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Injury of Neurons by Oxygen-Glucose Deprivation in Organotypic Hippocampal Slice Culture

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= Abstract =

Purpose : We intended to observe cell death and apoptotic changes in neurons in organotypic hippocampal slice cultures following oxygen-glucose deprivation (OGD), using propidium iodide (PI) uptake, Fluoro-Jade (FJ) staining, TUNEL staining and immunofluorescent staining for caspase-3.

Methods : The hippocampus of 7-day-old rats was cut into 350 μm slices. The slices were cultured for 10 d (date in vitro, DIV 10) and and exposed to OGD for 60 min at DIV 10. They were then incubated for reperfusion under normoxic conditions for an additional 48 h. Fluorescence of PI uptake was observed at predetermined intervals, and the cell death percentage was recorded. At 24 h following OGD, the slices were Cryo-cut into 15 μm thicknesses, and Fluoro-Jade staining, TUNEL staining, and immunofluorescence staining for caspase-3 were performed.

Results : 1) PI uptake was restricted to the pyramidal cell layer and DG in the slices after OGD. The fluorescent intensities of PI increased from 6 to 48 h during the reperfusion stage. The cell death percentage significantly increased time-dependently in CA1 and DG following OGD ($P<0.05$). 2) At 24 h after OGD, many FJ positive cells were detected in CA1 and DG. Some neurons had distinct nuclei and processes while others had fragmented nuclei and disrupted processes in CA1. TUNEL and immunofluorescent staining for caspase-3 showed increased expression of TUNEL labeling and caspase-3 in CA1 and DG at 24 h after OGD.

Conclusion : The numerous dead cells in the slice cultures after OGD tended to display apoptotic changes mediated by the activation of caspase-3. **(Korean J Pediatr 2008;51:1112-1117)**

Key Words : Hippocampus, Oxygen glucose deprivation, Hypoxia, Ischemia, Apoptosis, Immature, Rat, Brain

Introduction

Organotypic hippocampal slice culture is a suitable in vitro model for studying neural development, neural function and the pathophysioloy of neurodegenerative diseases[1-8]. In hippocampal slice cultures of 7-day-old rat brains, oxygen-glucose deprivation (OGD) can cause injury similar to hypoxic-ischemic injury of immature brains[9-15]. As well, it has been reported that, depending on the degree of OGD, necrosis or apoptosis of neurons can be readily seen in hippocampal slice cultures[9, 10].

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Apoptosis plays an important role in delayed neuronal death after hypoxic-ischemic injury to immature brains[16-18]. Apoptotic death of cells is deeply related to activation of the caspase system, which is commonly observed in neuronal or glial cell death following hypoxic-ischemia injury[19-21].

Thus, in this study, we aimed to maintain hippocampal slice cultures of 7-day-old rats brains for more than 10 days, and to observe cell death and the apoptotic changes of neurons in the hippocampal slice cultures following exposure to OGD, using propidium iodide (PI) uptake, Fluoro-Jade (FJ) staining, TUNEL [(TdT)-mediated biotin-16-dUTP nick-end labelling] staining and immunofluorescent staining for caspase 3.

Materials and Methods

1. Organotypic hippocampal slice culture

The experiments were performed in accordance to The

Guide of Dankook University for the Care and Use of Laboratory Animals. The brains of 7-day-old Sprague-Dawley rats were aseptically removed from the skull under anesthesia by ether and transferred into a Petri dish placed over ice. The hemispheres were separated from each other and the hippocampus was dissected. The separated hippocampus was cut vertically along its long axis into 350 μm slices using a MacIlwain tissue chopper (MacIlwain, Mickle Laboratory, Cambridge, UK). The hippocampal slices were transferred into cold (12-14℃) buffer solution (modified Krebs-Heseleit solution) and carefully separated from each other. Modified Krebs-Heseleit solution contains NaCl (120 mM), KCl (2 mM), $CaCl_2$ (2.0 mM), $NaHCO_3$ (26 mM), $MgSO_4$ (10 mM), KH_2PO_4 (1.18 mM), glucose (11 mM), sucrose (200 mM) with a pH of 7.4.

Following the protocol designed by Stoppini et al.[22] hippocampal slices were cultured using the membrane-interface technique, allowing the tissue to be maintained at the liquid/air interface. In brief, hippocampal slices were placed on a semi-porous membrane (Millicell, Bedford, MA, USA) in plastic inserts of a 6-well plate. Each well contained 1 mL of culture medium which was composed of 50% MEM (minimal essential medium), 25% HBSS (Hank's balance salt solution), 25% horse serum, 6.5 mg/mL glucose, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin with pH 7.2. Three to four hippocampal slices were placed in each insert and cultures were maintained for 10 days (date in vitro, DIV 10). Cultures were then exposed to OGD. The culture medium was changed every two days.

2. Oxygen-glucose deprivation (OGD)

Hippoampal slice cultures were exposed to OGD at DIV 10 using a modification of the submersion protocol of Frantseva et al.[11]. For OGD, glucose was excluded from buffered solution. Bubbling this solution for at least 30 minutes with a gas mixture containing 5% CO_2 and 95% N_2 resulted in a deoxygenated, glucose free OGD solution. After a single wash of the culture well with the OGD solution, 1 mL of OGD solution was placed below a semi-porous membrane. The culture trays were then placed in an air-tight anoxic bag (AnaeroGen^{TM}, Oxoid Ltd, UK) and placed in an incubator at 37℃ for 60 minutes. After exposure to OGD, the cultures were washed two times with horse-serum free culture medium. The cultures were then transferred to trays with horse-serum free oxygenated culture medium containing glucose and incubated for reperfusion under normoxic conditions, for additional 48 hours.

3. Propidium iodide (PI) uptake

For evaluation of cell death after exposure to OGD, two hours prior to OGD exposure the cultures and wells were washed two times with buffer solution. The slices were then cultured in horse serum free culture media containing 0.5 μg/mL of PI for two hours just prior to OGD exposure. Just before OGD insults, the basal fluorescent intensity of PI (0 hours, pre-OGD) was observed with an inverted fluorescence microscope (Olympus, Tokyo, Japan) using a rhodamine filter and recorded with a digital camera. After 60 minutes of exposure to OGD, slices were washed two times with horse-serum free culture medium. Afterwards, while cultured in horse-serum free culture medium containing PI, intensities of PI uptake were observed and recorded at 6, 12, 24, 48 hours following exposure to OGD during reperfusion under normoxic conditions. After 48 hours, the slices were refrigerated and incubated at 4℃ for 24 hours to kill all cells in the hippocampal slices, at which point the final PI uptake intensity was again observed and recorded. Pixel intensties of stored images were quantitatively measured at CA1 (cornu ammonis) and DG (dentate gyrus) using an image analyser (image-J, NIH). The percent of cell death was expressed as a percentage of final fluorescence (F_{fin}) minus basal fluorescence (F_0) as shown in the equation, % cell death=$(F_t-F_0)/(F_{fin}-F_0)\times100$, where F_t is the fluorescence of slices measured at 6, 12, 24, and 48 hour during reperfusion under normoxic conditions following OGD[11].

4. TUNNEL staining and immunofluorescent staining

Hippocampal slice cultures were fixed for one hour in 4% paraformaldehyde just before exposure to OGD and at 24 hours after exposure to OGD. Following immersion in sucrose and freezing at -70℃, the slices were cryocut into 15 μm thick slices. Different sets of sections were stained by FJ staining (Chemicon, Temecula, CA, USA), TUNEL staining (TUNEL Apoptosis Detection Kit, DNA Fragmentation/Fluorescence Staining, Upstate Co., USA), or immunofluorescent staining with caspase-3 antibody (Sigma, St. Louis, MO, USA) in accordance to the protocol provided by the manufacturer. After staining, sections were observed with a fluorescence microscope (Olympus, Tokyo, Japan) and a confocal laser microscope (Carl Zeiss, Thornwood, NY, USA).

5. Statistical anaylsis

One-way ANOVA was used for determining statistical significance (SPSS ver 11.0). P-value< 0.05 was considered as statistically significant.

Results

1. PI uptake following OGD

PI uptake could be seen as an indicator of OGD induced neural death due to the fact that PI uptake was restricted to the pyramidal cell layer and DG in hippocampal slices after exposure to OGD. The fluorescent intensities of PI increased from 6 to 48 hours during reperfusion under normoxic conditions following exposure to OGD (Fig. 1A). Percent cell death significantly increased time-dependently in CA1 and DG during reperfusion under normoxic conditions following exposure to OGD (Fig. 1B, P<0.05)

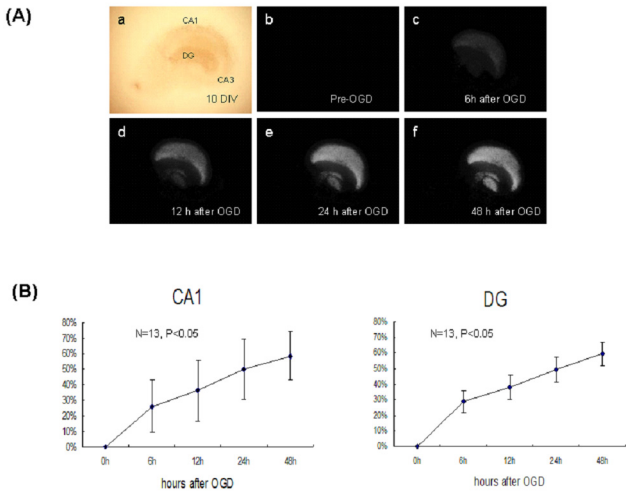

Fig. 1. Temporal analysis of neuronal death in P7 rat hippocampal slice culture. (A) The hippocampal slices were cultured for 10 d (a). The slices were then exposed to OGD for 60 min and cultured under reperfusion and normoxic conditions for an additional 48 h. Neuronal death was examined by assessing the PI uptake fluorescence at pre-OGD, 6 h, 12 h, 24 h, and 48 h after OGD (b-f). The PI uptake fluorescence was restricted to the pyramidal cell layer, and the DG and fluorescence intensities of PI increased from 6 h to 48 h during reperfusion and normoxia following OGD. (B) Percentage of cell death during reperfusion and normoxia following OGD increased time-dependently in CA1 and DG (P<0.05).

2. FJ staining, TUNEL staining and immunofluorescent staining for caspase-3

Fluoprescence of slices after FJ staining was not detected at pre-OGD (Fig. 2A), but, at 24 hours after exposure to OGD, many FJ positve cells suggesting neurodegeneration were detected in CA1 (Fig. 2B) and DG (Fig. 2C). Findings observed under confocal laser microscopy with high power magnification (Fig. 2D) showed some neurons with distinct nuclei and processes and other neurons with fragmented nuclei, and disrupted processes in CA1.

The results for TUNEL and immunofluorescent staining for caspase-3 showed increased expression of TUNEL labelling and caspase-3 in CA1 and DG at 24 hours following exposure to OGD (Fig. 3A-H).

Discussion

Organotypic hippocampal slice culture is a commonly used in vitro model for studying brain cell injury. There has been much research done on neural development and function, and the pathophysioloy of neurodegenerative diseases[1-8].

In 1981, Ghlwiler et al.[1] described in detail the cultivation of nervous tissue by means of the roller-tube technique. In

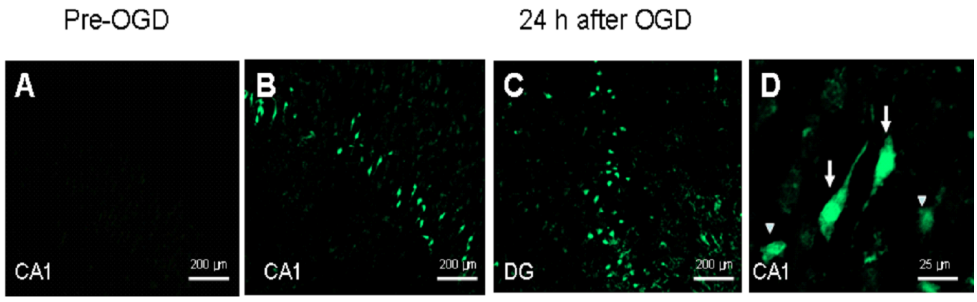

Fig. 2. Fluoro-Jade (FJ) staining of P7 rat hippocampal slices at pre-OGD and 24 h after OGD. Fluorescence after FJ staining was not detected at pre-OGD (A), but many FJ positive cells suggesting neurodegeneration were detected in CA1 (B) and DG (C) at 24 h after exposure to OGD (Mag. ×200). Confocal microscopic analysis (Mag. ×800) revealed some neurons with distinct nuclei and processes (arrow) and other neurons with fragmented nuclei, and disrupted process (arrow head) in CA1 (D).

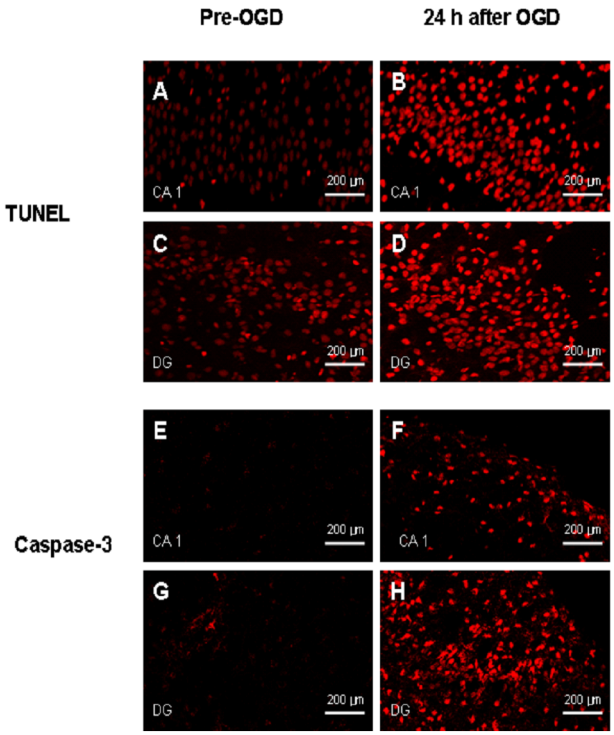

Fig. 3. TUNEL staining and immunofluorescent staining for caspase-3 in P7 rat hippocampal slices at pre-OGD and 24 h after exposure to OGD. Exposure of hippocampal slices to OGD increased the TUNEL labeling and the expression caspase-3 in CA1 and DG at 24 h after OGD.

1991 Stoppini et al.[22] described a simpler method for organotypic culture of nervous tissue different from the roller-tube technique. Hippocampal slices were placed on a semi-porous membrane and successfully cultured using the membrane-interface technique. Hippocampal slices for culture are usually prepared from rats aged 6-9 days old (P6-9) and cultures can be maintained for several weeks[11, 22]. However, it has been made known that long term stable slice cultures can be also prepared from younger rats as well as from adult rats (P4-P30)[6, 8, 10].

The method of submerging brain slice cultures in deoxygenated glucose-free medium can induce neuronal injury similar to that of hypoxia-ischemia[11, 23]. Exposure of hippocampal slice cultures to OGD induces consistent hypoxic-ischemic damage while preserving the selective regional vulnerability of different hippocampal cell layers[9-11, 23]. Within three to four hours after exposure of hipocampal slice cultures to OGD for a predetermined period, the CA1 area was first to receive injury, afterwards the CA3 and dentate gyrus (DG) also received injury[11, 23].

In this study, PI uptake after exposure to OGD was restricted to the pyramidal cell layer and DG in hippocampal slices, suggesting many neurons to be either dying or dead. Damage to the cell membrane is necessary for PI uptake to occur during OGD[14, 23]. Thus, injured areas in the hippocampus could be identified by increased PI fluorescence. The intensities of PI fluorescence increased time-dependently during reperfusion under normoxic conditons following OGD. Increased fluorescence due to PI uptake may be a crude but effective estimate of neuronal cell death[10, 11, 14, 23]. A reasonably linear relationship between fluorescence intensity and cell death was observed[10, 23]. In general, PI uptake might be an earlier and more sensitive marker than the LDH efflux. LDH, which is another cell death marker requires cell lysis while intracellular PI uptake requires only cell membrane damage[14, 23]. However, increased fluorescence of PI can not identify specific types of cells or show morphologic characters of cell death because it stains only the cell nuclei-spot.

FJ stain is a valid marker of neurodegeneration[24]. Following FJ staining of slices after OGD we observed many FJ positive cells on the CA1 and DG to be either dead or near death. FJ dye can not gain access to cell when cell membrane was intact[24]. Both PI and FJ stain are valid in detecting neuronal death in brain slice cultures[10, 12, 25, 26]. FJ stain has the advantage, when compared with PI stain, that FJ stain can be applied to non living fixed or unfixed tissue, while PI only stains a dying or dead cell in a culture medium. Also, FJ stain can be a useful marker of apoptotic cell death following exposure to the neurotoxin because FJ dye selectively stains degenerated neurons, as well FJ staining can display morphologic changes of nuclei, soma and axons of degenerated neurons[10, 12, 27]. In this study, FJ stain of cultures revealed some neurons with distinct nuclei and processes and other neurons with fragmented nuclei, and disrupted process in CA1. These findings suggested that many neurons injured by OGD might be undergoing apoptosis. As well, many TUNEL positive cells and increased expression of caspase-3 were observed on the CA1 and DG of hippocampal slice cultures at 24 hour after exposed to OGD. Activation of the caspase system following hypoxic-ischemic injury is a common findings in animal or human brains. However, there are only a few in-vitro reports using slice cultures that show similar results[20, 21, 28].

In this study, we maintained hippocampal slice culture of 7-day-old rat brains for more than 10 days. We observed many dead cells and caspase-3 mediated neuronal apoptosis

in hippocampal slice cultures following exposure to OGD using PI uptake, FJ staining, TUNEL staining and immunofluorescent staining for caspase-3.

요 약

뇌 해마조직 절편 배양에서 산소와 당 박탈에 의한 뇌신경세포 손상

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목 적 : 해마 절편 배양에서 산소-포도당 박탈(oxygen-glucose deprivation, OGD)에 의한 세포 사망과 신경 세포 사멸을 propidium iodide(PI) 섭취, Fluoro-Jade(FJ) 염색, TUNEL 염색, caspase-3 면역형광염색 방법으로 관찰하고자 하였다.

방 법 : 생후 7일된 Sprague-Dawley 흰쥐의 해마를 MacIlwain chopper로 350 μm 두께의 절편으로 절단하였다. 해마 절편을 6-well plate의 insert 내의 반 유공(sem-porous) 막 위에서 membrane-interface technique으로 10일 동안 배양하였다. 배양된 해마 절편에 산소-포도당 박탈을 60분 동안 가한 후 재산소-재관류하에 기초 배양액에서 48시간 배양하였다. 재산소-재관류 동안 PI 섭취 형광 정도를 시간에 따라 형광 현미경으로 관찰하고 세포사망 백분율(percent cell death)을 측정하였다. 산소-포도당 박탈 직전과 24 시간 후에 해마 절편을 15 μm 두께로 냉동 절단 후 FJ 염색, TUNEL 염색, caspase-3 면역형광염색을 시행하여 세포 사망을 관찰하였다.

결 과 : OGD 후 PI 섭취 는 해마 절편의 CA1과 DG에 한정되어있었다. OGD 후 재산소-재관류 동안 6시간에서 48시간까지 PI 섭취 형광 강도는 시간이 증가함에 따라 증가하였다. 세포 사망 백분율은 CA1과 DG에서 모두 OGD 후 재산소-재관류 시간이 증가함에 따라 의미 있게 증가하였다(P<0.05). OGD 후 24시간에 세포 변성을 의미하는 많은 FJ 염색 양성 신경 세포 들이 CA1과 DG에서 관찰되었다. 고배율 confocal laser 현미경으로 관찰한 CA1에서의 신경 세포들 중 일부는 명확한 핵과 돌기를 가지고 있는 것을 보여 주었으며, 다른 신경 세포들은 핵의 분절화, 돌기의 손실 등을 보여 주었다. TUNEL 염색과 caspase-3 염색은 OGD 후 24시간에 CA1과 DA에서 TUNEL 양성 발현을 증가시키고 caspase-3 발현을 증가시켰다.

결 론: 해마 절편 배양에서 산소-포도당 박탈 에 의한 다수의 세포 사망을 관찰할 수 있었다. 사망한 세포 들은 주로 신경 세포의 caspase-3 활성화에 의해 매개된 사멸을 보였다.

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