Short Communication

Bilobalide Protects Pheochromocytoma Cell from Oxygen-Glucose Deprivation/Reperfusion Induced Injury via Activating Wnt1/Beta Catenin Pathway

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To explore the protective effect of bilobalide on the pheochromocytoma cell injury induced by oxygen-glucose deprivation/reperfusion in vitro. To simulate ischemia-reperfusion condition, the pheochromocytoma cell injury was induced by oxygen-glucose deprivation/reperfusion in vitro. The cells were divided into control, oxygen-glucose deprivation/reperfusion and oxygen-glucose deprivation/reperfusion+bilobalide groups. The cell viability, proliferative capacity, malondialdehyde level, superoxide dismutase level, apoptosis, Wnt1 protein level and nuclear beta-catenin protein level were assayed. Compared with control group, the cell viability, proliferative capacity, superoxide dismutase level, Wnt1 protein level and nuclear beta-catenin protein level decreased in the oxygen-glucose deprivation/reperfusion group, malondialdehyde level and the percentage of apoptotic cells increased in the oxygen-glucose deprivation/reperfusion group, while the cells were treated with bilobalide in the oxygen-glucose deprivation/reperfusion+bilobalide group, all the above-mentioned observation indicators recovered to a certain extent, but still did not reach the level of the control group. Bilobalide protects pheochromocytoma cell from oxygen-glucose deprivation/reperfusion induced injury in vitro through inhibiting oxidative stress via activating Wnt1/beta-catenin pathway.

Key words: Bilobalide, ischemia, cell damage, apoptosis, neuroprotective agents

Cerebrovascular disease has become an important cause that threatens the health and quality of life of the middle-aged and elderly people. Cerebral ischemia-induced cerebral infarction is the most common and it is also a key factor causing disability and death in the elderly worldwide[1]. Cerebral ischemia can cause obstacles to the transport of substances such as sugar and oxygen, and induce nerve cell damage. Even if blood circulation in the brain tissue is restored after therapy, ischemia-reperfusion injury will occur[2]. Therefore, it is particularly important to find effective protective drugs. Neuroprotection is an important link in the treatment strategy of ischemic stroke, which refers to disrupting the neuronal death cascade by blocking ischemic signaling pathways. With the in-depth research on the pathophysiological mechanism of ischemic stroke, the development of neuroprotective agents has become a hot spot in the research and development of ischemic stroke treatment drugs. Bilobalide is an extract of Ginkgo biloba, the sesquiterpene lactone compound[3]. At present, the data results of multiple studies have shown that bilobalide has various biological functions, including anti-oxidative stress, anti-inflammatory, scavenging oxygen free radicals, improving energy metabolism, anti-excitatory amino acid toxicity, protecting mitochondria, inhibiting nerve cell apoptosis[4-6]. It has a wide range of neuroprotective effects. However, the role and mechanism of bilobalide in Cerebral Ischemia-Reperfusion Injury (CIRI) remain unclear. In this study, we used the Pheochromocytoma (PC12) cell to make the Oxygen-Glucose Deprivation/Reperfusion (OGD/R) model in vitro and the cell model was treated with bilobalide, then the cell viability, proliferative capacity, Malondialdehyde (MDA) level, Superoxide Dismutase (SOD) level, apoptosis, Wnt1 protein level and nuclear beta-catenin protein level were assayed. This

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study aims to clarify the protective effect of bilobalide on ischemia-reperfusion injury in vitro and the possible mechanism. The PC12 cell was purchased from Tongpal Biotechnology Co., Ltd (Shanghai, China) and cultured in the Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, United States of America (USA)). The cells were divided into control, OGD/R and OGD/R+bilobalide groups. The cell model was prepared based on a previous study[7]. The OGD/R cell model was prepared in glucose-free DMEM under 37°, 0.2 % Oxygen (O₂), 5 % Carbon dioxide (CO₂), 95 % Nitrogen (N₂) and cultured for 3 h. Then, the cells were cultured in glucose-containing DMEM with 10 % Fetal Bovine Serum (FBS) under 37°, 5 % CO₂ for 24 h. Cells in the control group were cultured normally. The bilobalide (MUST, China) solution was prepared with normal sodium containing 8 % ethanol. During the above cell culture, the medium of OGD/R+bilobalide group contained 25 mg/l bilobalide and the medium of control, OGD/R groups contained the equal concentration of ethanol. The dose selection of bilobalide was based on a previous study[9]. The PC12 cells (3×10⁵/ml) were cultured into 96-well plates according to the above grouping and culture methods. After the culture, 10μl of CCK-8 solution was added to the cells, incubated at 37° for 2 h and then the Optical Density (OD) value at 450 nm was measured with a microplate reader (BioTek microplate reader, USA). The cells of each group were blocked after the culture medium was removed, then the cells were incubated with primary antibody: rabbit anti-Ki67 (1:800, Abcam, UK) for 4 h in a humidified chamber at 37°, then the cells were incubated with second antibody: Alexa Fluor® 568-labeled goat anti-rabbit Immunoglobulin G (IgG) (1:1000, Abcam, UK) for 2 h in a humidified chamber at 37°. The Hoechst 33342 (1:2000, Beyotime, China) was used to stain the cells nuclei for 15 min at 37°. The Ki67 positive cells percentage of OGD/R+bilobalide group was more than that in the OGD/R group but was lower than that in the control group (p<0.05) as shown in fig. 1A. The results of Ki67 immunofluorescence showed Ki67 positive cells percentage of OGD/R group was the lowest, the Ki67 positive cells percentage of OGD/R+bilobalide group was more than that in the OGD/R group but was lower than that in the control group (p<0.05) as shown in fig. 1B. The results of TUNEL assay showed almost no apoptotic cells were seen in the control group, more apoptotic cells were seen in OGD/R group and fewer apoptotic cells were in OGD/R+bilobalide group (p<0.05) as shown in fig. 1C. Compared with control group, the MDA level increased and SOD level decreased in the OGD/R group (p<0.05), while the cells were treated with bilobalide in the OGD/R+bilobalide group, the MDA and SOD levels recovered to a certain extent, but still did not reach the level of the control group (p<0.05) as shown in fig. 2. Compared with control group, the Wnt1 and nuclear β-catenin protein levels significantly decreased in the OGD/R group (p<0.05), while the cells were treated with bilobalide in the OGD/R+bilobalide group, the Wnt1 and nuclear β-catenin protein levels recovered to a certain extent, but still did not reach the level of the control group (p<0.05) as shown in fig. 3. CIRI refers to a phenomenon in which the blood supply to the brain is...
restored after a period of ischemia and the brain function is not improved, but a serious disorder occurs, the key pathological process of the disease development. The occurrence of CIRI in the brain tissue further aggravates the brain injury, which is a pathological process involving multiple factors and multiple mechanisms. Current research believes that it mainly involves various mechanisms such as oxidative stress, inflammatory response, excitatory amino acid toxicity, intracellular calcium overload, energy metabolism disorders and free radical damage, which ultimately lead to nerve cell death. In this study, the PC12 cell injury induced by OGD/R in vitro, and the results showed the cell viability, proliferative capacity significantly decreased and the percentage of apoptotic cells increased in the OGD/R group, while the cells were treated with bilobalide in the OGD/R+bilobalide group, all the above-mentioned observation indicators recovered to a certain extent, but still did not reach the level of the control group. The results indicated that bilobalide can protect the PC12 cell from injury induced with OGD/R in vitro. The process of oxidative stress is an important role that cannot be ignored. Reducing oxidative stress injury may play a key role in reducing CIRI and downstream pathophysiological responses. After oxidative stress occurs, a large number of reactive oxygen species are generated. The final product of peroxidation reaction is MDA, and MDA can affect the activity of mitochondrial respiratory chain complexes and key enzymes in mitochondria, so MDA can reflect the degree of lipid peroxidation in the body and the degree of cellular oxidative damage. SOD is the main antioxidant enzyme and its level represents the antioxidant capacity in the body. In this study, the MDA level increased and SOD level decreased in the OGD/R group, while the cells were treated with bilobalide in the OGD/R+bilobalide group, the MDA and SOD levels recovered to a certain extent. The results indicated that bilobalide can reduce the oxidative stress level in the PC12 cell OGD/R injury in vitro. Wnt/β-catenin signaling pathway is one of the important regulatory signaling pathways in the body, which can affect many cell life activities in the body, such as regulating cell cycle, promoting cell division, affecting cell growth and apoptosis, etc. This signaling pathway is also involved in the pathophysiological process of various diseases such as tumors, cardiovascular and cerebrovascular diseases and aging. One of the hallmarks of the activation of the Wnt/β-catenin signaling pathway is the accumulation of β-catenin into the nucleus, which activates the downstream gene expression changes of this signal, thereby exerting biological effects. In this study, the Wnt1 and nuclear β-catenin levels decreased in the OGD/R group, while the cells were treated with bilobalide in the OGD/R+bilobalide group, Wnt1 and nuclear β-catenin levels recovered to a certain extent. The results indicated bilobalide can activate the Wnt1/β-catenin signaling pathway in the PC12 cell OGD/R injury in vitro. In summary, bilobalide protects PC12 cell from OGD/R induced injury in vitro through inhibiting oxidative stress via activating Wnt1/β-catenin pathway.

Fig. 1: The effect of bilobalide on the, (A): PC12 cell viability; (B): Proliferative capacity and (C): Apoptosis
Note: (*) vs. control group, p<0.05 and (#) vs. OGD/R group, p<0.05. Bar=20 μm
Fig. 2: The effect of bilobalide on the (A): MDA and (B): SOD in the PC12 cell
Note: (*) vs. control group, p<0.05 and (#) vs. OGD/R group, p<0.05

Fig. 3: The effect of bilobalide on the (A): Wnt1 and (B): Nuclear β-catenin protein levels in the PC12 cell
Note: (*) vs. control group, p<0.05 and (#) vs. OGD/R group, p<0.05

Ethical approval:
This study was approved by the Ethics Committee of Dongtai People's Hospital (2021-dtry-L-004).

Authors' contributions:
Xiaofeng Yu and Jie Hou have contributed equally to this work.

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Conflict of interests:
The authors declared no conflict of interest.

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