Comparison of the Effects of Digoxin and Bevacizumab on Oxygen-Induced Retinopathy Rat Model

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Research Article

Keywords: bevacizumab, digoxin, oxygen-induced retinopathy, vascular endothelial growth factor, tumor necrosis factor-α

Posted Date: April 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1551749/v1

Abstract

Purpose: To evaluate the effect of digoxin on oxygen-induced retinopathy (OIR) model and compare the results with bevacizumab treatment.

Methods: Twenty-eight newborn Sprague-Dawley rats were randomly divided into four groups:control group, normoxia+intraperitoneal (ip) normal saline (NS); OIR group, OIR+ip NS; OIR+digoxin group, OIR+ip 0.1 mg/kg of digoxin; and OIR+bevacizumab group, OIR+ip 2.5 mg/kg of bevacizumab. The rats were exposed to 50% oxygen for 24 h, followed by 10% oxygen for 24 h, to induce OIR. This cycle was repeated seven times until 14 days postnatal (P). In all groups, single-dose injections were administered on P15th day. Histopathological and immunohistochemical examinations were performed at the end of the study.

Results: The mean neovascular cell nuclei counts (NVCN) of the four groups were 9.00±3.16, 41.80±11.44, 19.38±2.20, and 16.00±2.62. The mean NVCN count was significantly reduced in the treatment groups compared to the OIR group ($p<0.001$). The mean NVCN count was similar between the treatment groups ($p=0.078$). In immunohistochemical staining, the immunoreactivity values of the vascular endothelial growth factor (VEGF) were 0.01±0.00, 1.65±0.30, 0.09±0.08, and 0.04±0.02, and the tumor necrosis factor alpha (TNF-α) values were 0.10±0.00, 1.12±0.18, 0.18±0.13, and 0.14±0.05. In the OIR group, VEGF and TNF-α immunoreactivity increased markedly compared to the control group ($p<0.001$). VEGF and TNF-α immunoreactivity of the treatment groups decreased significantly compared to the OIR group ($p<0.001$). VEGF and TNF-α immunoreactivity were similar between the treatment groups (VEGF:$p=0.752$; TNF-α:$p=0.099$).

Conclusions: Retinal neovascularization was significantly suppressed by digoxin treatment, and this effect was comparable to bevacizumab in the OIR model.

Introduction

Pathological ocular neovascularization (NV) is the main cause of visual loss in ischemic retinopathies, such as proliferative diabetic retinopathy (PDR), retinopathy of prematurity (ROP), age-related macular degeneration, and retinal vein occlusion (RVO) [1]. ROP is a vasoproliferative retinopathy characterized by an abnormal proliferation of new vessels that appear at the border of the vascular and non-vascular retina of premature infants. It is an important cause of blindness in children with a very low birth weight and gestational age. A biphasic hypothesis has been proposed to explain the pathogenesis of the disease. In the first phase, hyperoxia occurs because of the inhalation of either room air or supplemental oxygen, and it suppresses hypoxia-induced vascular growth factors (HIF), leading to a delay in vascular maturation [2]. In the second phase, retinal hypoxia results in the activation of hypoxia-inducible genes, which drive retinal angiogenesis and preretinal NV (4).

Although many mediators trigger ocular pathological NV, the most known of these is the vascular endothelial growth factor (VEGF) [3–5]. The inhibition of VEGF bioactivity is one of the main goals in the treatment of ocular neovascular diseases. Currently, anti-VEGF agents (e.g., bevacizumab, ranibizumab,

and aflibercept) are used as treatments for this purpose [6]. The first agent used, bevacizumab, is a human monoclonal antibody that binds all VEGF-A isoforms [7]. The effectiveness of bevacizumab has been shown in ocular neovascular diseases, such as PDR, RVO, neovascular glaucoma, and ROP [8–12]. The lack of efficacy of anti-VEGF therapies in the treatment of some cancers and neovascular ocular diseases [10, 16] is possibly due to the contribution of other proangiogenic factors, such as platelet-derived growth factor (PDGF-B), stromal-derived growth factor-1 (SDF-1), placental growth factor (PGF), and angiopoietin-2 (ANG 2). In view of the many proangiogenic factors that contribute to ocular NV, possible future approaches to effectively treating these conditions can rely on either combination therapy or blocking some critical modulators. In addition, the high cost of anti-VEGF treatment, the need for repeated injections, and the relative inefficiency of the response have led to the search for new treatments.

Digoxin is a cardiac glucoside used in the treatment of heart failure and arrhythmia. It inhibits the Na/K ATPase pump, which causes increased intracellular Na^{+} and Ca^{++} and decreased K^{+} concentrations. Experimental studies have shown that digoxin inhibits tumor growth by inhibiting HIF-lα transcription and VEGF mRNA expression [13]. Moreover, it can be used in the treatment of ocular neovascular diseases by suppressing angiogenesis. Digoxin suppresses retinal and choroidal neovascularization by reducing the HIF-1α level, which blocks several proangiogenic pathways. We could not find any study comparing the effects of VEGF inhibitors and digoxin in the treatment of retinal NV.

In this study, we evaluated the effect of digoxin on the oxygen-induced retinopathy rat model and compared our results with those of bevacizumab, which has been shown to be effective in the treatment of ROP.

Materials And 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 approved by the Experimental Animal Studies Ethics Committee of Fırat University (2019/45). In the current 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 provided ad libitum. All rats were born at term.

Twenty-eight newborn Sprague–Dawley rats were randomized into four groups: control group, normoxia+intraperitoneal (ip) normal saline (NS); OIR group, OIR+ip NS; OIR+digoxin group, OIR+ip 0.1 mg/kg of digoxin (Digoxin-Sandoz, Novartis, USA); and OIR+bevacizumab group, OIR+ip 2.5 mg/kg of bevacizumab (Altuzan, Roche, USA). Single-dose injections were made on the 15^{th} day after birth. The ROP model was created by Penn et al., as previously described [14]. In the 50/10 OIR model, rats within 4 h of birth were placed with their mothers in an oxygen-regulated environment where they were exposed to 50% oxygen for 24 h, followed by 10% oxygen for 24 h. This cycle was repeated seven times until the 14^{th} day after birth. Oxygen concentrations were monitored using a sensor placed in the incubator and regulated using an oxygen controller. Carbon dioxide in the incubator was also monitored daily and

flushed from the system by maintaining sufficient gas flow. It was checked at least three times daily during the oxygen exposure period. The rats were taken to normal room air for four days after the 14th day postnatal.

All animals were sacrificed with high-dose intracardiac anesthesia on the 18th day, when the OIR model was considered to be the most obviously produced. The enucleated eyes were fixed in a 10% formaldehyde solution for 12 h. After adequate fixation, the tissues were dehydrated by passing them through a series of graded ethanol concentrations. The tissues were then cleared in xylol and embedded in paraffin wax. All eyes were cut sagittally parallel to the optic nerve head, and sections were taken at 100-μm intervals from five 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. The sections were examined using a bright-field light microscope and photographed with an attached camera (BH-2, Olympus, Tokyo, Japan).

Histological quantification of retinal NV and digoxin toxicity

Tissue samples in all groups were stained using the standard hematoxylin–eosin technique. The neovascular cell nuclei (NVCN) on the vitreous edge of the internal limit membrane were counted from 10 sides of each eye at 400x magnification by the same blind observer. The mean number of NVCNs in each eye was calculated for all groups. The effect of digoxin treatment on the retina for toxic assessment was evaluated by measuring the thickness of six layers: outer segment layer, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, and ganglion cell layer. Thickness measurements were performed on the eye sections of all groups and compared with each other.

Immunohistochemical analysis of VEGF and tumor necrosis factor alpha (TNF-α)

VEGF (Bioss Inc., Massachusetts, USA) and TNF-α (Bioss Inc., Massachusetts, USA) were detected in rat eye tissue with OIR through immunohistochemical staining using rabbit polyclonal antibodies and the streptavidin–biotin peroxidase technique. The procedure was performed under identical conditions for all sections, as previously described [15].

The extensity of the staining was taken as the basis when immunohistochemical staining was evaluated. A histoscore was derived from the distribution (0.1: < 25%, 0.4: 26%–50%, 0.6: 51%–75%, 0.9: 76%–100%) and intensity (0: no staining; +0.5: very little staining; +1: little staining; +2: medium; +3: very strong) of staining immune reactivity (histoscore = distribution × intensity) [16, 17].

Statistical analysis

The SPSS statistical software package (version 25.0, SPSS Inc., Chicago, IL, USA) was used to analyze the data. A one-way variance analysis and post hoc Tukey tests were used for the analysis of the statistical data. The parameters were calculated as the average, standard deviation, and percentage. $P < 0.05$ was considered statistically significant.

Results

Quantitative assessment of retinal NV and toxicity

The mean NVCN numbers of all groups were 9.00 ± 3.16, 41.80 ± 11.44, 19.38 ± 2.20, and 16.00 ± 2.62 (Figure 1). The mean number of NVCN increased significantly in the OIR group compared to the control group ($p< 0.001$). The mean NVCN number was significantly reduced in the treatment groups compared to the OIR group ($p< 0.001$). No statistically significant difference was found in the mean NVCN number between the treatment groups ($p = 0.078$).

The retinal layers of all groups were evaluated, and no atrophy was observed. No statistically significant difference was found in the mean retinal thickness between the groups.

VEGF and TNF-α immunohistochemical analysis

The VEGF immunoreactivity values of all groups were 0.01 ± 0.00, 1.65 ± 0.30, 0.09 ± 0.08, and 0.04 ± 0.02 (Figure 2). The VEGF immunoreactivity of the OIR group increased significantly compared to the control group ($p< 0.001$). The VEGF immunoreactivity of the treatment groups was significantly reduced compared to the OIR group ($p< 0.001$). The treatment groups did not have a significant difference in VEGF immunoreactivity compared to the control group ($p = 0.470$). The VEGF immunoreactivity values of the treatment groups were similar ($p = 0.752$).

The TNF-α immunoreactivity values of all groups were 0.10 ± 0.00, 1.12 ± 0.18, 0.18 ± 0.13, and 0.14 ± 0.05 (Figure 3). In the OIR group, the TNF-α immunoreactivity increased significantly compared to the control group ($p= 0.0159$). In the treatment groups, it significantly reduced compared to the OIR group ($p< 0.0079$). The TNF-α immunoreactivity values were similar among the treatment groups ($p= 0.099$).

Discussion

This study aimed to compare the effects of digoxin and bevacizumab on a rat OIR model. The results showed that digoxin significantly suppressed retinal NV by reducing neovascular endothelial cell proliferation and VEGF and TNF-α immunoreactivity. The effects were comparable to those of bevacizumab.

The most important point in the pathogenesis of these diseases is vasoproliferative retinopathy, which occurs secondary to hypoxia. Choroidal and retinal hypoxia, for any reason, cause the release of

proangiogenic and anti-angiogenic factors. The products of genes induced by increased HIF-1α in the case of hypoxia are VEGF, ANG 2, PDGF-B, PGF-1, and erythropoietin (EPO) [18]. In experimental studies, increased levels of VEGF produced by retinal pigment epithelial cells have been shown to be a major risk factor for retinal NV [19]. Anti-VEGF agents are used as the current treatment for this purpose [6]. There are no randomized controlled studies on the use of bevacizumab, but its effectiveness has been shown in ocular neovascular diseases [8–12].

The demolition of the HIF-1α gene in hypoxic RPE cells causes VEGF expression to decrease vascular leakage and retinal NV [18, 19]. Digoxin and other cardiac glycosides inhibit HIF-lα transcriptional activity and suppress tumor xenograft growth by activating the ERK pathway in cultured cells. In one study, digoxin was shown to inhibit HIF-1α synthesis, and the idea that it could inhibit tumor angiogenesis was proposed [13]. HIF-lα is a transcription factor that regulates the expression of many genes, primarily VEGF, an important regulator of angiogenesis. By suppressing the HIF-lα level, the expression of proangiogenic factors, such as VEGF, PGF-1, PDGF-B, ANG 2, SDF-1, SCF, and EPO, decreased, and retinal and choroidal neovascularization decreased [20]. Digoxin also contributes to the prevention of inflammation by significantly reducing the number of macrophages in ischemic retinal cells [21]. In a study investigating Src and FAK activation with digitoxin in human umbilical vein endothelial cells, digitoxin was shown to inhibit angiogenesis by suppressing FAK activation, and the potential use of digitoxin as a broad-spectrum anti-angiogenic drug was proposed [22]. Studies have used various cardiac glycosides for therapeutic purposes in retinal NV animal models. In a previous study, honokiol, one of the cardiac glycosides, was shown to inhibit the HIF-la pathway-mediated expression of proangiogenic genes [23]. In an animal model of ischemic retinopathy, digoxin was shown to suppress retinal and choroidal neovascularization by preventing the upregulation of various proangiogenic factors in the ischemic retina [24]. Moreover, digoxin and some digoxin derivates inhibited the release of proinflammatory cytokines, such as TNF-α, interleukin 6 (IL-6), and IL-8, in human peripheral blood mononuclear cells [25]. Aside from reducing the production of proangiogenic and proinflammatory factors, digoxin has been shown to significantly reduce the number of macrophages in the ischemic retina [26, 27]. In this study, we compared the efficacy of digoxin therapy, which has been shown to reduce retinal NV in OIR, with that of proven bevacizumab therapy. Our results showed that neovascular endothelial cell proliferation and VEGF and TNF-α immunoreactivity decreased significantly in the OIR + digoxin group compared to the OIR group. In addition, the effects of digoxin on the OIR model were comparable to those of bevacizumab.

Aside from determining the side effects of digoxin, which has been a frequently used agent for a long time because of its affordability and easy access, obtaining results comparable to those for bevacizumab is promising for the future of ROP treatment. However, the intraperitoneal application of digoxin constitutes a limitation of the current study. Although some studies have reported that digoxin could cause retinopathy, we did not observe any side effects in our study [28, 29]. The digoxin dose that induces retinopathy in mouse models is 2 mg/kg, but it has been reported that three injections of a lower dose (up to 1 mg/kg) did not cause retinal degeneration [28]. Although digoxin toxicity affects photoreceptors, the toxicity of ganglion, bipolar, amacrine, and horizontal cells has not been determined [28]. We could not perform electroretinography, but no toxicity was observed at the histological level.

In conclusion, digoxin (0.1 mg/kg, single injection) may reduce retinal NV without causing any toxicity in the OIR model. Digoxin may have potential as a therapeutic agent in the treatment of ROP and ischemic retinopathies. In further studies, the optimal dosing protocol and efficacy of intravitreal administration should be determined.

Declarations

Author contributions:

MB and FU contributed to concept, design, intellectual content and drafted the article. HY and MCL contributed to design, concept and statistical analysis. SD acquired data and interpreted data. SD and MCL contributed to data analysis and intellectual content. SD acquired data, drafted the article and interpreted data. CAİ contributed to pathological and histological analysis, data analysis, intellectual content. All authors read and approved the final manuscript.

Funding:

No financial support was received for this study.

Compliance with ethical standards

Conflict of interest:

The authors declare that they have no conflict of interest.

Ethical approval and animal rights:

This study was carried out in accordance with the ARVO Statement of Ophthalmic and Vision Research on Animal Use. The Fırat University Experimental Animal Studies Ethics Committee approved the study protocols.

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Figures

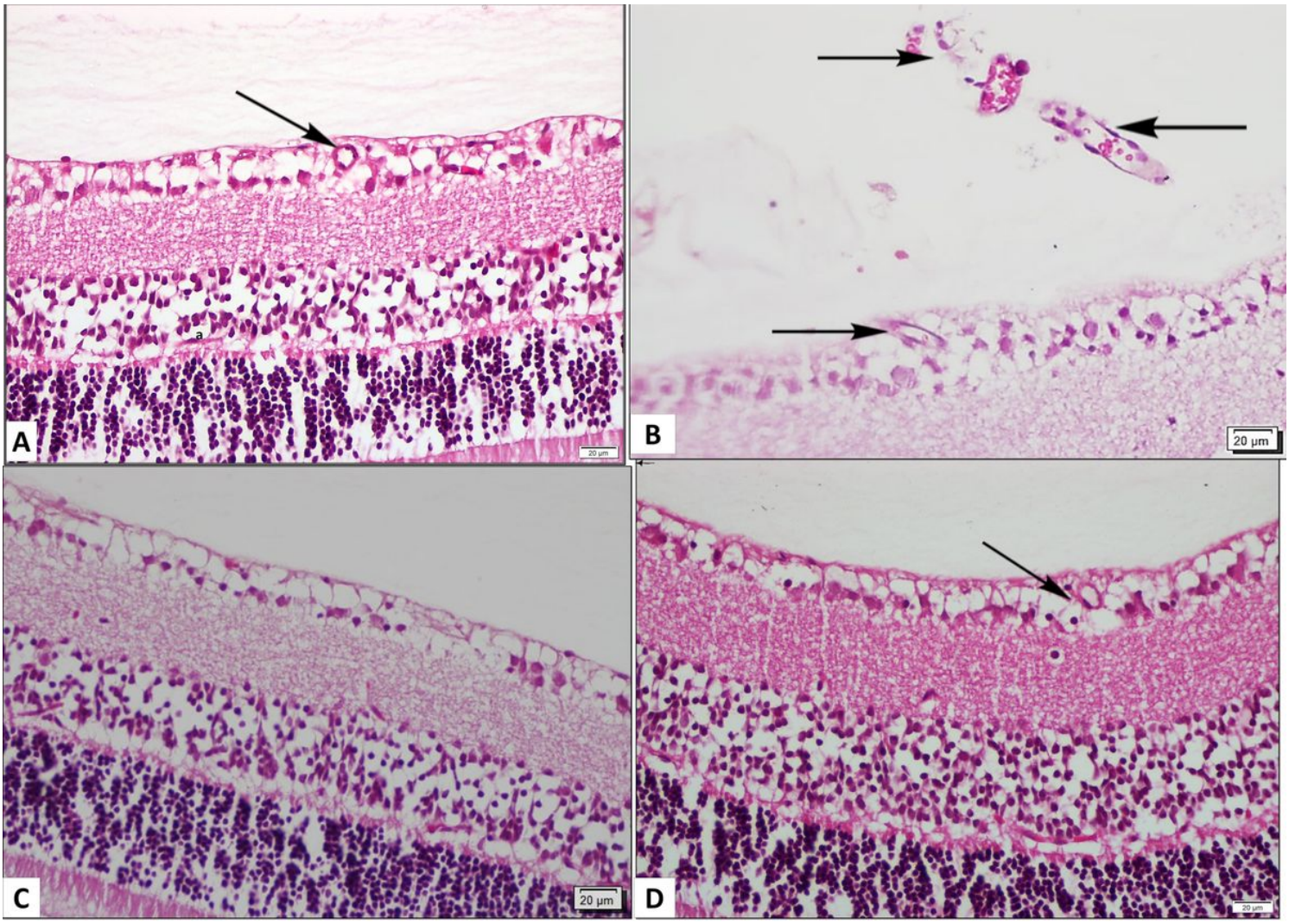

Figure 1

Histopathological changes in all groups. Normal retina in the control group (a). Clusters of neovascularizations in the OIR group (arrows) (b). Reduced number of vascularizations in the OIR + digoxin (c) and OIR + bevacizumab groups (d).

Figure 2

VEGF immunoreactivity in the vessel walls. No immune reactivity in the control group (a). Highly pronounced immune reactivity in the OIR group (b). Decreased or no immune reactivity in the OIR + digoxin and OIR + bevacizumab groups (c and d).

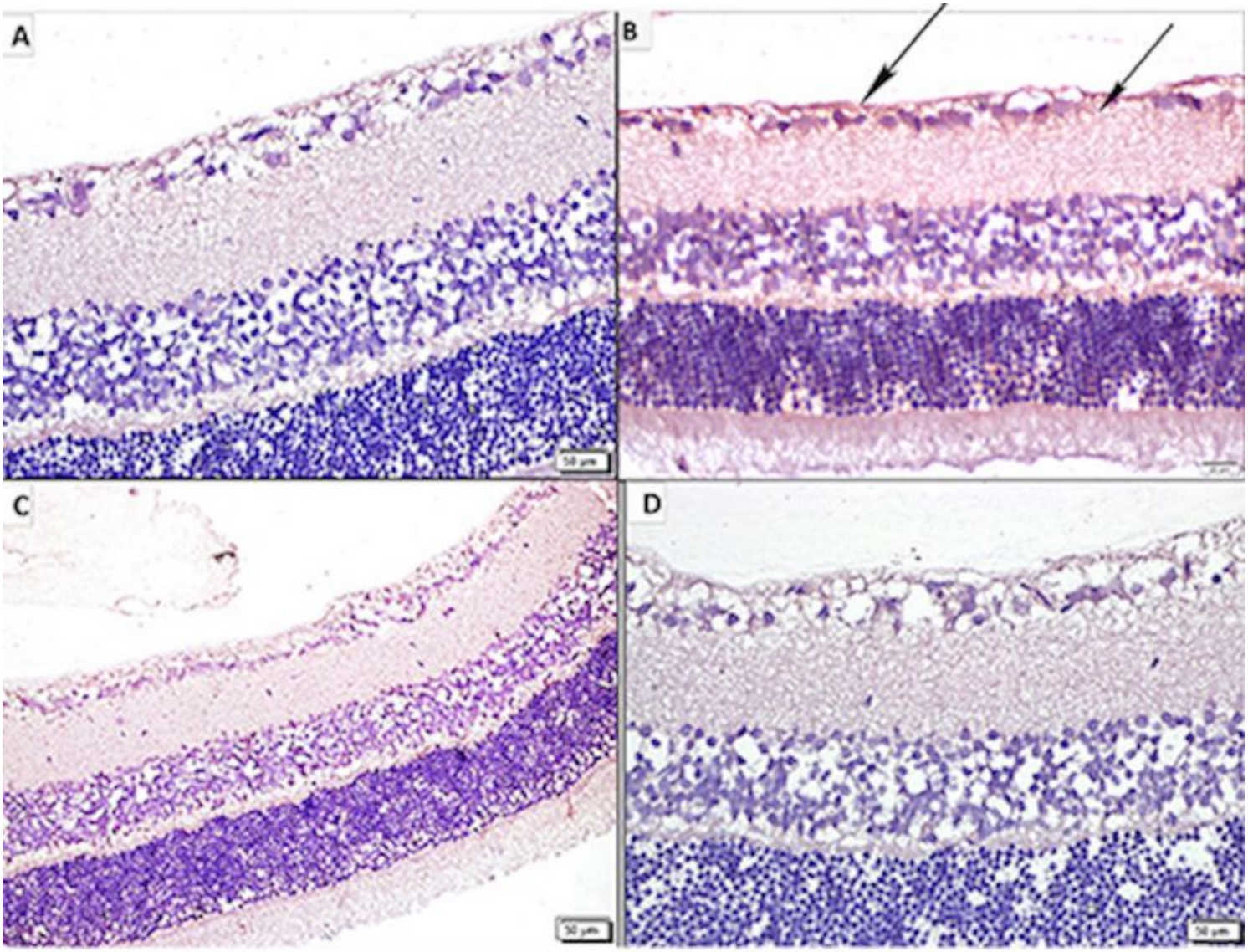

Figure 3

TNF-α immunoreactivity in the ganglion cell layer of all groups. Light intensity of the TNF-α immunoreactivity in the ganglion cell layer of the control group (a). Prominent TNF-α immunoreactivity in the OIR group (b). Decreased TNF-α immunoreactivity in the ganglion cell layer of the OIR + digoxin and OIR + bevacizumab groups (c and d).