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Gamma-Secretase Inhibitor, DAPT, Prevents the Development of Retinopathy of Prematurity in a Rat Model by Regulating the Delta-Like Ligand 4/Notch Homolog-1 (DLL4/Notch-1) Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Retinopathy of prematurity (ROP), or retrolental fibroplasia, affects premature infants who have undergone intensive care with oxygen therapy. This study aimed to investigate the inhibitory effect of the gamma-secretase inhibitor, DAPT, on neovascularization and its mechanism in a rat model of ROP.

Material/Methods: Sixty neonatal Sprague-Dawley (SD) rats included the control group (n=20), the model group (n=20), and the DAPT-treated group (n=20). The rat model of ROP was established using repeat cycles of oxygen inhalation. Enzyme-linked immunosorbent assay (ELISA) measured serum levels of vascular endothelial growth factor (VEGF), VEGF receptor-1 (VEGFR-1), and VEGFR-2. Histology of the retinal tissue included immunohistochemistry for the expression of Notch homolog-1 (Notch-1) and delta-like ligand 4 (DLL4). Retinal mRNA levels of DLL4, Notch-1, VEGF, VEGFR-1, and VEGFR-2 were evaluated with quantitative real-time polymerase chain reaction (qRT-PCR).

Results: The rat model of ROP showed increased serum levels of VEGF, VEGFR-1, and VEGFR-2 compared with the control group, which were decreased in the DAPT group. Histology of the retinal tissue in the model group showed degeneration of the retinal ganglion cells, and immunohistochemistry showed increased expression of Notch-1 and DLL4 compared with the control group and DAPT group. Retinal tissue in the model group had increased mRNA levels of DLL4, Notch-1, VEGF, VEGFR-1, and VEGFR-2 compared with the control group, and the DAPT group.

Conclusions: In a rat model, treatment with DAPT reduced the retinal changes associated with ROP with a mechanism that involved VEGF and its receptors through the DLL4/Notch-1 pathway.

MeSH Keywords: Receptor, Notch1 • Receptors, Vascular Endothelial Growth Factor • Retinopathy of Prematurity

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Background

Retinopathy of prematurity (ROP), or retrolental fibroplasia, is a bilateral eye disease that affects premature infants of low gestational age and low birth weight who have undergone intensive neonatal care that includes oxygen therapy [1,2]. ROP occurs in premature infants, especially in low birth weight infants and is characterized by retinal capillary dysplasia, retinal neovascularization, proliferative retinopathy, and retinal detachment, which may lead to permanent blindness [1]. Irreversible loss of vision can hinder cognitive and psychological development in children [1].

The pathogenesis of ROP is multifactorial and complex. Several cytokines are involved in the occurrence and development of ROP, including vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and hypoxia inducing factor-1α (HIF- 1α) [3]. In addition, the antioxidant system of premature infants is not well developed, and the retina is prone to damage by oxygen free radicals, which is believed to be a main cause of ROP [4,5]. Hypoxia-induced retinal neovascularization is the main pathological change resulting from ROP [6]. In an environment of hyperoxia, a high concentration of oxygen also promotes contraction and occlusion of retinal vessels in premature infants. Subsequent relative hypoxia of retinal tissues results in secretion of a series of angiogenic factors, leading to the pathological neovascularization that characterizes ROP. Abnormal retinal vascular proliferation can result in retinal degeneration and detachment, cataract formation, secondary glaucoma, strabismus, amblyopia, and other complications [7]. Retinal cryotherapy, laser treatment, scleral surgery, and vitrectomy are currently used to treat ROP [7,8]. However, long-term follow-up studies have shown that up to 50% of pediatric patients with ROP have a poor prognosis in terms of loss of visual acuity and reduced visual field, and other visual defects [8].

The Notch signaling pathway is highly evolutionarily conserved and is widely present in invertebrates and vertebrates. The Notch receptor activates the Notch pathway through binding to its corresponding ligand, regulating cell differentiation and tissue development, including angiogenesis [9,10]. The adjacent intercellular delta-like ligand 4 (DLL4) is an activator of the Notch pathway that exerts a key role in physiological and pathological angiogenesis [9,10]. Embryonic vascular development and tumor angiogenesis regulated by the DLL4/Notch-1 pathway have recently been studied. A significant role for the DLL4/Notch-1 pathway has been demonstrated in angiogenesis *in vitro*, including the regulation of endothelial cell number, cell pseudopod extension, neovascularization and maturation in three-dimensional cultured primary endothelial cell cultures, zebrafish embryos, and tumor-bearing mice models [11–15].

This study aimed to investigate the inhibitory effect of the gamma-secretase inhibitor, DAPT, on neovascularization and its mechanism in a rat model of ROP that used hyperoxia-induced retinal neovascularization and the specific function of DLL4/Notch-1 pathway.

Material and Methods

Construction of the rat model of retinopathy of prematurity (ROP)

This study was approved by the Animal Ethics Committee of Guangdong Medical University Animal Center. Sixty neonatal Sprague-Dawley (SD) rats included the control group (n=20), the model group (n=20), and the DAPT-treated group (n=20). The rat model of ROP was established using repeat cycles of oxygen inhalation. Newborn rats, within 12 hours of birth, who were born naturally on and on the same day were randomly assigned to the three groups. Rats in the model group and the DAPT group were placed in a semi-enclosed plastic oxygen chamber containing 80±2% oxygen. Twenty-four hours later, nitrogen was introduced into the chamber until the oxygen concentration was adjusted to 10±2%, which was maintained for 24 hours. The temperature in the chamber was maintained at 23±2°C. The rats inhaled the oxygen for 7 days and were then transferred to the normal environment for 5–7 days.

Following the development of the rat model of ROP, the rats in the DAPT group were injected four times a day with 1 mg/kg DAPT via the tail vein for two days. Rats in the control group and the non-treated model group were injected with normal saline.

Sample collection

Rats were euthanized with ether inhalation. The eyeballs were removed by excision of the orbital bone. The harvested eyeball was washed with phosphate buffered saline (PBS). After injection of 4% paraformaldehyde into the vitreous cavity of the eyeball, the whole eyeball was fixed for 24 hours. Serum samples of rats were collected and stored at 4°C for analysis.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were centrifuged and the supernatant was collected. Serum levels of vascular endothelial growth factor (VEGF), VEGF receptor-1 (VEGFR-1), and VEGFR-2 were determined using an ELISA assay kit (R&D Systems, Minneapolis, MN, USA).

Histological examination of the eye

After the removed eyes had been fixed for 24 hours, the lens and vitreous were removed, followed by further fixation and

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dehydration using graded ethanol. The eyes were embedded in paraffin wax and tissue sections were cut onto glass slides. The tissue sections were routinely stained with hematoxylin and eosin (H&E), and ten sections of each sample were selected to evaluate and count the number of vascular endothelial cells breaking through the retinal inner limiting membrane.

Retinal vascular analysis

Retinal vascular development of the rats in each group was observed dynamically using retinal smears and staining of blood vessels. Each retina was isolated and incubated in 0.5% Triton X-100 and 1% bovine serum albumin (BSA) at 4°C overnight. On the following day, the retina was washed and incubated with 1% Alexa Fluor™ isolectin GS-IB4 (Invitrogen, Carlsbad, CA, USA) overnight in the dark. The retinal tissue was sectioned onto poly-l-lysine coated glass slides, sealed with 50% glycerin, and stored at 4°C in the dark. The slides were observed using a fluorescence microscope. The retinal blood vessels were identified by green fluorescence, and the avascular zone was identified as a black area where the peripheral green fluorescence terminated and was not connected to the optic disc. The avascular zone and the total area of the retina were measured. The percentage of the area of the avascular zone to the total area of the retina was calculated.

Immunohistochemical staining

Retinal sections were dewaxed, dehydrated, and incubated with 3% H_2O_2 at room temperature for 10 min. Sections were blocked using the 5% normal goat serum in a 37°C water bath for 20 min. The tissue sections were incubated in primary antibodies to Notch homolog-1 (Notch-1) and delta-like ligand 4 (DLL4) and secondary antibodies, followed by color development using 3,3'-diaminobenzidine (DAB) (Solarbio, Beijing, China). The retinal tissue sections were observed using a light microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from retinal tissues, followed by quantitative real-time polymerase chain reaction (qRT-PCR) to determine the relative mRNA levels. The following primers were used:
DLL4: forward: 5'-TGCGGATAACCAACGACG-3';
DLL4: reverse: 5'-GCCCACAAAGCCATAAGGAC-3';
Notch-1: forward: 5'-TCGGAGTGGACAGGTCAGTA-3';
Notch-1: reverse: 5'-AGATACACGCATCGTTCAGG-3';
VEGFR-1: forward: 5'-GGCCACCACTCAGGACTACT-3';
VEGFR-1: reverse: 5'-GGCGCTTCCAAATcTCTAAC-3';
VEGFR-2: forward: 5'-GCGACCCCAAATTCCATTATG-3';
VEGFR-2: reverse: 5'-CTTCTGAGGCAAGGACCATCC-3';
β-actin: forward: 5'-GAGAGGGAAATCGTGCGTGA-3';
β-actin: reverse: 5'-GCCTAGAAGCATTTGCGGTG-3'.

Statistical analysis

Data were analyzed using the Statistical Product and Service Solutions (SPSS) version 20 software (IBM, Armonk, NY, USA). Data were expressed as the mean ± standard deviation (SD). An initial evaluation of normality and analysis of variance (ANOVA) between the two groups used the t-test or the F-test. A P-value <0.05 was considered to be statistically significant.

Results

Rat body weight and serum levels of vascular endothelial growth factor (VEGF), VEGF receptor-1 (VEGFR-1), and VEGFR-2

There was no significant difference in the gain in body weight of the 60 neonatal Sprague-Dawley (SD) rats in the control group (n=20), the rat model of retinopathy of prematurity (ROP) group (n=20), and the DAPT-treated group (n=20) from birth (P>0.05) (Figure 1A). Serum samples that were analyzed using an enzyme-linked immunosorbent assay (ELISA) showed significantly increased serum levels of VEGF, VEGFR-1, and VEGFR-2 in the model group compared with the control group, which were significantly decreased in the DAPT group (P<0.05) (Figure 1B–1D).

Histology of the retina in the ROP rat model group

Histology of the retinal layers of the eyes in the control group showed that they were intact (Figure 2A). In the model group, vacuoles and degeneration of the retinal ganglion cells were observed, the inner and outer nuclear layers were thin, retinal cells were disorganized, and the outer plexiform layer showed edema (Figure 2Aa, Ab). Cells in retinal ganglion cell layer in DAPT group were slightly swollen, and vacuoles were seen in the cells, but no other retinal lesions were found in DAPT group (Figure 2Ac).

Retinal vascular development

The retinal vascular development of neonatal rats in each group was observed dynamically by retinal smear and blood vessel staining. The growth of superficial blood vessels of the retina in the model group and the DAPT group lagged behind the control group (Figure 2B). The ratio of the retinal vascular area to the total retinal area in the model group and the DAPT group was significantly less than that of the control group (P<0.05) (Figure 2Ba–Bd). However, the DAPT group showed better retinal vascular development when compared with the model group.

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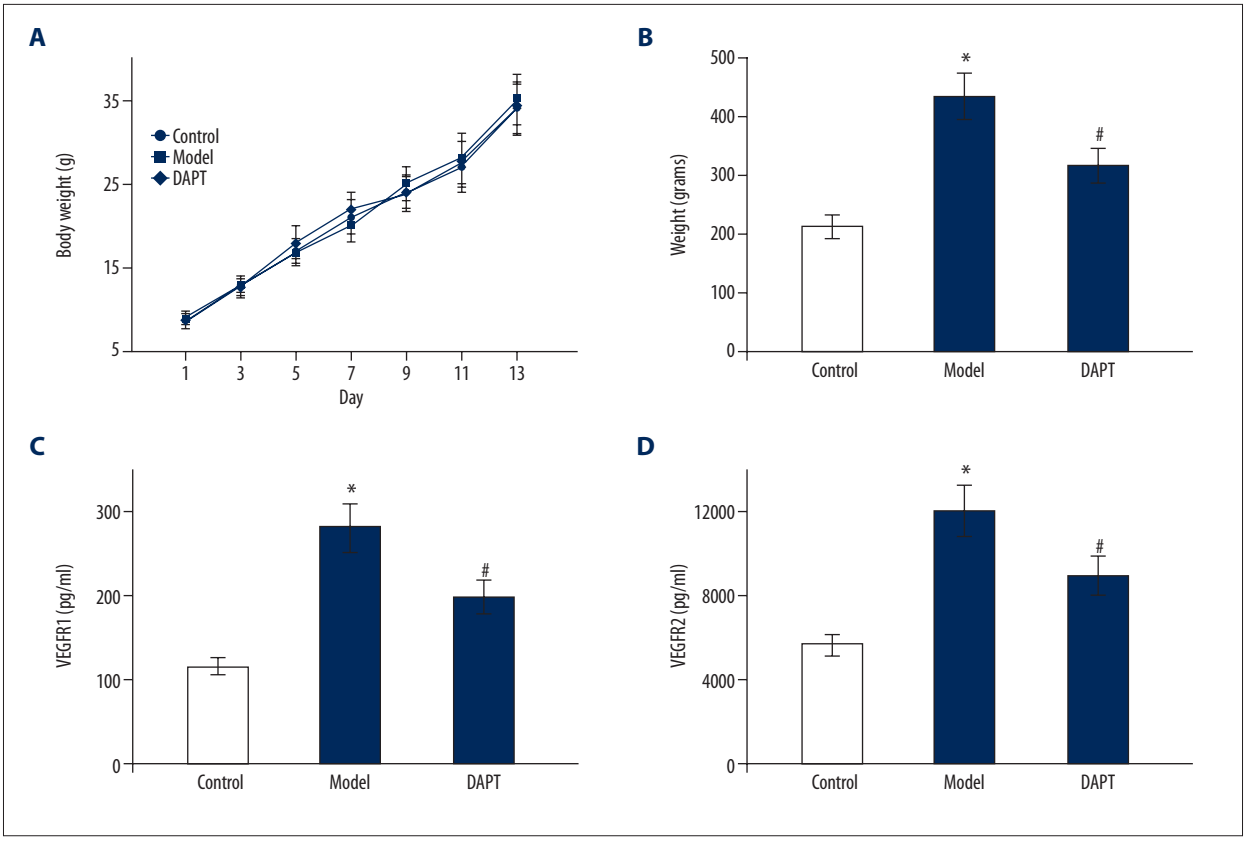

Figure 1. Rat body weight and serum levels of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-1 (VEGFR-1), and VEGFR-2 in the control group, the rat model of retinopathy of prematurity (ROP) group, and the DAPT-treated group (**A**) Body weight of rats in the control group, the model group, and the DAPT group. (**B**) Serum levels of vascular endothelial growth factor (VEGF) in rats in the control group, the model group, and the DAPT group. (**C**) Serum levels of vascular endothelial growth factor receptor-1 (VEGFR-1) in rats in the control group, the model group, and the DAPT group. (**D**) Serum level of VEGFR-2 in rats in the control group, the model group, and the DAPT group. * P<0.05, compared with the control group; # P<0.05, compared with the model group.

Immunohistochemical staining of the rat retina

Immunohistochemical staining of Notch homolog-1 (Notch-1) and delta-like ligand 4 (DLL4) in rat retina showed low levels of expression of Notch-1 in the retinal ganglion cell layer of neonatal rats in the control group (Figure 3Aa). By comparison, Notch-1 immunostaining was mainly seen in the retinal ganglion cell layer of neonatal rats in the model group, with low levels of expression in the inner nuclear layer with no immunostaining in other layers of the rat retina (Figure 3Ab). There were fewer Notch-1-positive cells in the retinal ganglion cell layer of neonatal rats in the DAPT group compared with the model group, and no positive immunostaining was found in the inner nuclear layer (Figure 3Ac). There was a significant difference in the percentage of Notch-1-positive cells between the three groups (P<0.05) (Figure 3Ad).

Immunostaining for DLL4 showed that positive cells were mainly in the retinal ganglion cell layer of rats in the control group, and were not found in other retinal layers (Figure 3Ba). Both the retinal ganglion cell layer and inner nuclear layer showed positive expression of DLL4 in rats in the control group (Figure 3Bb). DLL4 was only expressed in the retinal ganglion cell layer and inner nuclear layer, but was not expressed in vascular endothelial cells in the DAPT group (Figure 3Bc). The difference in the percentage of DLL4-positive cells among the three groups was statistically significant (P<0.05) (Figure 3Bd).

Relative expression of genes in the DLL4/Notch-1 pathway in rat retina

The relative expression levels of mRNA of the genes in the DLL4/Notch-1 pathway in each group were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Rats in the model group showed significantly higher mRNA levels of DLL4, Notch-1, VEGF, VEGFR-1, and VEGFR-2 compared with those of control group (P<0.05). However, DAPT treatment significantly decreased the expression of these genes when compared with the model group (P<0.05) (Figure 4A–4D).

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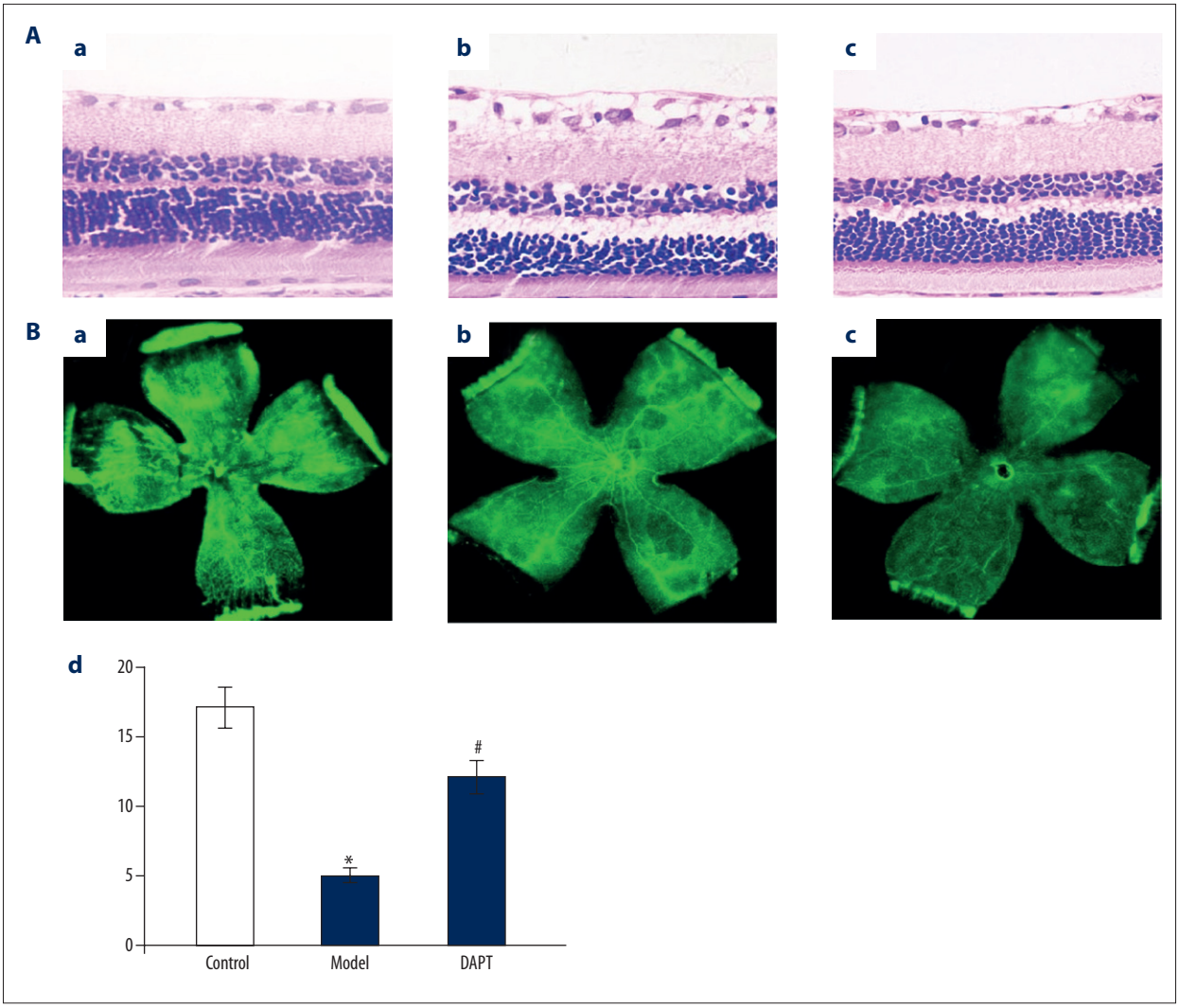

Figure 2. Photomicrographs of the rat retinal tissue in the control group, the rat model of retinopathy of prematurity (ROP) group, and the DAPT-treated group. (**A**) (**a**) Histology of the normal rat retina of the control group. (**b**) Histology of the retinal lesions in the rat model of retinopathy of prematurity (ROP) group. (**c**) Histology of the rat retina of the DAPT-treated group. Hematoxylin and eosin (H&E). (**B**) (**a**) Retinal vascular development in the control group. (**b**) Retinal vascular development in the model group. (**c**) Retinal vascular development in the DAPT group. (**d**) The percentage of the retinal vascular area to total retinal area. * P<0.05, compared with the control group; # P<0.05, compared with the model group.

Discussion

Retinopathy of prematurity (ROP), or retrolental fibroplasia, is a multi-gene disease, and its incidence increased with the development of premature infant monitoring. The main risk factors for ROP include prematurity, low birth weight, and oxygen treatment [16,17]. Unlike humans, retinal angiogenesis in neonatal rats begins after birth [18,19]. Normally, the increased demand for retinal oxygen due to growth leads to a relatively local hypoxic state resulting in secretion of vascular endothelial growth factor (VEGF) and blood vessel formation. Several pro-angiogenic factors are involved in the process of retinal neovascularization.

Delta-like ligand 4 (DLL4) was initially identified in arterial endothelial cells during embryonic development. Subsequent studies identified DLL4 expression in endothelial cell subpopulations, and recent studies have shown that vascular growth is precisely regulated by the feedback loop of VEGF and the Notch pathway [20–23]. VEGF can induce the expression of Notch homolog-1 (Notch-1) and DLL4, promoting the abnormal proliferation of tumor blood vessels by activating the DLL4/Notch-1 pathway [24,25]. As one of VEGF receptors, VEGFR-2 is abundantly expressed in endothelial cells of blood vessels, resulting in vascular leakage, the pathological proliferation of endothelial cells, and sprouting of new blood vessels. The binding of VEGF and VEGFR-2 can upregulate DLL4 expression

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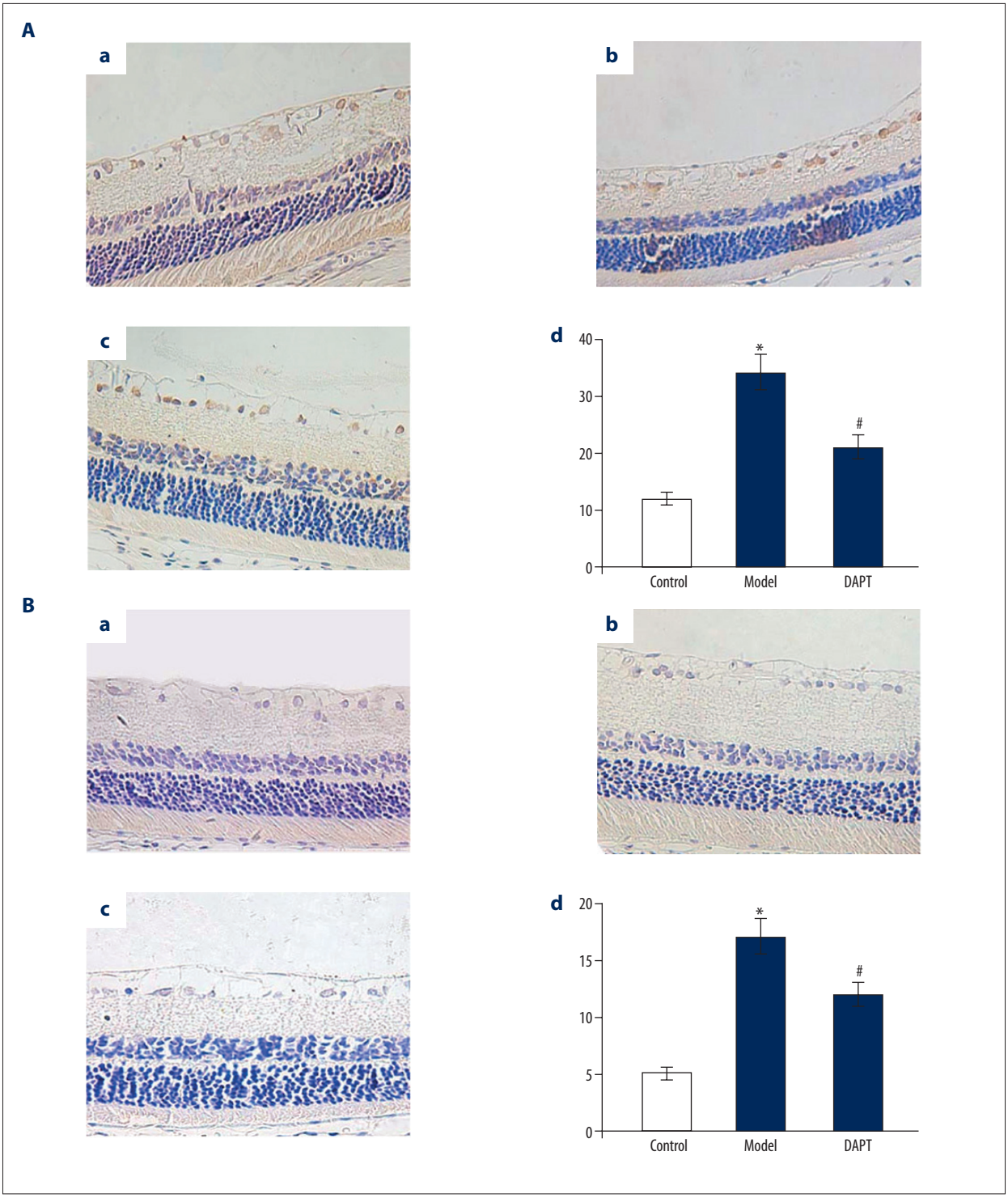

Figure 3. Immunohistochemical staining for Notch-1 and DLL4 in the rat retina. (**A**) (**a**) Immunohistochemical staining for Notch-1 in the rat retina of the control group. (**b**) Immunohistochemical staining for Notch-1 in the rat retina of the model group. (**c**) Immunohistochemical staining for Notch-1 in the rat retina of the DAPT group. (**d**) The percentage of Notch-1-positive cells. (**B**) (**a**) Immunohistochemical staining for DLL4 in the rat retina of the control group. (**b**) Immunohistochemical staining for DLL4 in the rat retina of the model group. (**c**) Immunohistochemical staining for DLL4 in the rat retina of the DAPT group. (**d**) The percentage of DLL4-positive cells. * P<0.05, compared with the control group; # P<0.05, compared with the model group.

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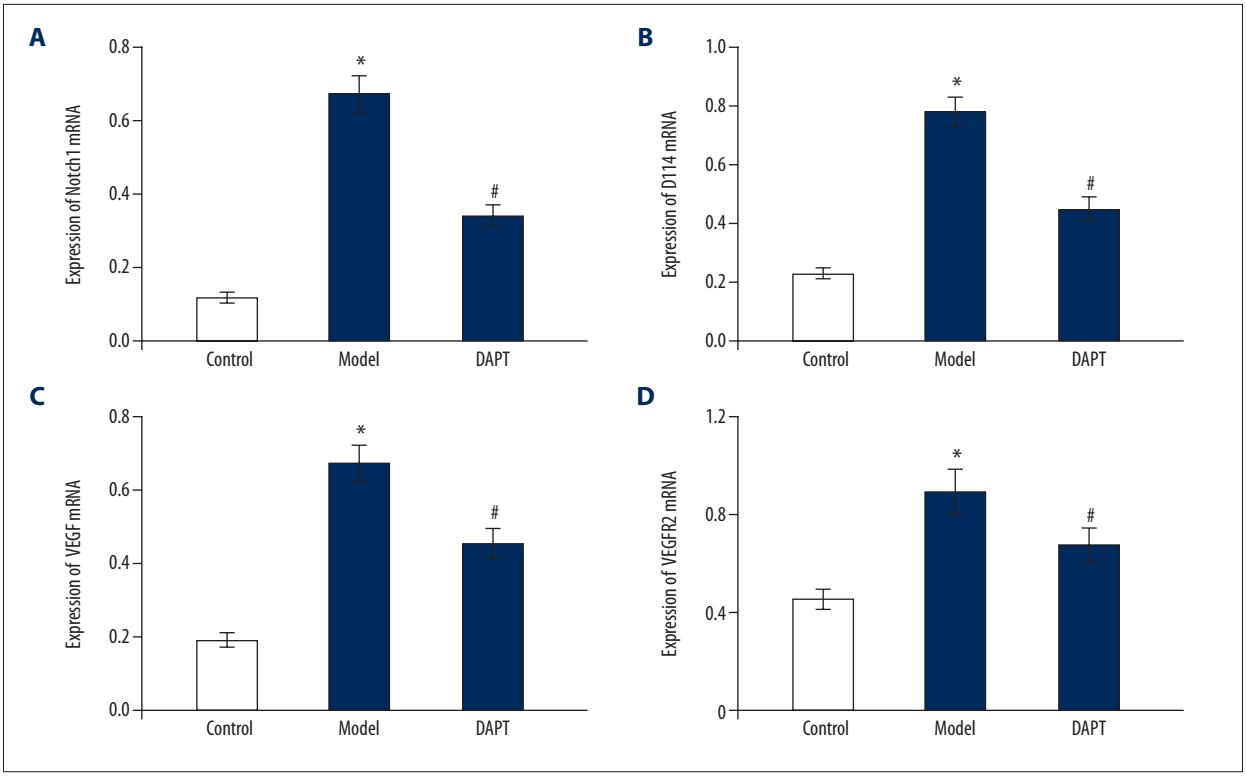

Figure 4. Relative expression of genes in the delta-like ligand 4/Notch homolog-1 (DLL4/Notch-1) pathway in the rat retina. (**A**) The mRNA level of Notch-1 in the rat retina of the control group, the model group, and the DAPT group. (**B**) The mRNA level of DLL4 in the rat retina of the control group, the model group, and the DAPT group. (**C**) The mRNA level of vascular endothelial growth factor-1 (VEGFR-1) in the rat retina of the control group, the model group, and the DAPT group. (**D**) The mRNA level of vascular endothelial growth factor-2 (VEGFR-2) in the rat retina of the control group, the model group, and the DAPT group. * P<0.05, compared with the control group; # P<0.05, compared with the model group.

in cultured endothelial cells *in vitro* [26]. During retinal vascular development, DLL4 is strongly expressed in VEGFR-2-enriched regions, and in a mouse model, DLL4 expression has been shown to reduce neovascularization following injection of a VEGF blocker into the vitreous cavity [27].

The gamma-secretase inhibitor, DAPT, is a specific blocker of the Notch receptor. DAPT inhibits the formation of soluble notch intracellular domain (NICD) protein by preventing the cleavage of gamma-secretase at the S3 site of the Notch receptor, preventing the transfer of NICD to the nucleus to block the Notch pathway [28,29]. Previously, gamma-secretase inhibitors have mainly been used in the treatment of Alzheimer's disease, but they have also been shown to inhibit tumor growth in a nude mouse model of acute T-cell lymphoblastic leukemia [30]. The effect of gamma-secretase on the inhibition of tumor cell growth may be attributed to the disrupted growth of tumor blood vessels [30]. Caiado et al. suggested that pretreatment with the gamma-secretase inhibitor, DAPT, in bone marrow-derived endothelial progenitor cells delayed wound healing [31]. Therefore, for the present study, a gamma-secretase inhibitor was included that could regulate angiogenesis by blocking the Notch pathway.

The findings of the present study demonstrated the expression of DLL4, Notch-1, VEGF, VEGFR-1 and VEGFR-2 by immunohistochemistry and quantitative real-time polymerase chain reaction (qRT-PCR). The results showed that in the control group, there was a low level of expression of these proteins in the ganglion cell layer, the inner nuclear layer, and the outer nuclear layer. In the ROP rat model group, there was strong expression of these genes in the ganglion cell layer, the inner nuclear layer, the inner plexiform layer, and the pigment epithelial layer, which were downregulated in DAPT group. Similar results were obtained for the mRNA levels of DLL4, Notch-1, VEGF, VEGFR-1, and VEGFR-2 in each group. These findings support the role of the gamma-secretase inhibitor, DAPT, in downregulating the expression of these pro-angiogenic genes in the rat model of ROP, and also support a role for the DLL4/Notch-1 pathway in ROP. Further studies are recommended to determine the therapeutic anti-angiogenic role of DAPT and other inhibitors of gamma-secretase in vascular retinopathy.

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Conclusions

In a rat model of retinopathy of prematurity (ROP), treatment with the gamma-secretase inhibitor, DAPT, reduced the retinal changes associated with vascular endothelial growth factor (VEGF) and its receptors through the delta-like ligand 4/Notch homolog-1 (DLL4/Notch-1) pathway.

Conflict of Interest

None.

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