Artesunate regulates the proliferation and differentiation of neural stem cells by activating the JAK-2/STAT-3 signaling pathway in ischemic stroke

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Abstract. Ischemic stroke is one of the most common causes of disability and death globally; therefore, the repair and reconstruction of the central nervous system (CNS) after stroke is very important. Neural stem/progenitor cells (NSPCs) may be the key to cell replacement therapy to treat CNS damage. It has previously been reported that artesunate (ART) is involved in the regulation of the biological functions of NSPCs; however, the mechanism of action of ART remains unclear. In the present study, different concentrations of ART were used to treat NSPCs following oxygen-glucose deprivation (OGD). Cell viability and apoptosis were analyzed using Cell Counting Kit-8 assay and flow cytometry, respectively, whereas immunofluorescence analysis was used to measure the expression levels of the differentiation-related molecule doublecortin (DCX) and proliferating cell nuclear antigen (PCNA). Western blotting was performed to analyze the expression levels of molecules related to the JAK-2/STAT-3 signaling pathway. The present results indicated that treatment with ART following OGD significantly promoted the viability of NSPCs, inhibited the apoptosis of NSPCs, and promoted the expression of PCNA and DCX. Moreover, ART significantly downregulated the protein expression levels of phosphorylated (p)-JAK-2 and p-STAT-3. Furthermore, activation of the JAK-2/STAT-3 signaling pathway and treatment with ART reversed the effects of ART on the proliferation, apoptosis and differentiation of NSPCs. In conclusion, the present data suggested that ART may promote the proliferation and differentiation of NSPCs, and reduce the apoptosis of NSPCs, by inhibiting the JAK-2/STAT-3 signaling pathway. ART may potentially be used for the treatment of ischemic stroke.

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Introduction

Ischemic stroke is one of the most disabling and fatal cerebrovascular diseases (1). In addition, >70% of survivors suffer from neurological dysfunction following a stroke (2). Improving neurological dysfunction after stroke has become an urgent medical and social issue. Neural stem/progenitor cells (NSPCs) are a group of pluripotent nerve cells with regenerative ability in the central nervous system (CNS) (3,4). NSPCs can self-renew through symmetrical division, or can generate neuronal progenitors and glial progenitors through asymmetric division, and can then differentiate into mature neurons, oligodendrocytes and astrocytes to maintain the homeostasis of CNS cell components (5-7). Therefore, it is important to promote the proliferation and differentiation of NSPCs to improve recovery after ischemic stroke.

Following CNS injury, NSPCs located in the subventricular zone and subgranular zone regions have been shown to proliferate, migrate and differentiate into neurons and glial cells, and serve therapeutic effects through nutritional support, regulation of inflammatory responses, directional differentiation for the replacement of neurons, reconstruction of neural circuits and functions, and release of paracrine nerve growth factors (8-10). These findings suggest that endogenous stem cells can repair CNS tissue damage. Notably, stem cell-based treatment of neurological diseases may be divided into exogenous stem cell transplantation and activation of endogenous neural stem cells (5).

Artesunate (ART), a water-soluble derivative of artemisinin with low toxicity (11), is an antimalarial drug that can easily penetrate the blood-brain barrier (12). ART has been demonstrated to have a potential role in cancer therapy, prevention of organ damage and dysfunction in hemorrhagic shock and trauma, regulation of immune and inflammatory responses, regulation of neurotransmission and treatment of type I diabetes (13-16). Artemisinin is insoluble in water, thus its application is severely inhibited. To overcome this issue, the water-soluble derivatives artelinic acid and ART have been

synthesized. Compared with ART, artemisinic acid has greater embryonic toxicity, neurotoxicity and nephrotoxicity (17). Moreover, ART can reach a high concentration in the brain; even if the level of ART drops significantly within 1 h in other tissues, it can still be detected in the brain, fat, intestines and serum (18). Therefore, ART may have a strong advantage in the treatment of neurological diseases. Dang *et al* (19) indicated that ART prolonged the survival of MRL/lpr mice, ameliorated symptoms of lupus nephritis and decreased the levels of pathogenic cytokines through activating the JAK-2/STAT-3 signaling pathway. Therefore, the present study investigated the effects of ART treatment on NSPCs following oxygen-glucose deprivation (OGD), in order to clarify the role of ART in ischemic stroke.

Materials and methods

Cell culture and establishment of an OGD model. A frozen aliquot of the third passage of NSPCs was donated by the Department of Neurosurgery and Key Laboratory of Neurotrauma, Southwest Hospital, Army Military Medical University (Chongqing, China) and was used for further experiments (20). As previously described, primary NSPCs were isolated from adult male C57BL/6 mice (age, 6-8 weeks; weight, 18-22 g) (20). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. Cells in the logarithmic growth phase were subjected to trypsinization and subculture with 0.25% trypsin (HyClone; Cytiva). The OGD model was established as previously described (20). Cells were incubated with glucose-free and FBS-free Earle's BSS buffer (Thermo Fisher Scientific, Inc.) at 37˚C for 8 h with 94% N_2, 5% CO_2 and 1% O_2. Different concentrations of ART (0.25, 0.5, 1, 2 and 4 μmol/l) and IL-6 (2 ng/ml) were added to medium to treat NSPCs for 48 h after ODG.

Cell counting kit-8 (CCK-8) assay. The viability of NSPCs was measured using the CCK-8 kit (Beyotime Institute of Biotechnology). CCK-8 reagent (10 μl) was added to the cultured NSPCs ($1x10^3$ cells per well) after OGD, followed by incubation at 37˚C for 2 h. The absorbance was determined at 450 nm using a microplate reader (BioTek Instruments, Inc.). The content of formazan dye was directly proportional to the number of live cells.

Flow cytometry. NSPCs ($1x10^6$) were harvested and washed three times with PBS. The apoptosis levels were measured using the Annexin V-FITC apoptosis detection kit (MilliporeSigma) according to the manufacturer' instructions. The cell suspension (190 μl) was incubated with 5 μl Annexin V-FITC and 5 μl propidium iodide. Subsequently, cells were incubated in the dark for 20 min at room temperature. Apoptotic cells were subsequently analyzed using a flow cytometer (BD Biosciences). The total apoptosis rate (early + late) was measured by ModFit LT 5.0 (Verity Software House) in this study.

Reverse transcription-quantitative PCR (qPCR). Total RNA was extracted from NSPCs using RNAiso Plus (Takara Bio, Inc.) and the A260/A280 ratio was calculated for RNA quantification. Total RNA was reverse transcribed into cDNA according to manufacturer's protocol (37˚C for 15 min and 85˚C for 5 sec) using the PrimeScript™ RT Master Mix (Takara Bio, Inc.). Subsequently, the SYBR Premix Ex Taq™ kit (Shanghai Yihui Biotechnology Co., Ltd.) was used to perform qPCR. The primer pairs for qPCR were designed using Primer Premier 6.0 software (PREMIER Biosoft), according to each gene sequence. The sequences were as follows: PCNA forward, 5'-CACCTTAGCACTAGTATTCGAAGCAC-3' and reverse, 5'-CACCCGACGGCATCTTTATTAC-3'; GAPDH forward, 5'-GACATCAAGAAGGTGGTGAAGC-3' and reverse, 5'-GAAGGTGGAAGAGTGGGAGTT-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 94˚C for 30 sec; 40 cycles at 94˚C for 15 sec and 60˚C for 60 sec. PCNA mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene GAPDH (21).

Immunofluorescence staining. NSPCs at 60-80% confluence were fixed in ice-cold acetone (Beyotime Institute of Biotechnology) at room temperature for 10 min, then washed three times with PBS and blocked with 5% normal goat serum (Boster Biological Technology) at room temperature for 1 h. Subsequently, cells were incubated with anti-PCNA (1:100 dilution; cat. no. ab18197; Abcam) and anti-DCX (1:400; cat. no. ab18723; Abcam) primary antibodies at 4˚C overnight. Following primary antibody incubation, the cells were washed with PBS and incubated with a fluorescence-labeled secondary antibody (1:100 dilution; cat. no. BA1032; Boster Biological Technology) at 37˚C for 1 h. A group only treated with secondary antibody was used as a negative control. The nuclei were counterstained with DAPI (Beyotime Institute of Biotechnology). The stained cells were observed under a Leica fluorescence DMLB microscope (Leica Microsystems GmbH), and the images were captured using a CCD camera and analyzed using Image-Pro Plus v6.0 (Cool-SNAP-Pro; Media Cybernetics, Inc.).

Western blot analysis. Total protein was extracted from NSPCs using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a BCA assay and proteins (40 μg per lane) were separated by SDS-PAGE on 10% gels. Subsequently, the proteins were transferred onto a PVDF membrane (MilliporeSigma). Then the membranes were blocked with skimmed milk (Shanghai Yifan Biotechnology Co., Ltd.) at room temperature for 2 h and incubated at 4˚C for 24 h with primary antibodies against cleaved-caspase-3 (1:1,000 dilution; 19 kDa; cat. no. ab214430; Abcam), DCX (1:1,000; 45 kDa; cat. no. ab18723; Abcam), phosphorylated (p)-JAK-2 (1:3,000; 120 kDa; cat. no. ab32101; Abcam), JAK-2 (1:3,000; 130 kDa; cat. no. ab108596; Abcam), p-STAT-3 (1:3,000; 98 kDa; cat. no. ab32143; Abcam), STAT-3 (1:1,000; 88 kDa; cat. no. ab68153; Abcam) and GAPDH (1:5,000; 36 kDa; cat. no. ab8245; Abcam). Following primary antibody incubation, the membrane was incubated with HRP-conjugated IgG secondary antibody (1:4,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. The protein bands were visualized using an enhanced chemiluminescence

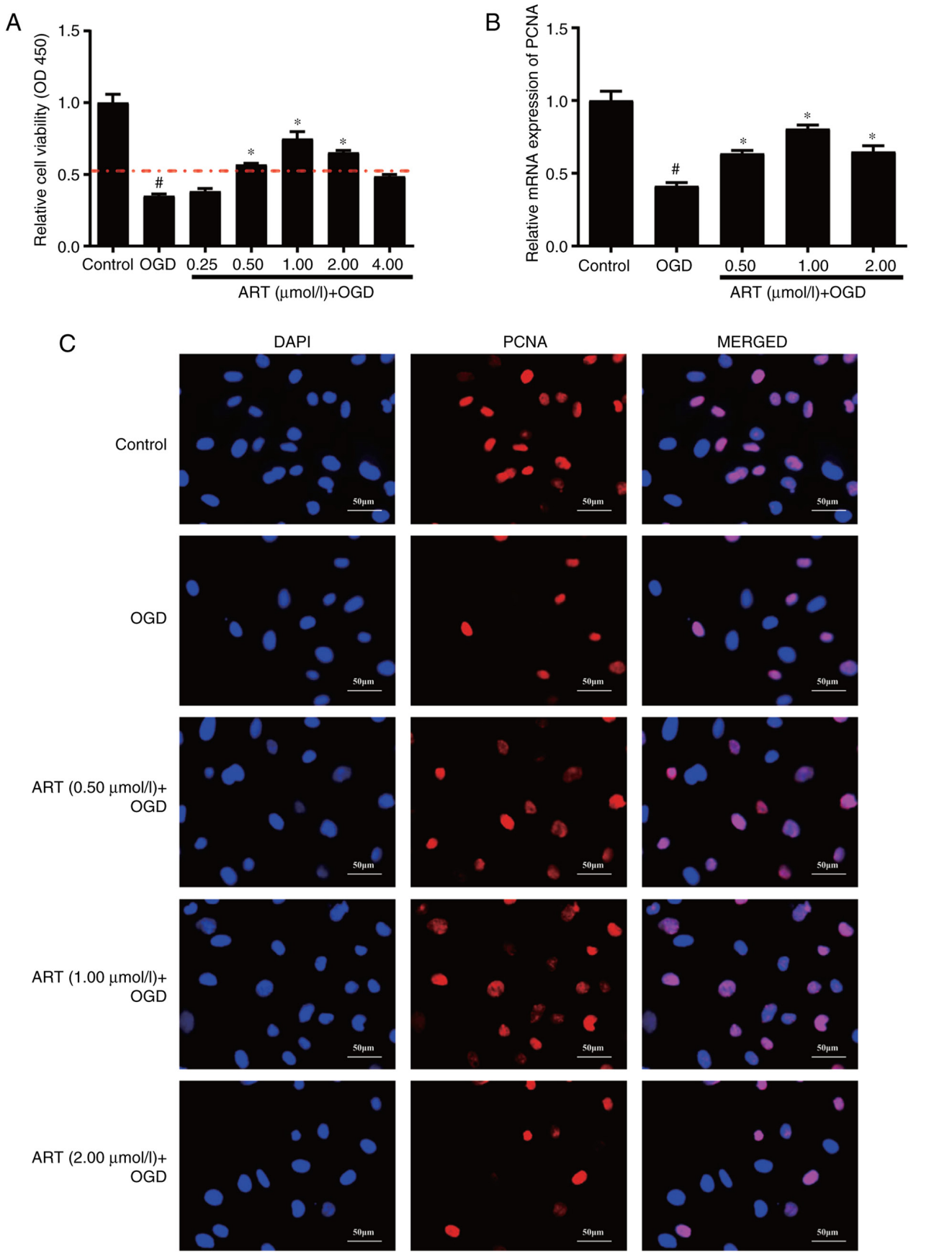

Figure 1. ART promotes the proliferation of NSPCs following OGD treatment. (A) Cell viability of NSPCs treated with ART after OGD was measured using the Cell Counting Kit-8 assay. (B) Reverse transcription-quantitative PCR analysis of the mRNA expression levels of PCNA in NSPCs treated with ART after OGD. (C) Immunofluorescence analysis of the expression levels of PCNA in NSPCs treated with ART after OGD (scale bar, 50 μm). $^{\#}$P<0.05 vs. control group; *P<0.05 vs. OGD group. The red dot-dashed line indicates OD450, 0.515, suggesting that ART (0.5, 1 and 2 μmol/l) promoted the viability of NSPCs after OGD. NSPC, neural stem/progenitor cell; ART, artesunate; OGD, oxygen-glucose deprivation; PCNA, proliferating cell nuclear antigen; OD450, optical density at 450 nm.

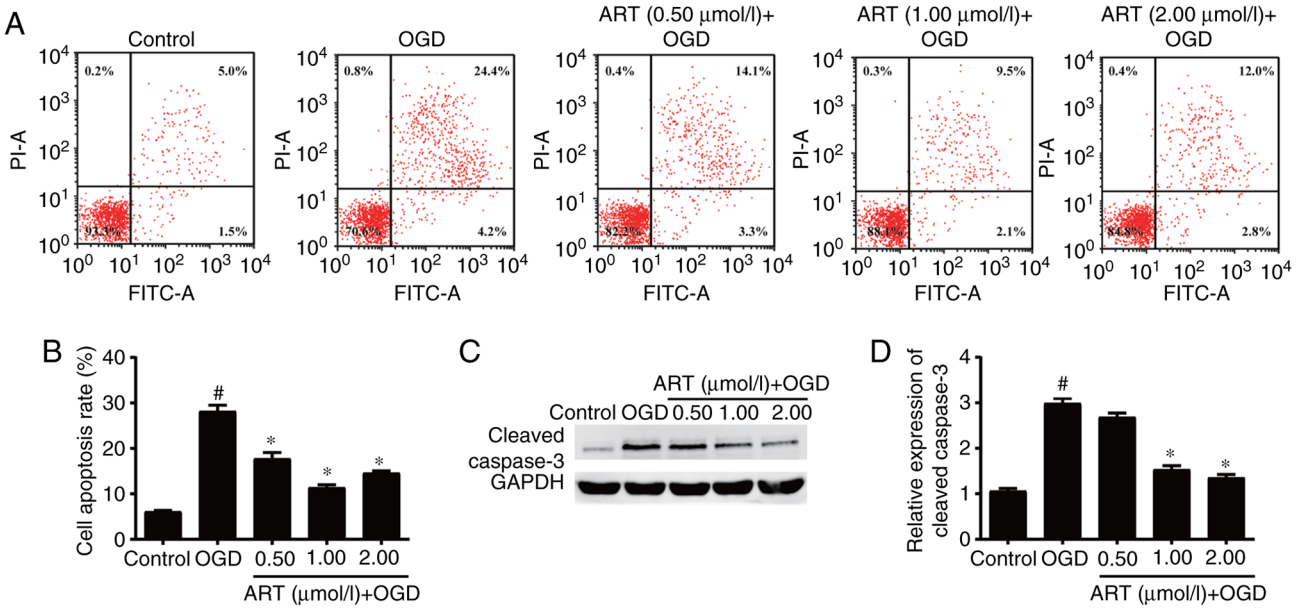

Figure 2. ART inhibits the apoptosis of NSPCs after OGD. (A) Flow cytometric analysis of the (B) apoptosis levels in NSPCs treated with ART after OGD. (C) Representative western blots and (D) expression levels of cleaved-caspase-3 in NSPCs treated with ART after OGD. #P<0.05 vs. control group; *P<0.05 vs. OGD group. NSPC, neural stem/progenitor cell; ART, artesunate; OGD, oxygen-glucose deprivation.

kit (cat. no. P1060-25; Applygen Technologies, Inc.). The expression levels were normalized to GAPDH using ImageJ version 1.49 software (National Institutes of Health).

Statistical analysis. Statistical analysis was performed using SPSS version 23.0 (IBM Corp.). All experiments were repeated at least three times. Data are presented as the mean ± standard deviation. One-way analysis of variance was used for multiple comparisons followed by Bonferroni correction or Student-Newman-Keuls post-hoc test. A P<0.05 was considered to indicate a significant difference.

Results

ART promotes the proliferation of NSPCs. To explore the effect of ART on the viability of NSPCs, different concentrations of ART (0.25, 0.5, 1, 2 and 4 μmol/l) were used to treat NSPCs for 48 h after ODG. The CCK-8 assay indicated that the viability of NSPCs after ODG was significantly decreased, whereas ART promoted the viability of NSPCs after OGD at concentrations of 0.5, 1 and 2 μmol/l (Fig. 1A). Therefore, these concentrations were used for further experiments. Moreover, ODG significantly inhibited the mRNA expression levels of PCNA in NSPCs compared with those in the control group (Fig. 1B), whereas ART treatment following ODG significantly increased the mRNA expression levels of PCNA in NSPCs compared with those in the ODG group (Fig. 1B). Furthermore, immunofluorescence staining indicated that ART enhanced the fluorescence intensity of PCNA in NSPCs after ODG (Fig. 1C). Taken together, these data indicated that ART promoted the proliferation of NSPCs after OGD.

ART inhibits apoptosis of NSPCs. The proliferation and apoptosis of NSPCs have an important role in repairing nerve function after ischemic stroke (7). Flow cytometry suggested that OGD promoted the apoptosis of NSPCs compared with that in the control group, whereas treatment with ART decreased the rate of apoptosis of NSPCs after OGD compared with that in the OGD group (Fig. 2A and B). Western blot analysis demonstrated that OGD significantly increased the expression levels of cleaved-caspase-3 in NSPCs compared with those in the control group, whereas ART treatment at concentrations of 1 and 2 μmol/l significantly decreased the protein expression levels of cleaved-caspase-3 in NSPCs after ODG compared with those in the ODG group (Fig. 2C and D). These results demonstrated that ART may inhibit the OGD-induced apoptosis of NSPCs.

ART enhances the differentiation of NSPCs. NSPCs are capable of self-renewal, and are able to differentiate into neurons, oligodendrocytes and astrocytes (3). NSPCs may be the key to cell replacement therapy following CNS damage. Immunofluorescence staining indicated that the fluorescence intensity of DCX was decreased in NSPCs following OGD compared with that in the control group, whereas ART treatment following OGD could partially restore the fluorescence intensity of DCX in NSPCs compared with that in the OGD group (Fig. 3A). Western blot analysis demonstrated that OGD significantly decreased the expression levels of DCX in NSPCs compared with those in the control group, whereas treatment with ART at 1 and 2 μmol/l following OGD significantly enhanced the expression levels of DCX compared with those in the OGD group (Fig. 3B and C). The present data indicated that ART may restore the differentiation of NSPCs impaired by OGD.

ART inhibits the JAK-2/STAT-3 signaling pathway in NSPCs. Activation of the JAK-2/STAT-3 signaling pathway is involved in the pathophysiological process of ischemic stroke and

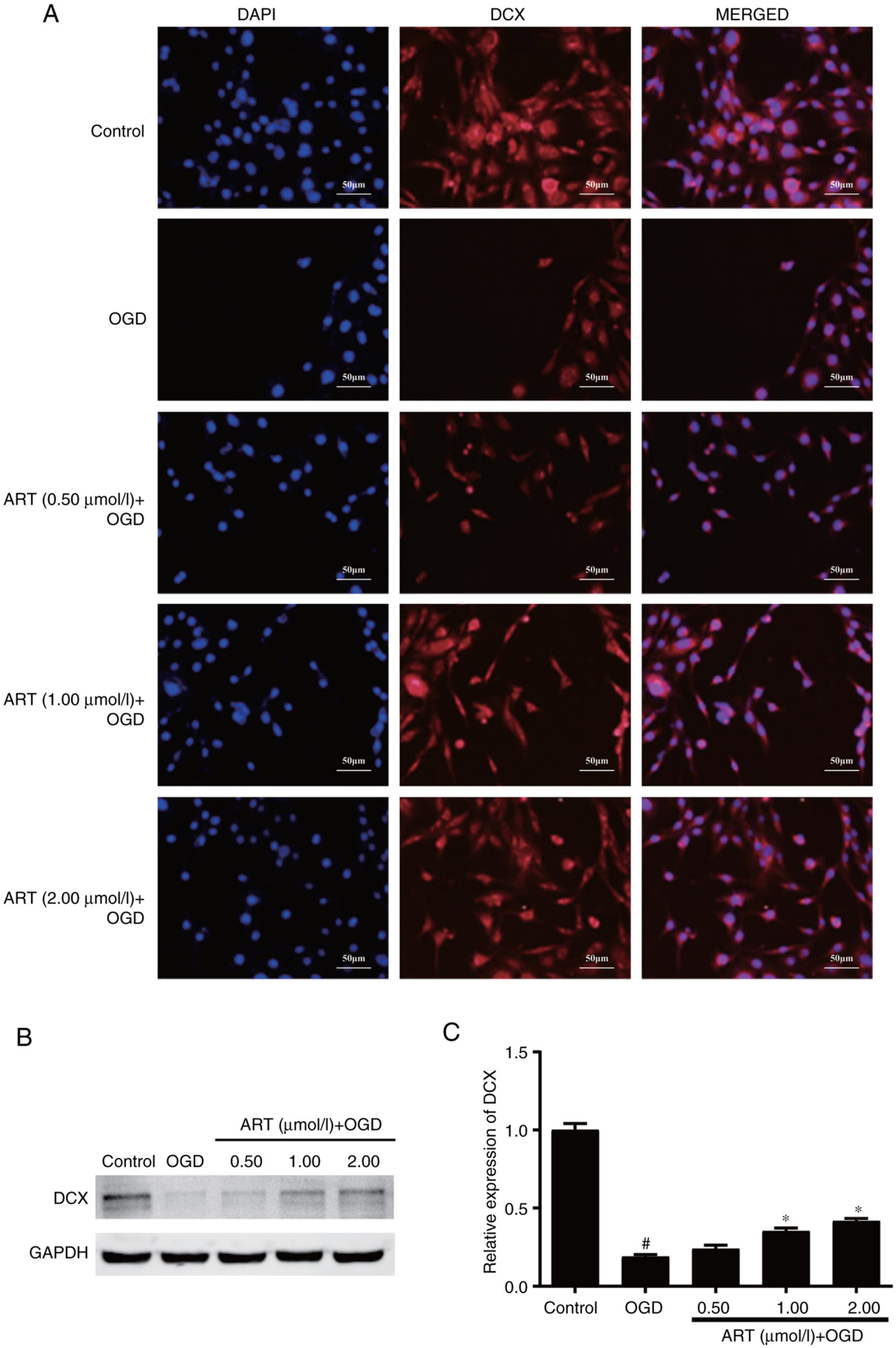

Figure 3. ART enhances the differentiation of NSPCs after OGD. (A) Immunofluorescence analysis of the expression levels of DCX in NSPCs treated with ART after OGD (scale bar, 50 μm). (B) Representative western blots and (C) relative expression levels of DCX in NSPCs treated with ART after OGD. $^{\#}$P<0.05 vs. control group; *P<0.05 vs. OGD group. NSPC, neural stem/progenitor cell; ART, artesunate; OGD, oxygen-glucose deprivation; DCX, doublecortin.

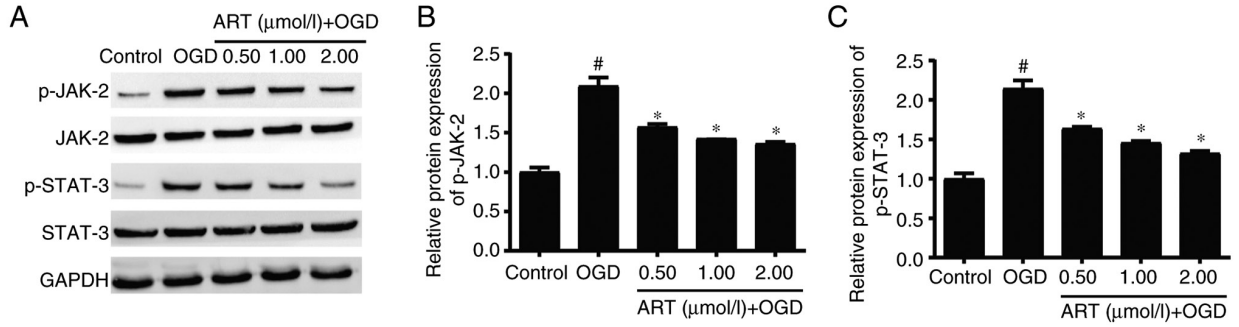

Figure 4. ART activates the JAK-2/STAT-3 signaling pathway in NSPCs after OGD. (A) Representative western blots of JAK-2, p-JAK-2, STAT-3, p-STAT-3 and relative expression levels of (B) p-JAK-2/total JAK-2 and (C) p-STAT-3/total STAT-3 in NSPCs treated with ART after OGD. $^{\#}$P<0.05 vs. control group; *P<0.05 vs. OGD group. NSPC, neural stem/progenitor cell; ART, artesunate; OGD, oxygen-glucose deprivation; p, phosphorylated.

affects the proliferation and differentiation of astrocytes, which is of great significance in the recovery of neurological function after ischemic stroke (22). Western blot analysis indicated that OGD significantly increased the expression levels of p-JAK-2 and p-STAT-3 in NSPCs compared with those in the control group, whereas ART treatment significantly downregulated the expression levels of p-JAK-2 and p-STAT-3 in NSPCs after OGD compared with those in the OGD group (Fig. 4A-C). These findings suggested that ART may regulate the biological functions of NSPCs through the JAK-2/STAT-3 signaling pathway.

ART regulates proliferation, apoptosis and differentiation of NSPCs after OGD through the JAK-2/STAT-3 signaling pathway. Previous studies have indicated that ART is able to inhibit the JAK-2/STAT-3 signaling pathway in different cells (23,24). In the present study, IL-6 was used to activate the JAK-2/STAT-3 signaling pathway. The results indicated that activation of the JAK-2/STAT-3 signaling pathway and treatment with ART reversed the effects of ART treatments on the proliferation (Fig. 5A), apoptosis (Fig. 5B-D) and differentiation (Fig. 5E and F) of NSPCs after OGD. Moreover, activation of the JAK-2/STAT-3 signaling pathway inhibited by ART may be reactivated by IL-6 in NSPCs after OGD (Fig. 5G-I). In summary, the present results indicated that ART may regulate proliferation, apoptosis and differentiation of NSPCs after OGD via inhibition of the JAK-2/STAT-3 signaling pathway.

Discussion

Ischemic stroke is a common cause of disability and death worldwide (25); notably, the repair and reconstruction of the CNS after stroke are very important (26). Following an ischemic stroke, NSPCs can accelerate proliferation and differentiation, migrate to the surrounding infarct area, differentiate into mature neurons or glial cells, and become part of the neuronal circuit (27). However, the proliferation of endogenous NSPCs caused by trauma or ischemia is not enough to induce nerve repair, which may lead to permanent disability in patients following a stroke (28). Zhu *et al* (29) reported that niche-dependent regulation of NSPC proliferation occurred following adult hypoxic ischemia injury via the novel RBM3/IMP2/IGF2 signaling pathway. Knotek *et al* (30) demonstrated that Wnt signaling regulated the biological function of NSPCs and could be considered useful in ischemic stroke therapy. Gan *et al* (31) indicated that long noncoding RNA H19 promoted NSPCs proliferation and differentiation via the p53 signaling pathway. During an ischemic stroke, due to damage to the blood-brain barrier, excitotoxicity and neuroinflammation destroy the cellular microenvironment to a great extent (32). The cell microenvironment presents an evident pathological environment in the damaged brain tissue, which is not conducive to the survival, neurogenesis and differentiation of endogenous NSPCs (33).

Recent studies have shown that ART has anti-neuritis, antioxidant and blood-brain barrier-protective functions, and multipotency in promoting neurogenesis (34,35). Liu *et al* (36) reported that ART inhibited neutrophil infiltration, microglial activation and inflammatory cytokines by suppressing the NF-κB pathway in the distal middle cerebral artery occlusion mouse model. Zhang *et al* (20) demonstrated that ART promoted NSPCs proliferation and reduced ischemia-reperfusion injury via the PI3K/Akt/FOXO-3a/p27 signaling pathway in ischemic stroke. The present study indicated that ART significantly promoted the proliferation of NSPCs after OGD, inhibited the apoptosis of NSPCs, and promoted the expression of PCNA and DCX. The present results indicated that ART may be considered a potential therapeutic drug for ischemic stroke.

JAK-2/STAT-3 is an important signaling pathway in the JAK/STAT family (37). JAK/STAT serves an important role in cell proliferation, differentiation and angiogenesis (38,39). After ischemic stroke, the expression of p-STAT-3 has been reported to increase significantly, and to participate in processes such as neuroinflammation and angiogenesis (40). Cheng *et al* (22) reported that endothelin-1 could promote the proliferation and differentiation of neural progenitor cells through the JAK-2/STAT-3 signaling pathway after transient middle cerebral artery occlusion. Furthermore, microRNA-101 has been shown to inhibit the apoptosis of neuronal cells and reduce ischemic brain injury through suppressing the JAK-2/STAT-3 signaling pathway in ischemic stroke (41). In addition, the JAK-2/STAT-3 signaling pathway has been shown to be activated in ischemic stroke, which may be closely related to cell apoptosis, angiogenesis, inflammatory response and oxidative stress in the pathogenesis of ischemic stroke (42). However, it remains unclear as to whether ART regulates the

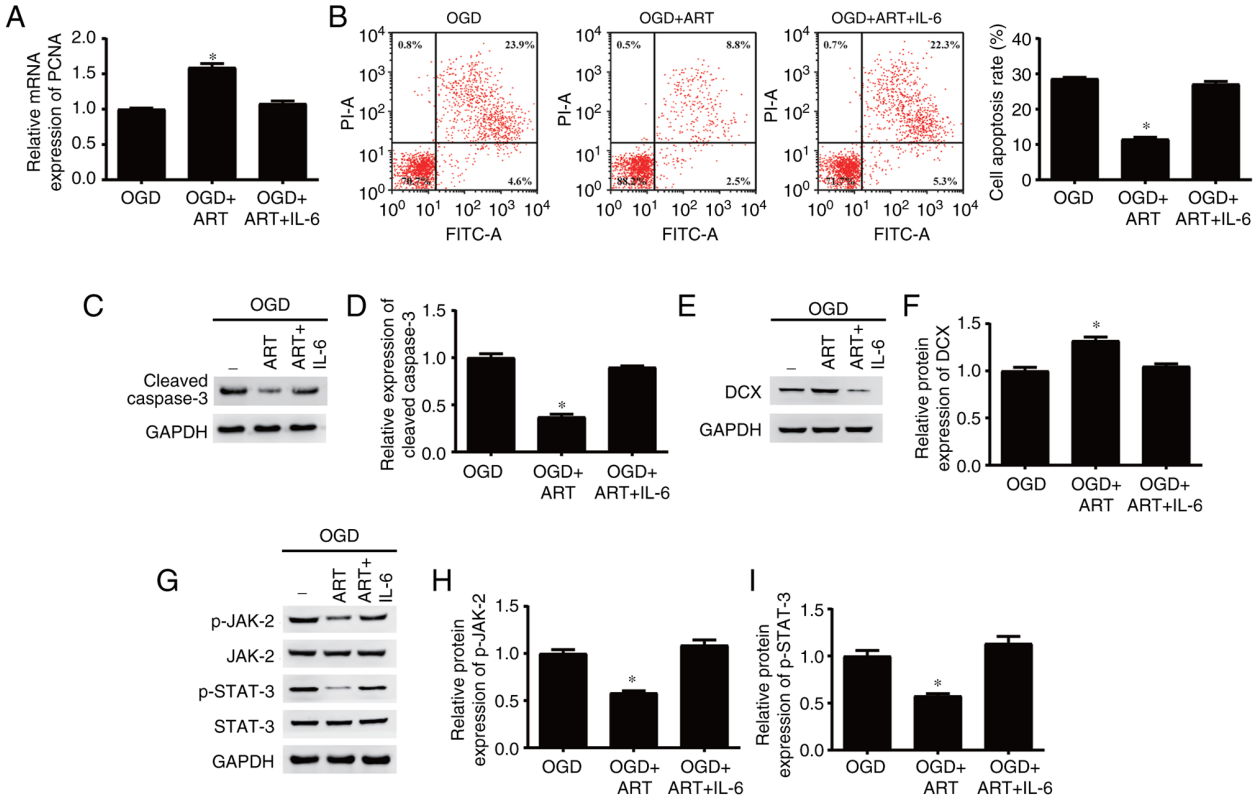

Figure 5. ART regulates proliferation, apoptosis and differentiation of NSPCs after OGD through activation of the JAK-2/STAT-3 signaling pathway. (A) Reverse transcription-quantitative-PCR analysis of the mRNA expression levels of PCNA in NSPCs treated with ART or ART + IL-6 after OGD. (B) Flow cytometric analysis of the apoptosis levels of NSPCs treated with ART or ART + IL-6 after OGD. (C) Representative western blots and (D) expression levels of cleaved-caspase-3 in NSPCs treated with ART after OGD. (E) Representative western blots and (F) expression levels of DCX in NSPCs treated with ART after OGD. (G) Representative western blots of JAK-2, p-JAK-2, STAT-3, p-STAT-3 and relative expression levels of (H) p-JAK-2/total JAK-2 and (I) p-STAT-3/total STAT-3 in NSPCs treated with ART or ART + IL-6 after OGD. *P<0.05 vs. OGD group; #P<0.05 vs. OGD + ART group. NSPC, neural stem/progenitor cell; ART, artesunate; OGD, oxygen-glucose deprivation; p, phosphorylated; PCNA, proliferating cell nuclear antigen.

JAK-2/STAT-3 signaling pathway in ischemic stroke. In the present study, OGD significantly increased the expression levels of p-JAK-2 and p-STAT-3 in NSPCs, whereas ART treatment significantly reduced the impact of OGD on the p-JAK-2 and p-STAT-3 expression in NSPCs. These results suggested that ART may regulate the biological functions of NSPCs through the JAK-2/STAT-3 signaling pathway.

IL-6 is an important inflammatory factor that promotes the phosphorylation of STAT-3 protein through JAK-2 (43). The IL-6/JAK-2/STAT-3 signaling pathway has been reported to be involved in various diseases, including tumors, ulcerative colitis, hyperuricemic nephropathy and chronic mild stress (44-47). In the present study, IL-6 was used to activate the JAK-2/STAT-3 signaling pathway. The present results indicated that the activation of the JAK-2/STAT-3 signaling pathway and treatment with ART could reverse the effects of ART on the proliferation, apoptosis and differentiation of NSPCs after OGD. Moreover, activation of the JAK-2/STAT-3 signaling pathway inhibited by ART could be reactivated by IL-6 in NSPCs after OGD. Therefore, these findings suggested that ART regulated the proliferation, apoptosis and differentiation of NSPCs after OGD by inhibiting the JAK-2/STAT-3 signaling pathway. Preliminary studies on cell migration (data not shown) indicated that ART had little effect on the migration of NSPCs; therefore, NSPCs migration should be further explored in future studies.

In conclusion, the present study demonstrated that ART could promote the proliferation and differentiation of NSPCs, and reduced the apoptosis of NSPCs by inhibiting the JAK-2/STAT-3 signaling pathway. Therefore, ART may be considered a promising therapeutic option for the effective treatment of ischemic stroke.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FW designed the experiments. YL and YB performed the experiments. YL and FW analyzed the data. YL, YB and FW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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