PrimoVert) with attached digital CCD camera was used to photograph the slides. NeuN- and Nestin-positive cells were counted in the cerebral cortex and SVZ. Each section was randomly divided into five areas, and the number of positive cells per defined square (µm²) was calculated in each area. All data were analysed using Statistical Package for Social Sciences, information analysis systems (Information Analysis Systems, SPSS, IBM, Armonk, NY, USA). All measurements of the control, PVL, and Injection groups were compared using ANOVA test. *p*-values <0.05 were considered statistically significant.

Results

Nestin immunoreactivity. In the SVZ, the number of nestin-positive cells in the PVL group was higher than that in the

control group (Figure 1). Interestingly, the number of nestin-positive cells in injection group was lower than PVL group (Figure 1). In the cerebral cortex, there was no difference in the number of nestin-positive cells between the control and PVL groups (Figure 2). The number of nestin-positive cells in Injection group was higher than PVL group in the cerebral cortex (Figure 2).

NeuN immunoreactivity. In the SVZ, there was no difference in the number of NeuN-positive cells between the control and PVL groups (Figure 3). The number of NeuN-positive cells in the cerebral cortex was lower in the PVL group than in the control group (Figure 4). The number of NeuN-positive cells in the injection group was significantly higher than that in the PVL group (Figure 4).

Discussion

We measured the number of nestin- and NeuN-positive cells in the cerebral cortex and SVZ. The number of nestin-positive cells in the SVZ was higher in the PVL group than in the control group. The number of NeuN-positive cells in the cerebral cortex was lower in the PVL group than in the control group. This reduction was achieved using IGF-1 injection.

Neuronal and glial progenitor cells, which are the sources of neurogenesis, were abundant in the SVZ (25). Especially, in the ischemic brain, these cells have been reported as important self-renewing sources for neurons and glial cells (23). The self-renewing processes, including neuron survival, axon sprouting, and neuronal enlargement and proliferation, aimed at recovering from brain injury, also known as the plasticity of the CNS, are observed in the ischemic region (26). Nestin is an intermediate neurofilament expressed by multipotential neural stem cells and precursors of neuronal and glial cells (27). In our study, the number of nestin-positive cells in the SVZ was higher in the ischemic group than in the non-ischemic group. These positive cells are considered to be sources for self-renewing processes in rats with PVL. Okoshi *et al.* showed strong nestin immunoreactivity of SVZ neurons in the white matter surrounding a lesion of PVL (28). It is suggested that the early stage of neurogenesis is triggered in the SVZ of the rats from the PVL group.

In our study, the numbers of early neuronal cells and nestin-positive cells were increased in the SVZ in the rats from the PVL group. The numbers of mature neuronal cells and NeuN-positive cells were reduced in the cerebral cortex in the rats from this group. The early neuronal cells in the SVZ migrated to the cerebral cortex; neurogenesis was triggered, but cell survival was not affected (29). This phenomenon was reversed by IGF-1 administration. As mentioned in the introduction, IGF-1 plays a major role in neuronal cell survival and maturation (14, 30). IGF-1 activates the MAPK pathway, especially, the extracellular

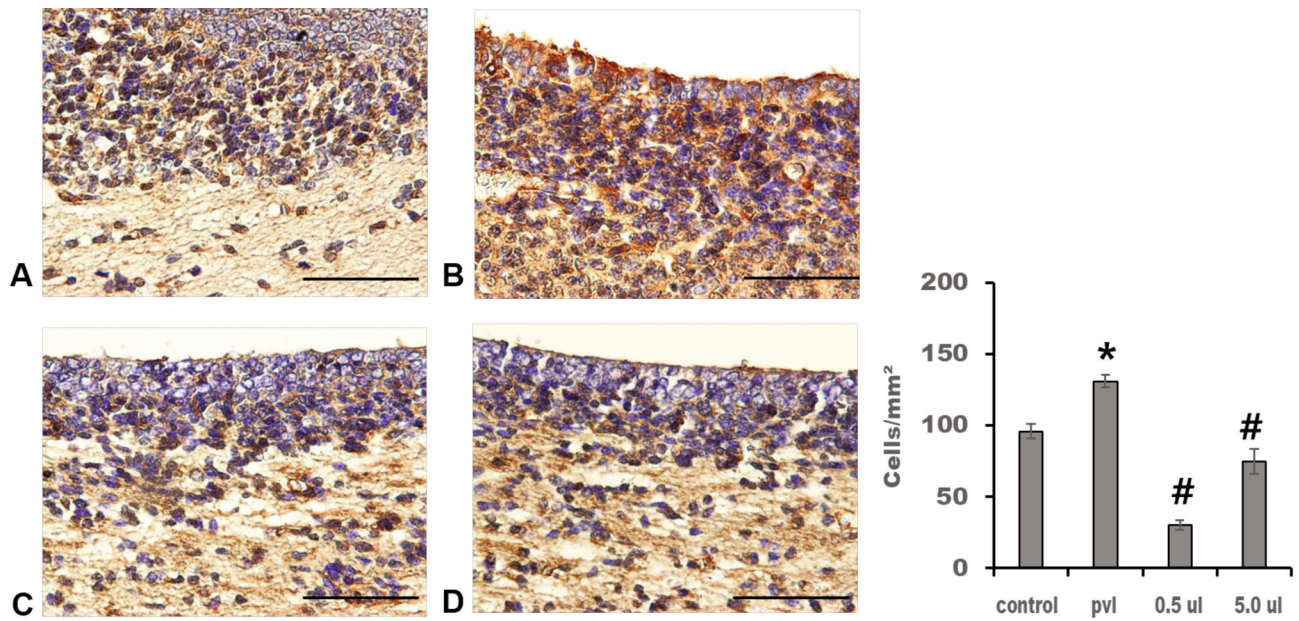


Figure 1. Representative photographs and density of nestin-immunoreactivity cells in the subventricular zone. (A): Control group, (B): PVL group, (C): injection group (0.1 $\mu\text{g}/\mu\text{l}$), (D): injection group (1 $\mu\text{g}/\mu\text{l}$). The data are expressed as mean and SEM values. * $p < 0.05$ (compared control), # $p < 0.05$ (compared PVL). Scale bars=100 μm .

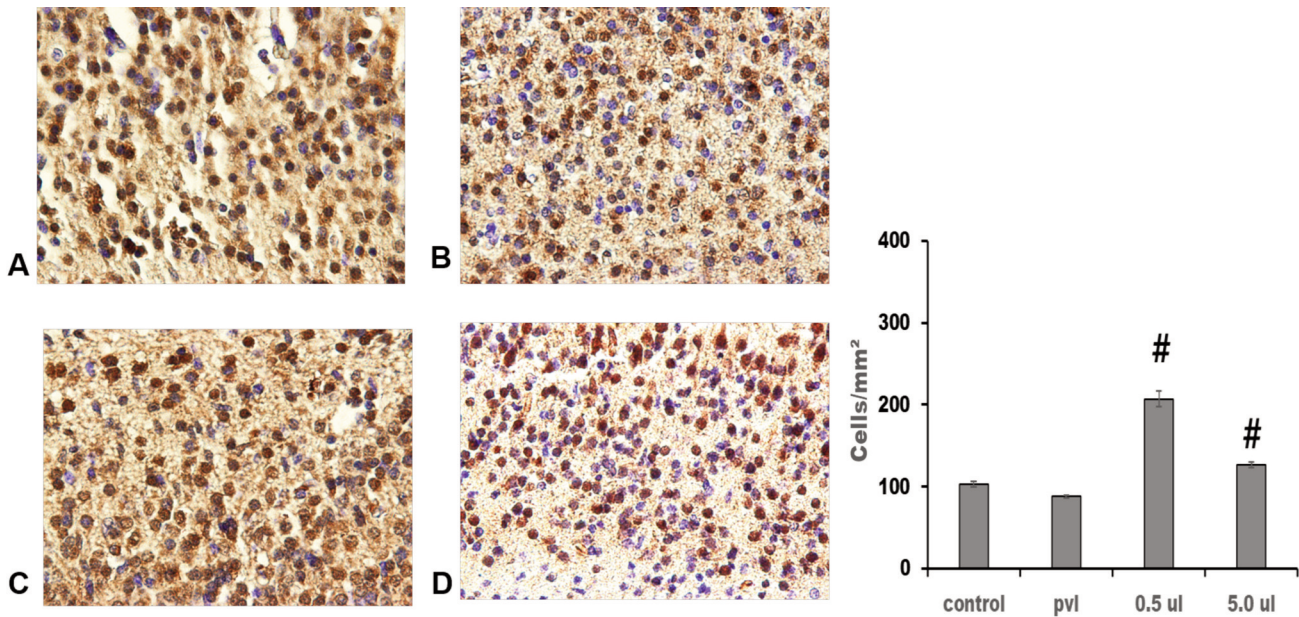


Figure 2. Representative photographs and density of nestin-immunoreactivity cells in the cerebral parietal cortex. (A): Control group, (B): PVL group, (C): injection group (0.1 $\mu\text{g}/\mu\text{l}$), (D): injection group (1 $\mu\text{g}/\mu\text{l}$). The data are expressed as mean and SEM values. * $p < 0.05$ (compared control), # $p < 0.05$ (compared to PVL). Scale bars=100 μm .

signal-regulated kinase (ERK) pathway, to regulate cell survival and neuronal plasticity (31). In some cells, the PI3K/Akt pathway is activated by IGF-1 to enhance cell survival (32).

We studied the effects of high and low doses of IGF-1 on PVL in rats. Interestingly, in our study, the effects of both high and low doses of IGF-1 on PVL were similar. The numbers of mature neuronal cells were induced in the

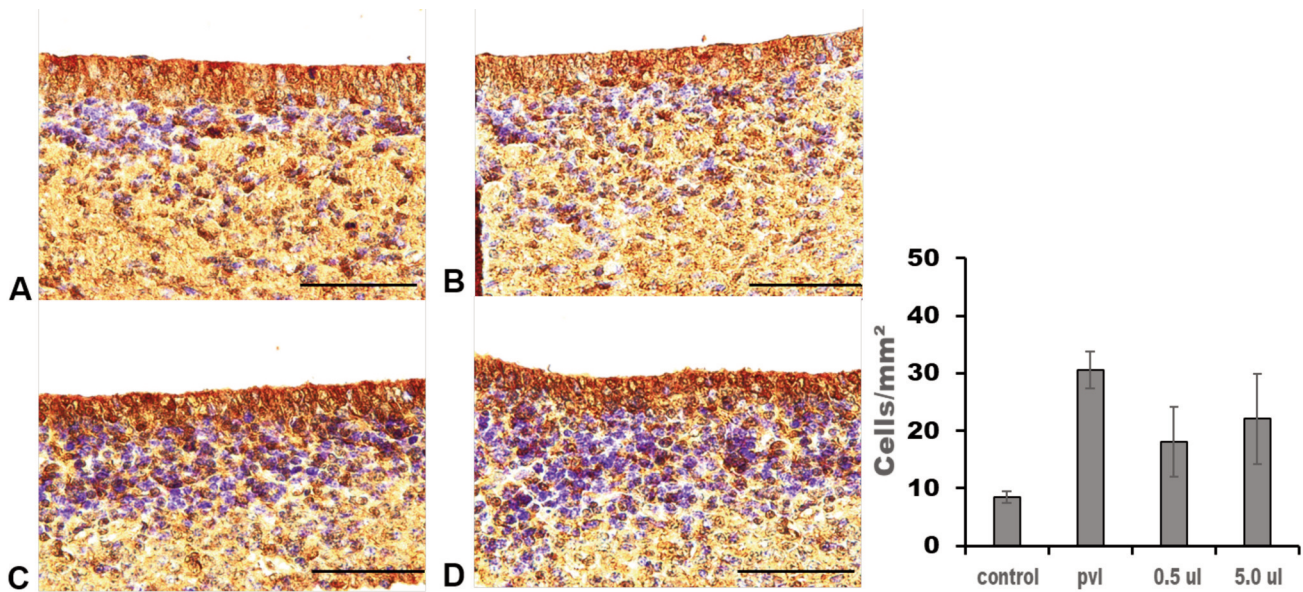


Figure 3. Representative photographs and density of NeuN-immunoreactivity cells in the subventricular zone. (A): Control group, (B): PVL group, (C): injection group (0.1 $\mu\text{g}/\mu\text{l}$), (D): injection group (1 $\mu\text{g}/\mu\text{l}$). The data are expressed as mean and SEM values. * $p < 0.05$ (compared to control), # $p < 0.05$ (compared to PVL). Scale bars=100 μm .

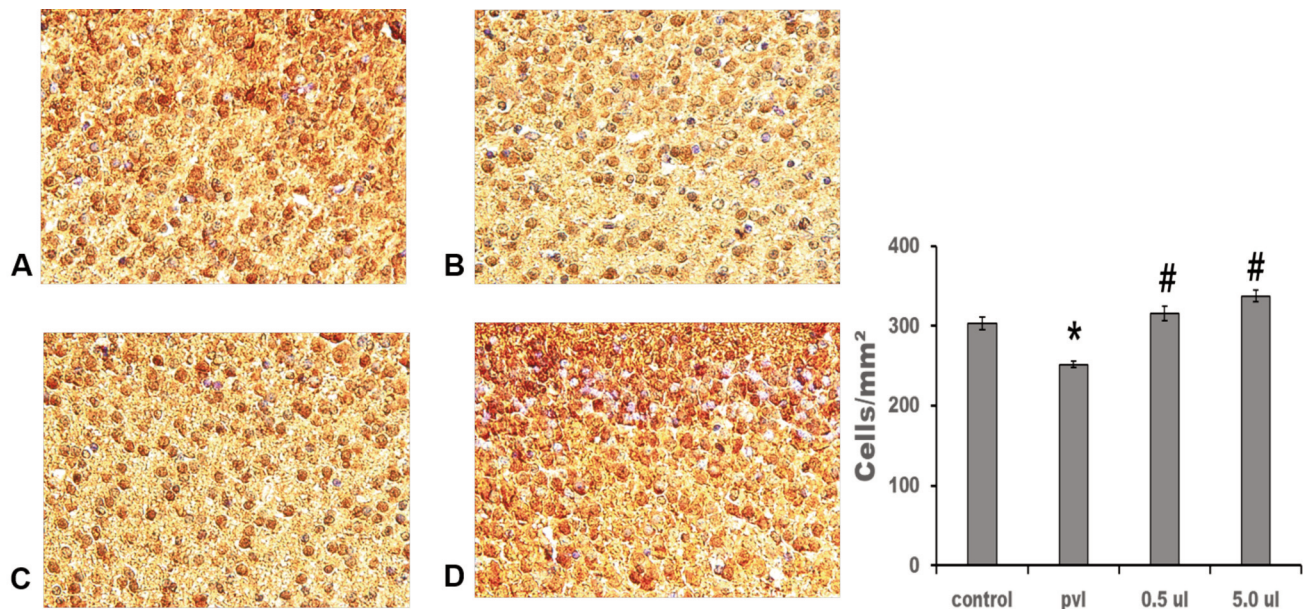


Figure 4. Representative photographs and density of NeuN-immunoreactivity cells in the cerebral parietal cortex. (A): Control group, (B): PVL group, (C): injection group (0.1 $\mu\text{g}/\mu\text{l}$), (D): injection group (1 $\mu\text{g}/\mu\text{l}$). The data are expressed as mean and SEM values. * $p < 0.05$ (compared to control), # $p < 0.05$ (compared to PVL). Scale bars=100 μm .

cerebral cortex in the rats from high and low doses of IGF-1 injection group. Several studies have revealed discrepant effects of IGF depending on its dose. Some studies have reported that IGF-1 protects against ischemic damage only

at a high dose (33). Controversially, Cao *et al.* showed that IGF-1 prevented the loss of myelin and glial cells after hypoxia in foetal sheep only at low doses (34). However, A previous study that was similar to our study showed that

hypoxia and infection co-existed and interacted with each other (35). Inflammatory responses caused by the intracerebral injection of lipopolysaccharide (LPS) is similar to the pathology observed in case of PVL (35). Several studies have reported that IGF-1 exhibits neuroprotective effects against ischemic damage and neuroinflammation (15, 36). We suggest that IGF-1 has protective effects against either hypoxia or inflammation in the developing rat brain.

PVL led to increase in the number of early neuronal cells in the SVZ and reduction of survival of mature neurons in the cerebral cortex. These effects were reversed by IGF-1 injection.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

YHJ designed the study. DJK and HLS participated in the surgical procedures. SYC, SUK, and DWJ analysed the obtained data. HIH performed the immunohistochemical analyses. All authors approved the final manuscript.

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