Special Focus on Anti-VEGF, Original Article

Effect of Decorin and Bevacizumab on oxygen-induced retinopathy in rat models: A comparative study

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Purpose: The aims of this study were to evaluate the effects of decorin (DCN) in rat oxygen-induced retinopathy (OIR) model and to compare the results with those of bevacizumab. **Methods:** Twenty-eight newborn Sprague–Dawley rats were randomly divided into four groups. Group I (control): normoxia plus intraperitoneal (ip) normal saline (NS), Group II (sham): OIR plus ip NS, Group III (DCN): OIR plus ip 0.1 mg/kg DCN, and Group IV (bevacizumab): OIR plus ip 2.5 mg/kg bevacizumab. The OIR model was induced by cycling the oxygen concentration between 50% and 10% every 24 h for 14 days following their birth. In all groups, injections were administered on postnatal day (PD) 15. All animals were sacrificed and their right eyes were enucleated on PD 18. The nuclei of neovascular endothelial cells on the vitreal side of the inner limiting membrane were counted, and vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF)-α immunoreactivity were detected in histopathological and immunohistochemical examinations. One-way analysis of variance and post hoc Tukey tests were used for statistical analyses of the data. **Results:** In Groups II, III, and IV, the mean neovascular cell nuclei counts were 13.14 ± 1.34, 6.57 ± 1.51, and 6.71 ± 1.49, respectively. The mean neovascular cell nuclei count was significantly reduced in treatment groups compared with sham group ($P < 0.001$). In immunohistochemical staining, the immunoreactivity of VEGF was 0.07 ± 0.02, 0.97 ± 0.21, 0.37 ± 0.12, and 0.23 ± 0.17, respectively. Likewise, immunoreactivity of TNF-α was 0.02 ± 0.02, 1.11 ± 0.36, 0.37 ± 0.13, and 0.62 ± 0.21, respectively. VEGF and TNF-α immunoreactivity increased markedly in the sham group compared with those in the control group ($P < 0.001$). VEGF and TNF-α immunoreactivity of treatment groups decreased significantly compared to sham group ($P < 0.001$). **Conclusion:** The beneficial effects obtained by DCN administration in OIR model were comparable to the effects of bevacizumab.

Key words: Bevacizumab, decorin, oxygen-induced retinopathy, retinopathy of prematurity, vascular endothelial growth factor

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Retinal neovascularization (RNV) is the main characteristic feature of ischemic retinopathies, which includes retinopathy of prematurity (ROP), diabetic retinopathy (DR), and central retinal vascular occlusion (CRVO), all of which may lead to irreversible visual loss. Vascular endothelial growth factor (VEGF) is one of the most important regulating agent of physiological and pathological angiogenesis.[1,2] Clinical studies have revealed a complex pathogenesis of RNV, in which inflammation caused by retinal hypoxia is a primary factor and inflammatory responses are tightly linked with pathological angiogenesis.[3]

ROP is one of the major causes of blindness in children who are born with very low birth weight and gestational age. A biphasic hypothesis has been proposed to explain the pathogenesis of this disease. In the first phase, inhalation of either room air or supplemental oxygen causes hyperoxia that suppresses hypoxia-induced vascular growth factors.[4] This results in delayed vascular maturation. In the second phase, retinal hypoxia causes the activation of hypoxia-inducible genes, leading to retinal angiogenesis and RNV.[4] The oxygen-induced retinopathy (OIR) model in animals that have similar vascular development to human is necessary to understand the pathophysiology of ROP and to provide the development of new ROP treatment protocols.

Nowadays, VEGF inhibitors have been used as a predominant treatment of RNV.[5] Bevacizumab is a humanized monoclonal antibody that can bind to all VEGF-A isoforms. It is used for treating various neovascular eye diseases such as DR, CRVO, neovascular glaucoma, and ROP.[6-8] However, all anti-VEGF treatments only offer temporary respite from vascular leakage with limited clinical success. Moreover, anti-VEGF agents could be associated with side effects and complications such as endophthalmitis, retinal atrophy, intraocular pressure

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elevation, as well as cardiac and cerebral vascular incidents.[9,10] Therefore, alternative treatments need to be explored.

Decorin (DCN) is a small leucine-rich proteoglycan that possesses powerful antiangiogenic and antimetastatic properties. It functions as a paracrine inhibitor of receptor tyrosine kinases, including epidermal growth factor receptor and other ErbB family members, insulin-like growth factor receptor (IGF)-I, and hepatocyte growth factor receptor.[11-13] The action of DCN differs between normal and transformed cells. DCN differentially regulates the action of IGF-I receptor activation in both physiological and pathological cell models.[14,15] Further, it has a multikinase inhibitory property that causes it to suppress VEGF receptors through various mechanisms.[16] Previous studies have shown the effect of DCN on neovascularization and angiogenesis by using animal models of corneal and choroidal neovascularization.[17,18] Moreover, DCN suppresses scar formation by acting like a transforming growth factor (TGF)-β inhibitor. In rabbit traumatic proliferative vitreoretinopathy model, DCN treatment was shown to reduce the progression of fibrosis and tractional retinal detachment.[19,20]

In this study, our aim was to evaluate the effects of DCN in rat OIR model. Further, the obtained results are compared with those of bevacizumab treatments that have been proven to be effective for ROP.

Methods

This study was performed in accordance with the guidelines of the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The study was also approved by the Experimental Animal Studies Ethics Committee of the University (2018/20/186). In this study, newborn pups obtained from eight pregnant Sprague–Dawley rats were used. All pregnant animals were maintained under a 12 h light/12 h dark cycle with food and water were provided ad libitum.

Twenty-eight newborn Sprague–Dawley rats were randomly divided into four groups of seven rats each. The OIR model was created in Groups II, III, and IV. Specifically, the treatments applied were as follows: Group I (control); normoxia plus intraperitoneal (ip) normal saline (NS), Group II (sham); OIR plus ip NS, Group III (DCN); OIR plus ip 0.1 mg/kg DCN[21] (D-8428; Sigma-Aldrich, USA), Group IV (bevacizumab); OIR plus ip 2.5 mg/kg bevacizumab[22] (Avastin, Genentech, USA).[22] In all groups, injections were administered as a single dose on postnatal day (PD) 15. OIR model was induced in newborn rats as described in a previous study.[23] In brief, in the 50/10 OIR model, rats within 4 h of birth were placed with their mothers into an oxygen regulated environment in which they were exposed to 50% oxygen for 24 h followed by 10% oxygen for 24 h. This cycle was repeated seven times until PD 14. Oxygen concentrations were monitored using a sensor placed inside the cage and regulated using an oxygen controller. The amount of carbon dioxide inside the cages was also monitored daily. Sufficient gas flow was maintained by flushing it from the system. At PD 14, the rats were returned to standard room air for 4 days.

All animals were sacrificed by intracardiac high dose anesthesia. Their right eyes were enucleated on PD 18, which was considered the most prominent in OIR model. The eyes were fixed in 10% formaldehyde solution for 12 h. After fixation, the tissues were dehydrated by passing them through a series of graded ethanol concentrations. Then, the tissues were cleared in xylol and embedded in paraffin. All eyes were cut sagittally parallel to the optic nerve head, and sections were taken at 100 μm intervals from 10 areas on each side of the optic disc. The tissue blocks were sectioned at a thickness of 6 μm to perform immunohistochemical and histopathological staining.

Histologic quantification of RNV

Tissue samples of all groups were stained by using standard hematoxylin and eosin (H and E) technique. The nuclei of neovascular endothelial cells on the vitreal side of the inner limiting membrane (ILM) were counted in 10 sections of each eye at x400 magnification by a blinded independent observer, as described in previously.[24-26] The mean number of neovascular endothelial cell nuclei of each eye in all groups was calculated.

Immunohistochemical analysis of VEGF and TNF-α

VEGF and TNF-α were detected in the rat eye tissue with OIR by immunohistochemical staining using rabbit polyclonal antibodies (VEGF; bs-0279R, TNF-α; bs-2081R, Bioss, USA) and the streptavidin–biotin peroxidase technique. The procedure was performed as described previously[27] and identical conditions were applied for all tissue sections.

Extensity of the staining was taken as the basis for evaluation of immunohistochemical staining. A histoscore was calculated by multiplying the distribution (0.1: <25%, 0.4: 26%–50%, 0.6: 51%–75%, 0.9: 76%–100%) by intensity (0: no staining; +0.5: very little staining; +1: little staining; +2: medium; +3: very strong) to evaluate the immune reactivity (Histoscore = distribution × intensity).[28]

Statistical analyses

The SPSS statistical software package (version 25.0, SPSS Inc., Chicago, IL, USA) was used for data analyses. One-way analysis of variance and post hoc Tukey tests were used for statistical analyses of the data. The parameters were presented as mean ± standard deviation and percentage. A statistical significance was considered if $P < 0.05$.

Results

Histopathology

Histologic examinations indicated that vascular structures were present only in inner retina. In the control group, these blood vessels did not form clusters and were indistinctly distributed with a small diameter. By contrast, in the sham group, the blood vessel diameter increased and formed clusters in retina and vitreous. This group was also characterized by persistent thick-walled hyaloid artery clusters in the optic nerve head. Further, in the treatment groups, the blood vessel diameter was similar to or slightly lower than that in the control group [Fig. 1].

Quantitative assessment of RNV

There were no neovascular cell nuclei breaching the ILM in the control group. In Groups II, III, and IV, the mean neovascular cell nuclei count was 13.14 ± 1.34, 6.57 ± 1.51, and 6.71 ± 1.49, respectively [Fig. 2]. The mean neovascular cell nuclei count was significantly increased in Groups II, III, and IV compared with control group ($P < 0.001$ for all). The mean neovascular cell nuclei count was significantly decreased in the treatment groups compared with that in the sham group ($P < 0.001$ for both). The mean neovascular cell nuclei count was also similar between the two treatment groups ($P = 0.097$).

Immunohistochemical analyses of VEGF and TNF-α

VEGF immunoreactivity in Groups I, II, III, and IV was 0.07 ± 0.02, 0.97 ± 0.21, 0.37 ± 0.12, and 0.23 ± 0.17, respectively [Fig. 3]. VEGF immunoreactivity was increased markedly in Groups II, III, and IV compared to control group ($P < 0.001$, 0.005 and 0.203, respectively). In treatment groups, VEGF immunoreactivity was decreased significantly compared to sham group ($P < 0.001$ for both). Moreover, the VEGF immunoreactivity was similar between the two treatment groups ($P = 0.354$).

TNF-α immunoreactivity in Groups I, II, III, and IV was 0.02 ± 0.02, 1.11 ± 0.36, 0.37 ± 0.13, and 0.62 ± 0.21, respectively [Fig. 4]. In Groups II, III, and IV, TNF-α immunoreactivity was increased markedly compared to control group ($P < 0.001$, 0.033 and <0.001, respectively). In the treatment groups, TNF-α immunoreactivity was decreased significantly compared to sham group ($P < 0.001$ for both). Moreover, the TNF-α

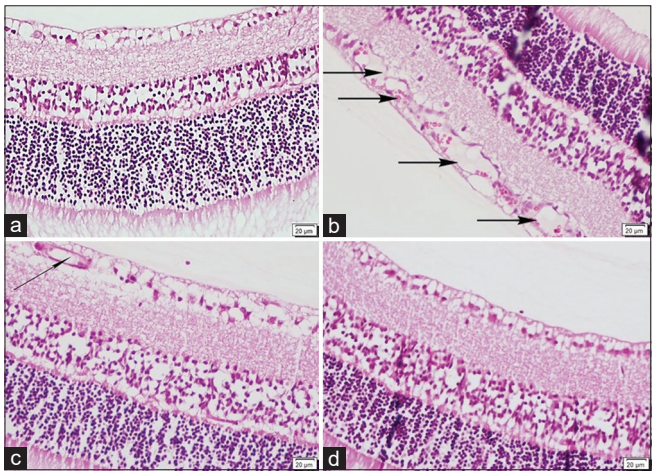

Figure 1: Representative histopathological changes in groups. Normal retina in control group, scarce or no vascularization (a). In sham group, clusters of neovascularizations (arrows) (b). Reduced number of vascularization in treatment groups (c and d)

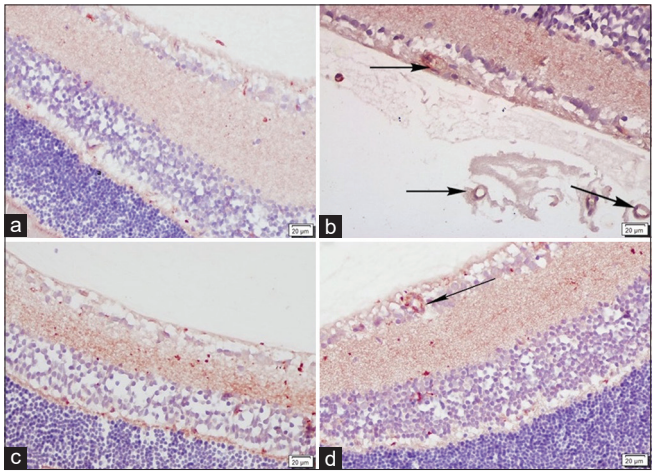

Figure 3: VEGF immunoreactivity in the vessel walls. No immune reactivity in control group (a). Highly pronounced immune reactivity in sham group (b). Decreased or no immune reactivity in treatment groups (c and d)

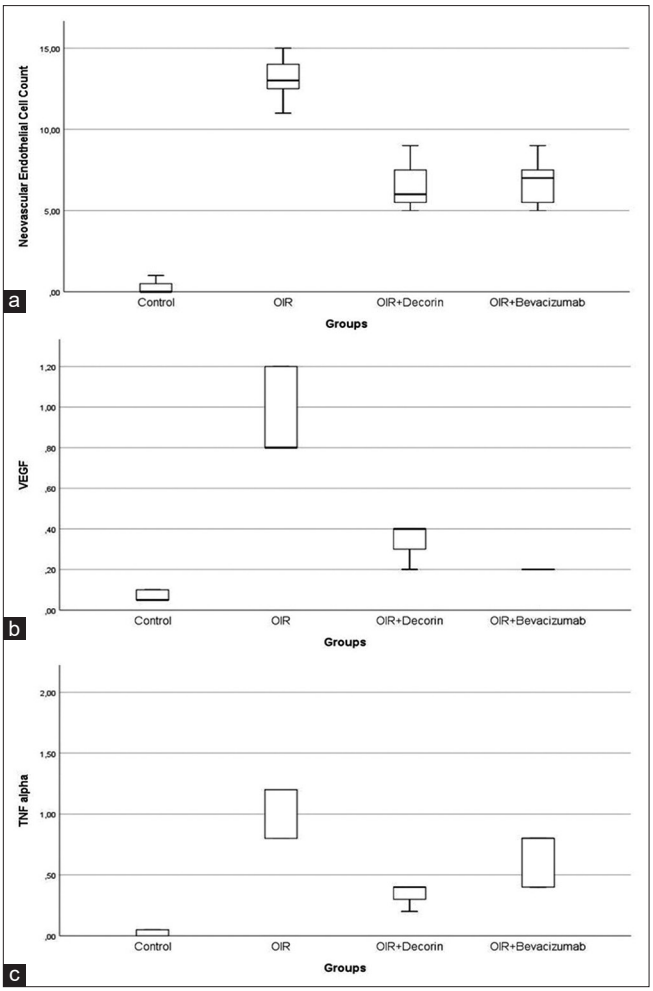

Figure 2: Boxplot representations of the studied parameters. (a) Neovascular endothelial cellular examination in all groups. (b) VEGF immunoreactivity examination in all groups. (c) TNF-α immunoreactivity examination in all groups

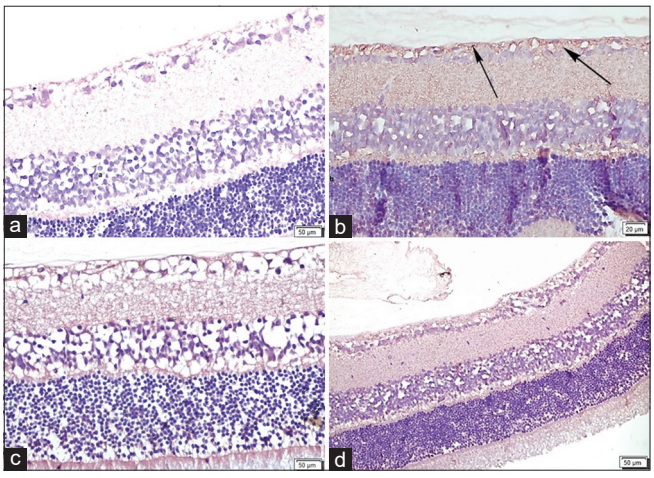

Figure 4: TNF-α immunoreactivity in the ganglion cell layer of all groups. TNF-α immunoreactivity in the ganglion cell layer of the control group showed light intensity (a). TNF-α immunoreactivity is very prominent in sham group (b). Decreased TNF-α immunoreactivity in the ganglion cell layer of treatment groups (c and d)

immunoreactivity was similar between the two treatment groups ($P = 0.161$).

Discussion

This study, the effects of systemic administration of DCN in rat OIR model were investigated. The results showed that DCN significantly suppressed RNV by reducing neovascular endothelial cell proliferation, VEGF, and TNF-α immunoreactivity, and the effects were comparable to bevacizumab.

The potential of VEGF to increase endothelial cellular proliferation and vascular permeability has been well documented.[29] A previous in vivo study showed that an overexpression of DCN suppressed angiogenesis in tumor cells by inhibiting the production of VEGF and suggested the use of DCN as a potential treatment of diseases associated with neovascularization.[30] Moreover, the ability of DCN to suppress VEGF receptors through several different mechanisms shows that it may be an alternative treatment strategy for pathologies associated with angiogenesis. As a multikinase inhibitor, DCN was shown to be an antagonistic ligand for VEGFR-2 in endothelial cells.[31] Therefore, it was suggested that DCN could not only reduce hypoxia-induced VEGF expression but also it may suppress retinal angiogenesis through its specific antagonistic effects on VEGFR-2 receptors.

DCN inhibits Met activation that is common in many cancers to suppress the proliferation of tumor cells and angiogenesis.[13,32,33] In vitro studies with ARPE-19 cells investigating the impact of DCN on choroidal NV showed its antiangiogenic effects.[17] DCN provides these effects by suppressing the Met pathway, inhibiting the expressions of hypoxia associated Rac1 and hypoxia inducible factor-1α, and decreasing VEGF levels. Moreover, activation of TGF-β/Smad signal transduction pathway is associated with increased levels of retinal VEGF and TNF-α following the formation of choroidal NV.[17] The potential effects of TGF-β on choroidal NV could be explained by overexpression of VEGF through RPE. Nevertheless, overstimulation of TNF-α should also be considered. In the study of Du *et al*.[17] administration of DCN as a natural TGF-β inhibitor showed antiangiogenic effects through inhibition of Smad 2-3 phosphorylation and expression of VEGF and TNF-α in laser-induced choroidal NV mouse models. In this study, VEGF and TNF-α immunoreactivity were evaluated. The results suggested a significant reduction of VEGF and TNF-α immunoreactivity following the administration of DCN. Interferons (IFN) are well known as angiogenic molecules. Previous studies have shown that DCN decreased inflammation-associated angiogenesis by reducing IFN activity, especially the activity of IFN-γ.[34] Therefore, DCN has a dual role in inflammation-associated angiogenesis. Park *et al*.[35] reported a decrease in DCN at the retinal ganglion cell layer and ILM in a rat OIR model. Moreover, DCN simultaneously suppresses expression of the two proangiogenic proteases, matrix metalloproteinase (MMP)-2 and MMP-9, and their enzymatic activities. In the study of Barnet *et al*.,[36] the partial role of metalloproteinases in retinal angiogenesis has been shown. To our knowledge, this is the first study to investigate the effects of DCN treatment using an OIR model. Our results showed that DCN treatment significantly decreased neovascular endothelial cell proliferation, VEGF, and TNF-α immunoreactivity in the ganglion cell layer. Moreover, the effects of DCN were comparable to that of bevacizumab. Previous studies suggested that anti-VEGF injection in ROP treatment could cause a progression in tractional retinal detachment through increased fibrovascular contraction (especially via membrane contraction at the stages 4a and 4b).[37,38] Therefore, the antifibrotic properties of DCN could be superior to those of anti-VEGF agents. In this study, no side-effects were observed in animals following ip administration of DCN.

There were several limitations in this study. Effects of DCN were only evaluated through ip administration. However, it is very promising that our results are comparable to bevacizumab, which has been proven to be effective in the treatment of ROP. The dose response relationship, pharmacokinetic and functional analyses, and other quantitative analyses like Western blot and flow cytometry were not evaluated. Additionally, the modulatory effect of DCN on IGF-IR axis in rat OIR model was not evaluated. Therefore, future studies are required to determine the optimal dosing protocol through intraocular administration and elucidate mechanisms of DCN action in different experimental models.

Conclusion

In conclusion, this study showed that the effects of DCN treatment in OIR models were comparable to the effects of bevacizumab treatment. DCN decreased the immunoreactivity of both VEGF and TNF-α in the rat OIR model. Results of this study suggested the potential therapeutic effects of DCN on RNV using the rat OIR model. However, future studies are required to elucidate the precise mechanistic action of DCN in RNV and other ocular neovascularization.

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Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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