The protective effect of Wnt3a on inflammatory response in oxygen-glucose deprivation/reoxygenation (OGD/R) astrocyte model

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Abstract
Involving in the immune response after cerebral infarction, astrocytes could secrete large amounts of pro- and anti-inflammatory factors. The aim of this study is to investigate the effect of Wnt3a intervention on the inflammatory response of oxygen-glucose deprivation (OGD) followed by reoxygenation (OGD/R) astrocyte model, and to provide a new target for immunoprotective treatment of cerebral infarction. We constructed the OGD/R rat astrocyte model, the astrocytes were treated by different concentrations of glucose (25, 50, 100 mM) intervened with/without Wnt3a (25 μg/ml). Microscope was used to observe the cell survival in rat astrocytes. The relative expression of inflammatory factors (TNF-α, IL-6, HIF-α) in rat astrocytes was detected by qRT-PCR. The expression of inflammatory factors such as TNF-α, IL-6 and HIF-α in rat astrocytes was increased after OGD/R treatment. The Wnt3a intervention promoted cell survival and decreased the expression of inflammatory factors in rat astrocytes induced by OGD/R. There is a neuroprotective effect that Wnt3a intervention could reduce inflammatory response in the OGD/R rat astrocyte model.

Key words: Wnt3a, astrocyte, oxygen-glucose deprivation (OGD/R), inflammatory factors.

Introduction
Stroke is the second leading cause of death worldwide and is characterized by a high rate of morbidity, mortality, and disability [8]. Among them, acute ischemic stroke (AIS), which accounts for 87% of the total incidence of stroke, is characterized by the sudden stop of oxygen and blood supply due to localized arterial occlusion in the brain tissue [31]. Patients with AIS are always affected by accompanied aphasia, hemiplegia and consciousness disorders. Due to poor prognosis and high mortality, the increasing morbidity of AIS in China resulted in a huge social economic burden [30]. Increasing evidence indicated that the post-ischemic inflammation accounts for the secondary progression of brain damage, and the severity of stroke outcome under comorbidities depends on the extent of this...
inflammatory response [24]. Astrocytes, as the most plentiful subtypes of glial cells, play an essential role in AIS and can cause exacerbated brain damage due to their participation in the inflammatory response [9]. What is more, involved in the immune response after cerebral infarction, astrocytes could secrete lots of pro-inflammatory and anti-inflammatory factors [18]. Immunomodulatory therapy for cerebral infarction is an urgent task to solve the problem of nerve cell damage during cerebral infarction.

As a member of the Wnt1 family of Wnt/β-catenin signalling pathway, Wnt3a plays an anti-inflammatory role and participates in regulation through Wnt/β-catenin signalling pathway [12]. Wnt3a proteins can increase the number of GFAP-positive astrocytes and participate in the regulation of astrocyte-mediated inflammatory responses [20]. Studies have shown that Wnt3a plays an important inhibitory role in the inflammatory response of various diseases. Lü et al. found that Wnt3a can inhibit the inflammatory response in periodontal disease [19]. Chen et al. found that Wnt3a suppresses *Pseudomonas aeruginosa*-induced inflammation by reducing the production of pro-inflammatory cytokines and by promoting apoptosis in macrophages [6]. Zhang et al. found that intranasal Wnt3a could ameliorate toxic responses of microglia and astrocytes in ischemic brain injury [35]. Taken together, we speculate that Wnt3a may suppress neuroinflammation in AIS. To verify the above inferences, we constructed an oxygen-glucose deprived (OGD) rat astrocyte model and evaluated the role of Wnt3a in the inflammatory response of OGD/R rat astrocytes stimulated by different concentrations of glucose. These experiments may provide a new target for immunoprotective treatment of AIS.

**Material and methods**

**Isolation of primary rat astrocytes and construction of the OGD/R rat – astrocyte model**

To clarify the role of Wnt3a in the inflammatory response of OGD/R astrocytes, we isolated astrocytes from SD rats and constructed an OGD/R astrocyte model. This study was approved by the Animal Ethics Committee of Affiliated Hospital, Sun Yat-sen University (No. 2020-067-01) and performed in accordance with approved guidelines. Primary astrocytes were prepared from the cortical tissue of neonatal Sprague-Dawley rats according to the method described by Wang et al. [28]. Rat astrocytes which were cultured in glucose-free DMEM medium were transferred into the anoxic-sealed chamber filled with anoxic mixture (95% N₂ + 5% CO₂) for 6 h at 37°C. The astrocytes were allowed to recover under normoxic conditions (OGD restoration) for 24 h. Astrocytes maintained in DMEM without oxygen deprivation served as the normal group. The cell growth of astrocytes was observed by an electronic light microscope. When the cell density was halved, it means that the OGD/R model has been established.

**Grouping and treatment of rat astrocytes**

OGD/R rat astrocytes were divided into 6 groups according to the concentration of glucose in this experiment: 1) Low glucose + OGD/R (25 mM glucose + astrocyte OGD/R model); 2) Low glucose + OGD/R + Wnt3a (25 mM glucose + astrocyte OGD/R model + 25 μg/ml Wnt3a); 3) Medium glucose + OGD/R (50 mM glucose + astrocyte OGD/R model); 4) Medium glucose + OGD/R + Wnt3a (50 mM glucose + astrocyte OGD/R model + 25 μg/ml Wnt3a); 5) High glucose + OGD/R (100 mM glucose + astrocyte OGD/R model); 6) High glucose + OGD/R + Wnt3a (100 mM glucose + astrocyte OGD/R model + 25 μg/ml Wnt3a). Wnt3a was purchased from Novoprotein Inc. Wnt3a was first added into PBS (2 μl) buffer for preparation, and then added into astrocytes in each group for 7 h. Then the astrocytes were intervened by OGD/R with different concentrations of glucose as previously mentioned.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Rat astrocytes were seeded at 1 × 10⁵ cells per well in six-well plates, and cells were harvested at indicated time points after corresponding processing. Total RNA from astrocytes was extracted by TRIzol reagent (Sigma). Total RNA concentration and purity were detected using Nanodrop. The corresponding cDNA was synthesized by a reverse transcription kit (Takara), and the expression level of the inflammation-related genes was evaluated by real-time-PCR with SYBR Green (Takara) according to the manufacturer’s instructions. GAPDH served as the PCR control. Each sample was run in duplicate in PCR to determine gene expression of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and hypoxia-inducible factor 1α (HIF-1α). The primer sequences of the above genes were shown in Table I. The results were analysed using the 2⁻ΔΔCT method.

**Statistical analysis**

All experiments were repeated at least three times and the average data were calculated. Measurement data were expressed as mean ± standard deviation (SD). SPSS 22.0 was used for statistical analysis, two groups were compared using t test, and multiple groups were compared using one-way ANOVA. The statistical software used was SPSS 22.0 (IBM, USA). The level of statistical significance was set at p < 0.05.
compared using one-way analysis of variance. $P < 0.05$ was considered as statistically different.

Results

OGD/R induced inflammatory response in rat astrocytes

To clarify the role of Wnt3a in the inflammatory response of oxygen-glucose deprived (OGD/R) astrocytes, we isolated astrocytes from SD rats and constructed an OGD/R astrocyte model by depriving of glucose and oxygen (OGD/R). The qRT-PCR results showed that the mRNA expression of TNF-α was significantly increased in the Low glucose + OGD/R group compared with the Low glucose group ($P < 0.01$), unexpectedly decreased in the Medium glucose + OGD/R group compared with the Medium glucose group ($P < 0.01$), unchanged in the High glucose + OGD/R and High glucose group (Fig. 1A). Differently, compared with the control group with a different concentration of glucose, IL-6 and HIF-1α mRNA expression were increased in the OGD/R model group with all the different concentration of glucose ($P < 0.05$) (Fig. 1B, C). Thus, we suggested that the astrocyte OGD/R model with high inflammatory response was successfully constructed.

Wnt3a intervention promoted cell survival in OGD/R rat astrocytes

To clarify the role of Wnt3a in the OGD/R rat astrocyte survival, we intervened OGD/R rat astrocyte with Wnt3a for 7 h. The result is shown in Figure 2. These images showed that the density of astrocytes in the OGD/R model group was significantly lower than that in the normal group, while the astrocyte density in the OGD/R + Wnt3a group was significantly higher than that in the OGD/R group (Fig. 2). This result suggested that Wnt3a can significantly improve the survival rate of OGD/R rat astrocytes.

Wnt3a intervention inhibited inflammatory response in OGD/R rat astrocytes

In addition, we also found that the expression of TNF-α in OGD/R rat astrocytes stimulated by low glucose was decreased by Wnt3a intervention, but there were no statistically significant differences (Fig. 3A). As for IL-6, it was significantly decreased in OGD/R rat astrocytes stimulated by medium or high glucose intervened with Wnt3a compared with OGD/R rat astrocytes stimulated by different glucose concentrations ($P < 0.05$) (Fig. 3B). What is more, Wnt3a intervention decreased the expression of HIF-1α in OGD/R rat astrocytes stimulated by medium glucose (Fig. 3C). We suggested that Wnt3a intervention inhibited inflammatory response in OGD/R rat astrocytes stimulated by different concentrations of glucose.

Table I. The primer of target genes

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<thead>
<tr>
<th>Gene</th>
<th>Sequence of primer (5’ to 3’)</th>
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<tr>
<td>TNF-α</td>
<td>F: CATGATCCGGAGATGGAAGCT R: TCAACAGAACAGTGGCAAGAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: CTAGGAAAGACTGGCAATATG R: AAACATCCTGGCTAGTAAGA</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>F: CGAAGAACTCTCAGCACCAG R: AGACTCGTGTCCTCACAGATTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: CGACCCCCCCTATTGACCTCAACTACATG R: CCCCGGCCCCTCCATGGTGTAAGAC</td>
</tr>
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Fig. 1. OGD/R induced inflammatory response in rat astrocytes. A-C The mRNA expression of TNF-α (A), IL-6 (B) and HIF-1α (C) in rat astrocytes was detected by qRT-PCR. $* p < 0.05$, $** p < 0.01$, comparison of inflammatory factors in astrocytes with or without OGD/R intervention.
Acute ischemic stroke is a common complication of diabetes mellitus, and hyperglycaemia is one of the major risk factors for AIS, leading to aggravated neuronal damage after cerebral ischemia/reperfusion (I/R) [17]. OGD/R is widely used as an in vitro model for stroke, can simulate the process of ischemia reperfusion, showing similarities with the in vivo models of brain ischemia [27]. In our study, we explored the role of Wnt3a in the AIS inflammatory response by constructing an OGD/R rat astrocyte model and stimulating with low, medium and high concentrations of glucose to simulate the effects of different degrees of hyperglycaemia in AIS. We found that cell growth in the OGD/R group was significantly inhibited, while the levels of cellular inflammatory factors were significantly increased. The results of Zhu et al. also showed that the protein expression of NLRP3, ASC, CL-caspase-1, IL-1β and IL-18 were significantly increased in HT-22 cells of the OGD/R group compared with the control group [37]. These results indicated that we constructed an OGD/R rat astrocytes model.

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**Fig. 2.** Wnt3a intervention promoted cell survival of OGD/R rat astrocytes. Scale bar = 100 μm.

**Fig. 3.** The expression of inflammatory factors in the OGD/R model stimulated by different glucose concentrations (with/without Wnt3a intervention). A-C) The mRNA expression of TNF-α (A), IL-6 (B) and HIF-1α (C) in rat astrocytes was detected by qRT-PCR. *p < 0.05, comparison of inflammatory factors in astrocytes with or without Wnt3a intervention.
It is known that Wnt signalling pathways include the classical Wnt/β-catenin pathway, the planar cell polarity (PCP) pathway, and the Wnt-5a/Ca\(^2+\) pathway [10,14,26]. The research showed that the above pathways are important signalling pathways in the genesis, development and maturation of the central nervous system [23]. The experiments proved that Wnt3a promoted the repairing or regeneration of nerve cells and vascular endothelial cells in cerebral ischemia rats by regulating Wnt/β-catenin signalling pathway [3]. Inoue et al. and Yoshinaga et al. pointed out that Wnt3a may accelerate neuronal regeneration by shortening the time and period required for cell proliferation, thereby promoting neuronal repair, regeneration and proliferation [11,34]. It has been shown that β-catenin can enter the nucleus and induce cell survival-related gene expression to promote cell regeneration. β-catenin in the cytoplasm can bind to the axIN1/APC/GSK-3β complex and be phosphorylated and degraded. Therefore, extracellular Wnt that binding with its receptor could destroy the above-mentioned complex, promote the accumulation of more β-catenin and enter the nucleus for regeneration regulation [15,22]. The Wnt/β-catenin pathway not only regulates neuronal regeneration, but also may be involved in inducing stem cell differentiation. Studies which used rat neural stem cells to simulate hypoxic-ischemic encephalopathy (HIE) and treated with hyperbaric oxygen, suggested some neural stem cells (NSC) can differentiate into oligodendrocytes or even neurons through Wnt3/β-catenin pathway and BMP2 signalling pathway [5,7]. However, few studies have been reported on astrocyte regeneration after OGD/R that simulate ischemic hypoxia.

In our research, astrocyte survival was increased and the expression of inflammatory factors was decreased by Wnt3a intervention in OGD/R rat astrocytes. So it could be considered that Wnt3a has a protective effect on the OGD/R astrocyte model stimulated by different glucose concentrations. However, the OGD/R astrocyte model in this experiment was observed only by electron light microscope, which was a qualitative detection and might have certain errors. Further experiments are needed to reduce the error, such as the immunohistochemical combination of TUNEL staining method and NeuN (Mouse anti-neuronal nuclei (NeuN) to reveal the breaking of DNA strands of astrocytes. Comparing to the control group, we were able to find whether the number of astrocytes with DNA chain rupture after Wnt3a intervention changing, and these results further confirmed whether Wnt3a intervention could reduce apoptosis and promote cell regeneration in OGD/R rat astrocytes under different glucose concentrations. The specific mechanism is still unclear and further experimental investigation is needed to explore these.

Wnt signalling pathway plays a crucial role in regulating cellular homeostasis and energy balance from hypothalamus to metabolic organs [21]. It is currently believed that the occurrence of type 2 diabetes mellitus is related to the inflammatory response caused by abnormal lipid metabolism, and Wnt signalling pathway plays an important role in adipocyte formation [4,13]. By promoting non-classical Wnt signalling pathway and inhibiting typical Wnt pathway, abnormal adipocyte development and adipocyte formation can be initiated [1]. Studies have suggested that one of the mechanisms of type 2 diabetes is that the Toll-like receptors (TLRs) and NOD-like receptors (NLRs) pathways lead to activation of NF-κB, promote the release of pro-inflammatory cytokines by immune cells [25]. Then macrophages in adipose tissue are recruited and differentiated into M1 (classical activation) and M2 (alternative activation) subtypes, which ultimately leads to chronic low-grade inflammation and interferes with insulin signalling in metabolic tissues, leading to type 2 diabetes [2,16]. In addition, more and more in vitro and animal experiments have confirmed that the molecular mechanism of anti-diabetic agents may be related to the pathophysiological transformation of neurons in many diseases such as Alzheimer’s disease and Parkinson’s disease, and even active hypoglycaemic treatment may promote the repair and regeneration of neurons [32,33]. A large number of animal experiments have proved that immunomodulatory therapy for cerebral infarction is effective [29,36]. Therefore, we hypothesized that if the formation of adipocytes is inhibited by regulating the Wnt signalling pathway, the number of macrophages recruited and stimulated to differentiate would be further reduced, thus reducing the inflammatory response from the upstream of the metabolic link and further reducing the possibility of diabetes. Controlling diabetes or hypoglycaemic treatment plays a neuroprotective role.

In conclusion, Wnt3a intervention can significantly improve the survival rate of astrocytes in OGD/R rats, and inhibit the expressions of inflammatory factors TNF-α, IL-6 and HIF-α induced by OGD/R. Wnt3a may play an anti-neuroinflammatory role in AIS by participating in the canonical Wnt signalling pathway.

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**Disclosure**

The authors report no conflict of interest.
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