

# Effects of Low-dose Triamcinolone Acetonide on Rat Retinal Progenitor Cells under Hypoxia Condition

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## Abstract

**Background:** Retinal degenerative diseases are the leading causes of blindness in developed world. Retinal progenitor cells (RPCs) play a key role in retina restoration. Triamcinolone acetonide (TA) is widely used for the treatment of retinal degenerative diseases. In this study, we investigated the role of TA on RPCs in hypoxia condition.

**Methods:** RPCs were primary cultured and identified by immunofluorescence staining. Cells were cultured under normoxia, hypoxia 6 h, and hypoxia 6 h with TA treatment conditions. For the TA treatment groups, after being cultured under hypoxia condition for 6 h, RPCs were treated with different concentrations of TA for 48–72 h. Cell viability was measured by cell counting kit-8 (CCK-8) assay. Cell cycle was detected by flow cytometry. Western blotting was employed to examine the expression of cyclin D1, Akt, p-Akt, nuclear factor (NF)- $\kappa$ B p65, and caspase-3.

**Results:** CCK-8 assays indicated that the viability of RPCs treated with 0.01 mg/ml TA in hypoxia group was improved after 48 h, comparing with control group ( $P < 0.05$ ). After 72 h, the cell viability was enhanced in both 0.01 mg/ml and 0.02 mg/ml TA groups compared with control group (all  $P < 0.05$ ). Flow cytometry revealed that there were more cells in S-phase in hypoxia 6 h group than in normoxia control group ( $P < 0.05$ ). RPCs in S and G2/M phases decreased in groups given TA, comparing with other groups (all  $P < 0.05$ ). There was no significant difference in the total Akt protein expression among different groups, whereas upregulation of p-Akt and NF- $\kappa$ B p65 protein expression and downregulation of caspase-3 and cyclin D1 protein expression were observed in 0.01 mg/ml TA group, comparing with hypoxia 6 h group and control group (all  $P < 0.05$ ).

**Conclusion:** Low-dose TA has anti-apoptosis effect on RPCs while it has no stimulatory effect on cell proliferation.

**Key words:** Apoptosis; Cell Cycle; Hypoxia; Retinal Progenitor Cells; Triamcinolone Acetonide

## INTRODUCTION

Retinal degenerative diseases result in death of photoreceptors and are the leading causes of blindness in developed world.<sup>[1]</sup> Currently, no restorative clinical treatment exists. Some studies have shown that adult human retina retains retinal progenitors to reprogram cells to proliferate and differentiate into neuron-like cells *in vitro*.<sup>[2-4]</sup> Retinal progenitor cells (RPCs) have the potential to produce various types of cells. RPCs actively generate the mature neurons and Müller glia of the neural retina during eye development. A large body of evidence has now shown that RPCs can be isolated and cultured *in vitro*.<sup>[5,6]</sup> Our previous study indicated that retinal progenitor and photoreceptor precursor cell transplantation rescued the retinal structure and function in rats with retinal ischemia-reperfusion injury.<sup>[7]</sup> An experiment in mouse also indicated that cultured RPCs integrated in proximity to the ganglion cell layer and acted to preserve retinal

function as assessed by manganese-enhanced magnetic resonance imaging, optokinetic responses, and ganglion cell counts.<sup>[8]</sup> In addition to the direct ability to generate differentiated cells, RPCs may also maintain the survival of photoreceptors. RPCs can deliver the neurotrophic factor which is neuroprotective for the retinal neurons.<sup>[6]</sup> A study showed that transplantation of human neural progenitor cells enhanced retinal ganglion cell survival. Neuronal progenitor cells may be used as vehicles for local production and delivery of neurotrophic factors into the retina.<sup>[9]</sup> Therefore,

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how to protect RPCs in retina has become a hotspot in the treatment of retinal degenerative diseases.

Hypoxia is one of the main pathogeneses of retinal degenerative diseases, such as glaucoma, proliferative diabetic retinopathy (PDR), and age-related macular degeneration (AMD).<sup>[10]</sup> A study shows oxygen tension has emerged as a major regulator of stem cells. Low-oxygen concentrations that are toxic to mature cells can confer advantage to stem cells and early progenitors.<sup>[11]</sup> Low-oxygen culture conditions act to maintain both multipotency and self-renewal properties of human RPCs *in vitro*.<sup>[12]</sup> Hypoxia-induced metabolic stress in retinal pigment epithelial cells is sufficient to induce photoreceptor degeneration.<sup>[13]</sup>

Intravitreal glucocorticoid and anti-vascular endothelial growth factor (VEGF) therapies are widely used for the treatment of macular edema, neovascularization caused by retinal degenerative diseases, such as PDR, central retinal vein occlusion (CRVO), and AMD. Glucocorticoid possesses anti-edematous and anti-inflammatory effects. A growing number of researches suggest that glucocorticoid promotes the differentiation of embryonic stem cells.<sup>[14]</sup>

Triamcinolone acetonide (TA), a selective glucocorticoid receptor agonist, has been reported to reduce blood-retinal barrier breakdown, inhibit VEGF secretion, and prevent osmotic swelling of Müller cells.<sup>[15,16]</sup> Commonly used intra-articular corticosteroid has a dose-dependent, profound, and differential effect on mesenchymal stem cells *in vitro*.<sup>[17]</sup> In an early diabetic rat model, intravitreal injection of TA activates the glucocorticoid receptor and exerts neural protective effects on retinal neurons. Inhibition of the p38 mitogen-activated protein kinase pathway plays a critical anti-apoptotic role in retinal neurons of diabetes following TA treatment.<sup>[18]</sup> Although glucocorticoids are known from clinical practice to be effective, they may also have frequent and serious side effects, including secondary glaucoma and infections.<sup>[19]</sup> An experiment that subretinal injection of human RPCs under cyclosporin treatment showed that the RPCs gained a better survival rate in mice.<sup>[20]</sup> Hypoxia promotes the proliferation of monkey choroid-retinal endothelial cells while TA has the opposite effect in both normoxia and hypoxia conditions.<sup>[21]</sup> Current clinical applications of TA for the treatment of retinal degenerative diseases still lack a clear description of its mechanism; moreover, little is known about how low-dose TA interacts with RPCs in hypoxia condition. This study investigated the effects of TA on proliferation, apoptosis, and cell cycle of RPCs in hypoxia condition, which provided the experimental basis for the further study of cell therapy of retinal diseases.

## METHODS

### Primary cell culture

All experiments in this study were performed in Institute of Neurobiology, Medical School of Xi'an Jiaotong

University. One-day-old newborn Sprague-Dawley rats were used in this study. Rats were supplied by the center of experimental animals, Medical School of Xi'an Jiaotong University. RPCs from neural retinas were isolated and cultured in serum-free Dulbecco's modified Eagle medium/F12 medium containing N-2 supplement, B-27 serum-free supplement, epidermal growth factor, basic fibroblast growth factor (Gibco, USA), and 100 U/ml penicillin and streptomycin (Hyclone, USA). Cells were incubated in 25 cm<sup>2</sup> culture bottles at 37°C in 5% CO<sub>2</sub> environment. Neurospheres were formed after 2–3 days of culture, were disaggregated with TrypLE™ Select (Gibco) at 37°C for 3 min, and cultured in 6-well cell plates. The hypoxia groups were cultured under the condition of 37°C, 94% N<sub>2</sub> + 5% CO<sub>2</sub> + 1% O<sub>2</sub> for 6 h, which was used in previous experiments.<sup>[22]</sup> RPCs were observed under inverted phase contrast microscope (Leica DMI3000B, Leica Microsystems GmbH, Wetzlar, Germany).

### Immunofluorescence staining

Cells ( $5 \times 10^4$ /well) were seeded on 8 mm × 8 mm poly-L-lysine-coated slides in 24-well plates, incubated for 24 h to 48 h. Adherent RPCs were fixed with 4% paraformaldehyde for 20 min and washed three times with phosphate buffer solution (PBS). Cells were permeabilized in PBS with 0.3% Triton X-100 for 5 min. Then, samples were blocked with 5% normal goat serum for 1 h. Primary mouse anti-nestin antibody (1:1000, Abcam, UK) and rabbit anti-Pax-6 antibody (1:1000, Abcam) were incubated with the cells overnight at 4°C. After being rinsed extensively, cells were exposed to fluorescent secondary antibody (1:600, Alexa Fluor 488; 1:800, Alexa Fluor 594; Invitrogen, USA) in the dark for 60 min. The coverslips were rinsed again with PBS for three times and mounted with 4',6-diamidino-2-phenylindole for 5 min as a nuclear stain. Finally, cells were observed under fluorescent microscope (DP71, Olympus, Tokyo, Japan).

### Cell counting kit-8 assay

Cell viability was determined with cell counting kit-8 (CCK-8, 7Sea Pharmatech, China). TA (Kunming JIDA Pharmaceutical Co., Ltd., China) was sufficiently mixed and diluted proportionately by culture medium to different concentrations. RPCs were planted into 96-well plate ( $5 \times 10^3$ /well), divided into 8 groups as follows: control group (normoxia culture); hypoxia 6 h group; and 0.01, 0.02, 0.03, 0.05, 0.10, and 0.50 mg/ml concentrations of TA group (hypoxia 6 h culture). After 48 h and 72 h, 10 μl CCK-8 reagent was added to each well. Cells were incubated at 37°C for 4 h. The absorbance of each well was read with a plate reader at 490 nm. Each data point was obtained as an average of five values from five wells. The experiments were performed at least in triplicate.

### Cell cycle analysis

For the cell cycle analysis, RPCs were seeded in 25 cm<sup>2</sup> culture bottles at a density of  $1 \times 10^6$  cells/bottle. After incubation at 37°C for 24 h, cells were divided into four groups (normoxia

control group, hypoxia 6 h group, and 0.01 and 0.02 mg/ml TA under hypoxia 6 h group). Then, cells were cultured with TA for 72 h under normoxia condition. RPCs were collected and the cell suspension was added into 75% ice-cold ethanol, fixed overnight. Cells were washed twice with PBS and resuspended in 400  $\mu$ l of solution containing propidium iodide (100  $\mu$ g/ml) and RNase A (0.10 mg/ml, Sigma-Aldrich, USA) and then incubated in the dark for 20–40 min. Finally, the Cell cycle distribution was detected by flow cytometry, and cells were analyzed using FACSCalibur and FACSsort CellQuest software (BD Biosciences, New Jersey, USA).

### Western blotting analysis

The cells were lysed in RIPA buffer (Sigma-Aldrich, USA) supplemented with 1% protease inhibitor cocktail (Roche, Basel, Switzerland). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membranes were blocked in Tris-buffered saline and Tween 20 (TBST) containing 5% skim milk at room temperature for 3 h. Following this, the membrane was exposed to the primary antibodies (rabbit anti-cyclin D1 antibody, 1:1000, Abcam; anti-Akt antibody, 1:1000, Cell Signaling Technology, USA; anti-p-Akt antibody, 1:1000, Cell Signaling Technology; anti-nuclear factor [NF]- $\kappa$ B p65 antibody, 1:1000, Cell Signaling Technology; anti-caspase-3 antibody, 1:1000, Cell Signaling Technology) in a sealed bag overnight at 4°C, and then washed three times for 15 min each in TBST. Then, the membranes were incubated with secondary antibody (goat polyclonal secondary antibody to rabbit IgG-horseradish peroxidase, Abcam), diluted at 1:100,000 in TBST for 2 h at room temperature. The membranes were washed three times in TBST for 20 min

each time. An enhanced chemiluminescence substrate solution (Genshare, China) was placed on membranes for 2 min. Then, the membranes were placed in an LAS-3000 Fuji Film Intelligent Dark Box (GE, Connecticut, USA). The illuminated bands were detected and the image captured using LAS-3000 image reader software (GE). Western blotting analysis was performed at least on three separate occasions.

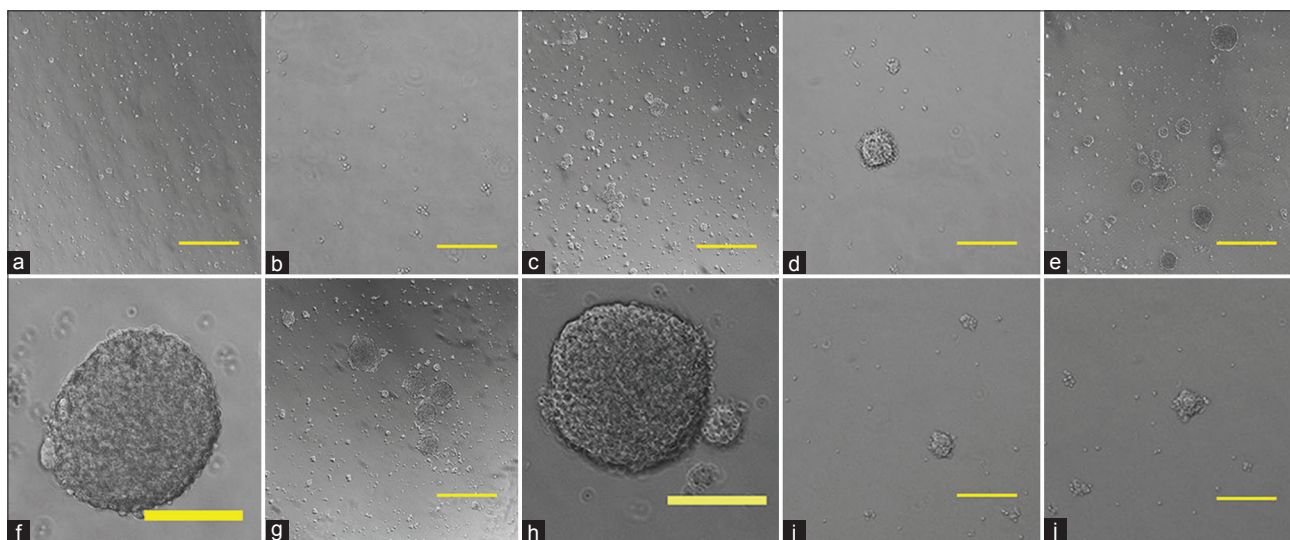
### Statistical analysis

All of the data are presented as means  $\pm$  standard deviation (SD). The statistical analyses were conducted using SPSS version 18.0 for Windows (SPSS Science Inc., Chicago, IL, USA). The statistical comparisons were performed using Student's *t*-test or analysis of variance. *P* < 0.05 was considered statistically significant.

## RESULTS

### Cell morphology of retinal progenitor cells under normoxia and hypoxia culture

P0 generation of RPCs appeared in individual cells and showed clear outline, abundant and bright cellular plasma, suspended in the medium [Figure 1a and 1b]. After 2 days of culture, the cells formed mulberry-like cell balls with strong refraction [Figure 1c and 1d]. P1 generation cells were cultured under hypoxia for 6 h and then continuously cultured for 48 h. The neurospheres were observed to be enlarged with the cellular plasma dark, and cell boundaries were found to be unclear [Figure 1e and 1f]. After 72 h, the neurospheres showed the trend to aggregation with each other with some of them dead and sunk to the bottom of the medium [Figure 1g and 1h]. Simultaneously, 0.01 and 0.02 mg/ml TA had no influence on the cell morphology after 24 h [Figure 1i and 1j].



**Figure 1:** Cell morphology of RPCs was observed under inverted phase contrast microscope. (a) P0 generation RPCs cultured in bottles. Scale bar = 200  $\mu$ m. (b) P0 generation RPCs. Scale bar = 100  $\mu$ m. (c) Mulberry-like cell balls formed after 2 days. Scale bar = 200  $\mu$ m. (d) P0 generation RPCs after 2 days. Scale bar = 100  $\mu$ m. (e and f) P1 generation RPCs in hypoxia 6 h group after cultured for 48 h. Scale bars = 100  $\mu$ m. (g and h) Hypoxia 6 h group after 72 h. Scale bars = 100  $\mu$ m. (i) 0.01 mg/ml TA group after 24 h. (j) 0.02 mg/ml TA group after 24 h. Scale bars = 100  $\mu$ m. RPCs: Retinal progenitor cells.

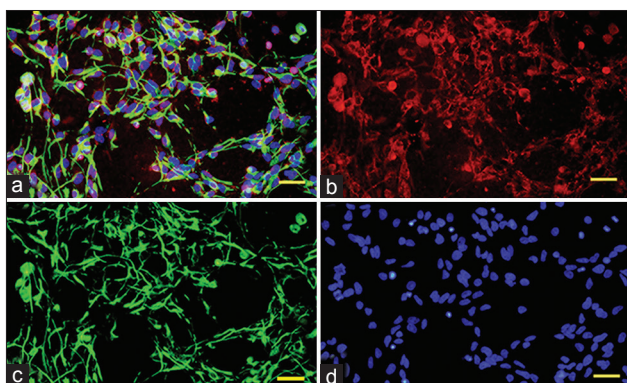


## Identification of retinal progenitor cells by cytoimmunofluorescence

The expression and distribution of nestin and Pax-6 in P1 generation RPCs were detected by immunofluorescence. P1 generation cells showed positive staining of nestin and Pax-6 [Figure 2a]. Under the physiology condition, Pax-6 (red) was expressed throughout the cytoplasm [Figure 2b] and nestin (green) was also localized in the cytoplasm in RPCs [Figure 2c]. Nuclei were stained blue with DAPI [Figure 2d].

## Cell viability

CCK-8 assays indicated cell viability in Hypoxia 6 h group and 0.01 mg/ml TA group were strengthened after 48 h, comparing with normoxia control group ( $P < 0.05$ ). However, no significant difference between hypoxia 6 h group and 0.01 mg/ml TA group was observed [Figure 3a]. After 72 h, the viability of cells treated with 0.01, 0.02 mg/ml TA in hypoxia group were improved and decreased when



**Figure 2:** Identification of retinal progenitor cells by immunofluorescence staining under the fluorescent microscope. (a) Merged images of nestin, Pax-6, and DAPI staining. (b) Pax-6 staining (red). (c) Nestin staining (green). (d) DAPI staining of cell nucleus (blue). Scale bars = 50  $\mu\text{m}$ .

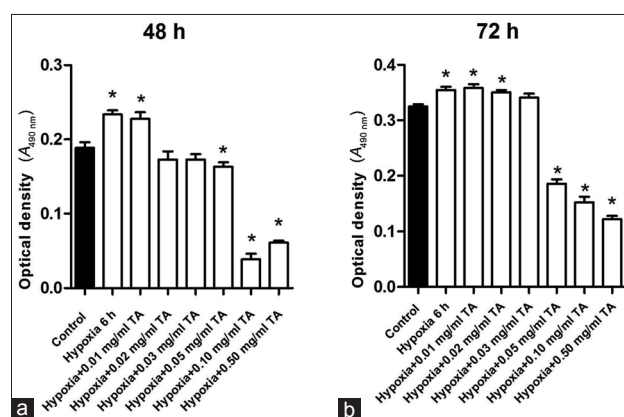
the concentrations of TA were above 0.05 mg/ml, comparing with normoxia control group [ $P < 0.05$ , Figure 3b].

## Effects of triamcinolone acetonide on cell cycle of retinal progenitor cells under hypoxia condition

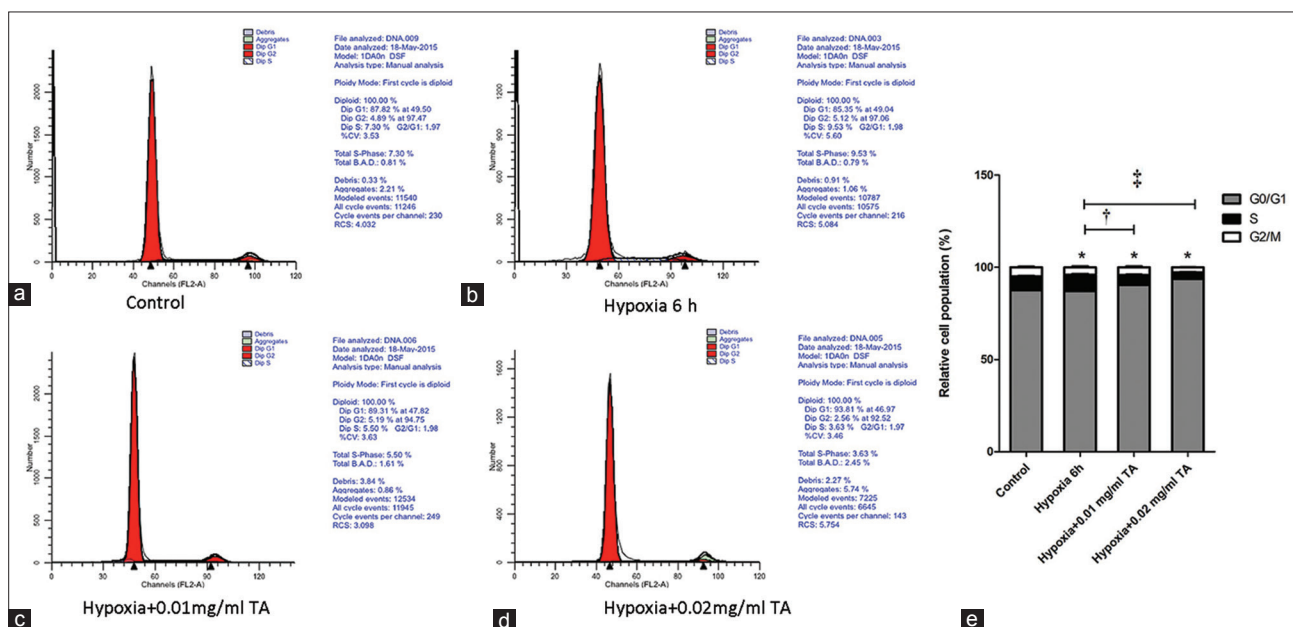
Flow cytometry revealed that there were more cells in S-phase in hypoxia 6 h group than normoxia control group ( $P < 0.05$ ). RPCs in S and G2/M phases decreased in groups given TA, comparing with hypoxia 6 h group and normoxia control group (all  $P < 0.05$ ) [Figure 4].

## Effects of triamcinolone acetonide on the protein expressions of Akt, p-Akt, cyclin D1, nuclear factor- $\kappa\text{B}$ p65, and caspase-3 in retinal progenitor cells under hypoxia

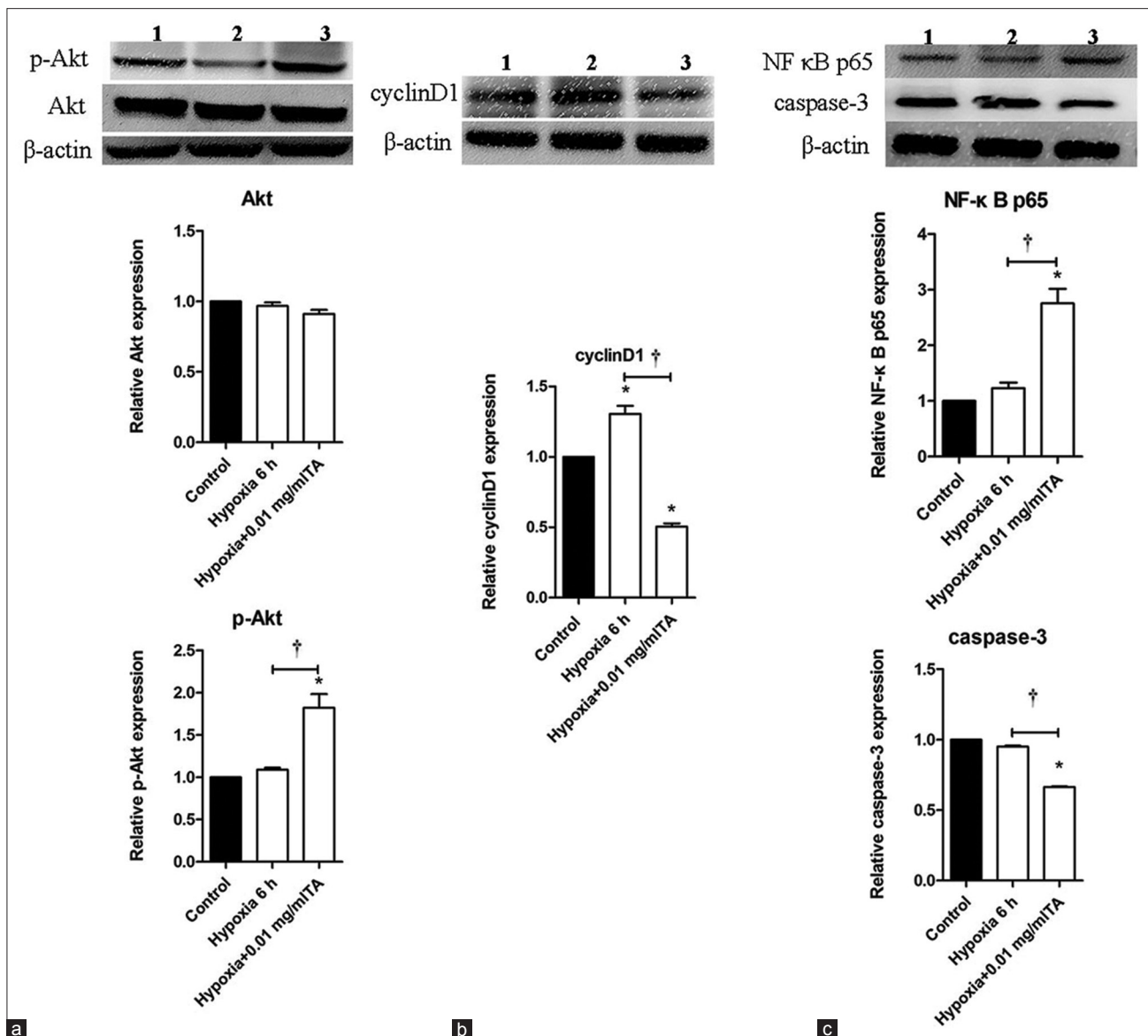
Total Akt expression has no significant difference among different groups while upregulation of p-Akt [Figure 5a]



**Figure 3:** Cell viability of retinal progenitor cells was measured using cell counting kit-8. (a) The absorbance of different groups after 48 h. (b) The absorbance of different groups after 72 h,  $*P < 0.05$ , vs. control group.



**Figure 4:** Cell cycle distribution of retinal progenitor cells was detected by flow cytometry. Effects of TA on cell cycle after 72 h. (a-d) Representative images of flow cytometry in different groups. (e) Cell cycle distribution of RPCs in different groups,  $*P < 0.05$ , compared with control group,  $\dagger P < 0.05$ , compared with hypoxia 6 h group. TA: Triamcinolone acetonide; RPCs: Retinal progenitor cells.



**Figure 5:** The protein expressions of Akt, p-Akt, cyclin D1, NF-κB p65, and caspase-3 in retinal progenitor cells were determined by Western blotting. (a) The expression of total Akt and p-Akt; (b) the expressions of cyclin D1; (c) the expressions of NF-κB p65 and caspase-3 in different groups (1: control group, 2: hypoxia 6 h group, 3: hypoxia + 0.01 mg/ml TA group), \* $P < 0.05$ , compared with control group; † $P < 0.05$ , compared with hypoxia 6 h group. TA: Triamcinolone acetonide; NF-κB: Nuclear factor-κB.

was observed in 0.01 mg/ml TA group compared with hypoxia 6 h group and normoxia control group (all  $P < 0.05$ ). Hypoxia 6 h stimulation improved the expression of cyclin D1, comparing with normoxia culture group [ $P < 0.05$ , Figure 5b]. However, in 0.01 mg/ml TA group, cyclin D1 expression was significantly reduced [ $P < 0.05$ , Figure 5b]. Upregulation of NF-κB p65 expression [Figure 5c] and downregulation of caspase-3 expression [Figure 5c] were observed in 0.01 mg/ml TA group compared with hypoxia 6 h group and normoxia control group (all  $P < 0.05$ ).

## DISCUSSION

RPCs are localized in several different tissues in the eye, including the ciliary marginal zone, neural retina, retinal

pigmented epithelium, and the iris. Being isolated from explants of neonatal rat retinas, RPCs have an identical morphology to nervous system progenitor cells. They are positive for nestin and showed photoreceptor cell markers.<sup>[23,24]</sup> We have isolated and cultured human fetal and rat RPCs successfully in our previous research.<sup>[7]</sup> Nestin is considered as a marker of nerve stem cell and Pax-6 directly controls multipotent state of RPCs.<sup>[25]</sup> In this study, the culture medium we used was neuron stem cells specific medium; meanwhile, the results of immunofluorescence, the morphology and growth of the cells proved the cells we cultured were RPCs.

Ischemia, hypoxia, and ischemia-reperfusion injuries are the leading causes of retinal degenerative diseases such as AMD, PDR, and CRVO. Effects of hypoxia environment on the

RPCs have direct relation to the prognosis of these diseases. In our previous study, under the hypoxia 4 h, RPCs were swelling and the boundaries were obscure. After 8 h, cell death occurred. After 12 h, most of the cells died and sunk to the bottom of the culture medium. We found RPCs had stronger anti-hypoxia ability than that of neural stem cells. In this study, hypoxia 6 h was taken as a stimulus to RPCs so that the injuries to the cells were slight and reversible. After being cultured under hypoxia condition for 6 h, RPCs were cultured under normoxia condition for 48 h to 72 h, in presence or absence of TA.

In our study, CCK-8 assays indicated that low-dose TA (0.01 mg/ml) in hypoxia condition increased RPCs activation, while in cell cycle analysis, S and G2/M phases were not improved as expected. S-phase increased in hypoxia group without TA, which was consistent with previous research.<sup>[26]</sup> Low-oxygen culture conditions act to maintain both multipotency and self-renewal properties of human RPCs *in vitro*.<sup>[12]</sup> According to our results, no significant difference between hypoxia 6 h group and 0.01 mg/ml TA group was observed. Therefore, low-dose TA alone had no proliferation stimulating activity on the RPCs while a short period of hypoxia can stimulate the proliferation of rat RPCs and a long time of hypoxia would lead to cell death.

Cyclin D1 is a cell cycle regulatory protein which plays a key role in cell proliferation and cell cycle transition from G1 phase to S-phase. Study with protein cyclin D1 achieved the same result of cell cycle test, which revealed that TA would not promote RPCs to enter S-phase from G1 phase. The experiments above suggested that TA had no stimulative effect on cell proliferation.

The serine/threonine kinase Akt is a potent regulator of cell survival. Akt may activate the transcription factor NF- $\kappa$ B functions to block apoptosis.<sup>[27]</sup> NF- $\kappa$ B family consists of five members, namely, p65, RelB, c-Rel, p50, and p52. The p65/p50 heterodimer is the main active form of NF- $\kappa$ B and the p65 subunit is indispensable for cell survival. As a ubiquitous nuclear transcription factor, NF- $\kappa$ B is one of the most important substrates of the activated Akt.<sup>[28]</sup> Simultaneously, the activation of caspase-3 is responsible for the cleavage of p65 and is considered as the terminal event preceding cell death that plays an extremely important role in neuronal apoptosis.<sup>[29-31]</sup> Therefore, we speculated that activation of Akt and inhibition of caspase-3 took functions together to upregulate NF- $\kappa$ B p65 expression. We measured the expression level of Akt, p-Akt, NF- $\kappa$ B p65, and caspase-3 by Western blotting. Upregulation of p-Akt and NF- $\kappa$ B p65 and downregulation of caspase-3 were observed in TA groups, which confirmed our speculation.

In conclusion, we found that short-term hypoxia had the enhancement effect on the viability of RPCs. Low-dose TA had anti-apoptosis effects through NF- $\kappa$ B signaling pathway while it had no stimulation effect on cell proliferation. Meanwhile, the limitations of our study

should be pointed out. The molecular mechanisms of NF- $\kappa$ B signaling pathway need to be further detected. Fully explication of the complex molecular interaction among this pathway will be explored in subsequent experiments.

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### Conflicts of interest

There are no conflicts of interest.

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