·Basic Research·

Protective effect of basic fibroblast growth factor on laser induced retinopathy

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Abstract

• **AIM: To investigate the side effects of the commonly used laser treatment along with testing the neuroprotective effect of bFGF on a potential retinal impairment.**

• **METHODS: To do this, 30 chinchilla pigmented adult male rabbits were divided into the control and experimental groups. The control and experimental groups underwent both laser application and bFGF treatment. The retinal tissue impairment and its renewal rate were tested under the light and electron microscopical levels.**

• **RESULTS: The focal laser application on rabbit eyes caused morphological alterations both in the application region and in the neighbouring areas. In the damaged areas, the outer nuclear layer of the neural retina was almost disappeared, retina pigment epithelium was interrupted, the retina pigment epithelium migrated intraretinally, and the damaged region along with neighbouring areas seemed to be not separated. bFGF application just after the laser photocoagulation, revealed better results in application areas.**

• **CONCLUSION: It could be suggested that the bFGF application following laser photocoagulation might have protective, repairing and wound healing effects on the retina.**

• **KEYWORDS:** retina; bFGF; laser photocoagulation; rabbit; light microscopy; electron microscopy
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INTRODUCTION

The usage of laser technology in scientific research, industry and in military areas has become rather common. The ophtalmic laser is oftenly used in the therapy of retinal diseases, microaneurysms bleedings, exudates caused by diabetes, macula degenerations in elderly people and in retinal tumours [1]. The usage of laser is accompanied commonly by occupational eye accidents. The application of laser photocoagulation to the perifoveal retina often causes sight loss[2-5]. This is an unavoidable situation which is a result of an indirect adverse effect of laser discharge on the neighbouring healthy tissues. Protecting/preserving the neighbouring healthy tissues and performing a reliable perifoveal photocoagulation increases the efficiency of the therapy. The retinal photocoagulation lesions cause primary and secondary degenerations followed by a partial healing. In recent years, there have been researches on the development of novel therapy protocols for accidental retinal burns that occur during the laser therapy[6-12].

Retinal glial cells, Müller cells, behave like stem cells and undergo reactive gliosis following acute retinal damage and chronic neuronal stress. The Müllerian cell gliosis is characterized by proliferation, changes in cell shape caused by an increase in the intermediate filament production, changes in ion transport characteristics and, secretion of signal vascular endothelial growth factor (VEGF)[12]. Growth factors and cytokines take part in the repair of retinal damage. Basic fibroblast growth factor (bFGF) acts in the central nervous system as a growth factor with proliferative, regenerative and functioning properties in the survival of cells. The application of exogenus bFGF, induces the proliferation of non-neuronal cells in retinal damages which increase the expression of intermediate filament proteins in retinal glial cells, prevents or delays photoreceptor cell degeneration and loss, leads the axons to regenerate and induces the regeneration of the whole retina in embryos in which the eyes were taken from[8-12].

The aim of the present study was to investigate the possibility of degeneration in retinal neurons, glial cells and matrix following the experimental retinal damage caused by diot laser application as well as the role of Müllerian cells on regeneration. Another goal of the present study was to determine the neuroprotective effect of bFGF on the retina just after the laser photocoagulation at under the light and electron microscopical levels.

MATERIALS AND METHODS

Materials Thirty adult male chinchilla, pigmented rabbits each of which weighing 1 500-2 700g were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and the experiments were approved by the Local Ethics Committee for the Animal Experiments, Cumhuriyet University, Sivas, Turkey. Animals were given a standard pellet diet of green vegetables and tap water *ad libitum*.

Surgical Procedure and Experimental Design Before the surgical procedure, all animals were anaesthetized by intramuscular injections of xyliazine (15mg/kg body weight) and ketamine chloride (50mg/kg body weight) by injecting into the front left leg muscle. Throughout the all experimental procedures, animals were lulled in which they breathed spontaneously. A subcutaneous 4mg/kg rimadyl injection was administered to each animal along the 3d period following the experimental procedures, in order to prevent any pain. Under general anaesthesia, rabbits were given laser shots to their cornea starting at 0.02s to 1.0s at 50-1 500mW power supply, at 50-1 000 calibre by using a diot laser device (Oculight Symphony Diot Laser, Italy). Retinal destruction was approved by retinal eye examination (Topcon Slit Lamp Biomicroscope SL07, Japan).

Thirty chinchilla pigmented adult male rabbits were divided into the control and the experimental groups. The experimental group underwent both laser application and bFGF therapy.

Group 1(n=10):While diot laser application were performed on their left eyes, their right eyes received no application.

Group 2 (n=10): Similarly the right eyes received no application, whereas diot laser application was applied to their left eyes which was followed by bFGF injection.

Group 3 (n=10): While the right eyes were injected by the buffer solution that was used for the preparation of the final bFGF solution, the left eyes were injected by bFGF solution, following diot laser application.

Ten days after all experimental applications, the right and left eyes from each animal were taken and processed for light and electron microscopical investigations.

Tissue Preparation A general body perfusion (cardiac perfusion) containing 2.5% glutaraldehyde+1.5% formaldehyde in 0.1mol/L sodium phosphate (pH: 7.4) at 38℃ was performed to rabbits[13]. Following partial fixation of the eyes by cardiac perfusion, the eyes were further fixed in the same fixative solution for 24h. Tissues for light microscopical evaluation were then fixed in buffered neutral formaldehyde for 24h and were dehydrated in the increasing concentrations of ethanol. They were cleared by xylene and were embedded in paraffine. Sections (5-7μm thick) were cut from those paraffine embedded tissues and those sections were stained by haematoxylene - eosin in order to find out the lesion areas.

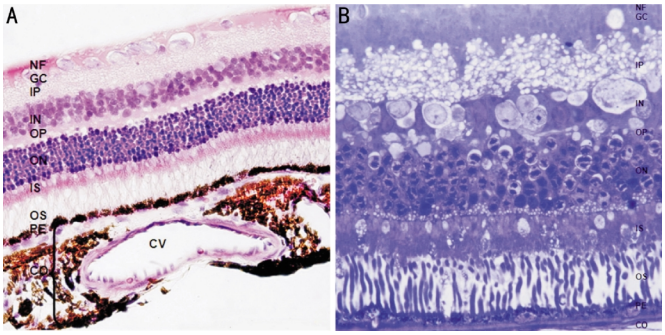

Figure 1 The light microscopy of retina and choroid layers The general view of the retina and choroid in paraffin (A) and semi-thin resin (B) sections of the control group. Choroid (CO), choroidal blood vessels (CV), pigmented retinal epithelium (PE), outer (OS) and inner (IS) segments of photoreceptors, outer nuclear (ON), outer plexiform (OP), inner nuclear (IN), inner plexiform (IP), ganglionic cell (GC) and neuronal fiber (NF) layers. A: H&E, ×40; B: Toluidin-blue, ×100.

Convenient fields of views were photographed by using a light microscope (Olympus BX 51, Tokyo, Japan). For electron microscopy, tissues were fixed in 3% glutaraldehyde in phosphate buffer at 4℃ for 5-8 d and postfixed in 1% aqueous osmium tetroxide for 2h. Tissues were then dehydrated through increasing concentrations of ethanol. Tisues were embeded in Agar 100 resin (Agar, USA). Semi-thin sections were cut by using an ultramicrotome (LKB, Sweden) and stained by toluidin blue in order to determine the lesion areas. Following the determination of appropriate lesion regions, ultra-thin silver-gold tissue sections were taken on 50 mesh copper grids and were stained by uranyl acetate, saturated in 70% methanol for 20min and were further stained by lead citrate for a minute. Selected fields of views from those sections were evaluated under a transmission electron microscope (EFTEM Libra 120) and electron micrographs were taken from convenient fields of views.

RESULTS

Non-laser Applied and the Buffer Application Control Groups Groups were evaluated together since the same findings were observed in the both control groups. All retinal layers were seen in their normal morphology. The highly vascularized pigmented loose connective tissue, choroid layer, was evident in its normal morphology beneath the pigment epithelium (Figure 1A). The semi-thin sections of the eye tissue revealed that all histological layers of the retina could be seen regularly. While lower cuboidal, retina pigment epithelial cell nuclei near the choroid layer were euchromatic and had distinctive nucleoli, their melanin granules seemed to be apically located and were dark basophilic (Figure 1B). The outer nuclear layer cell nuclei were rather large and seemed to fill almost the whole cytoplasm along with distinct heterochromatin patches at the electron microscopical level

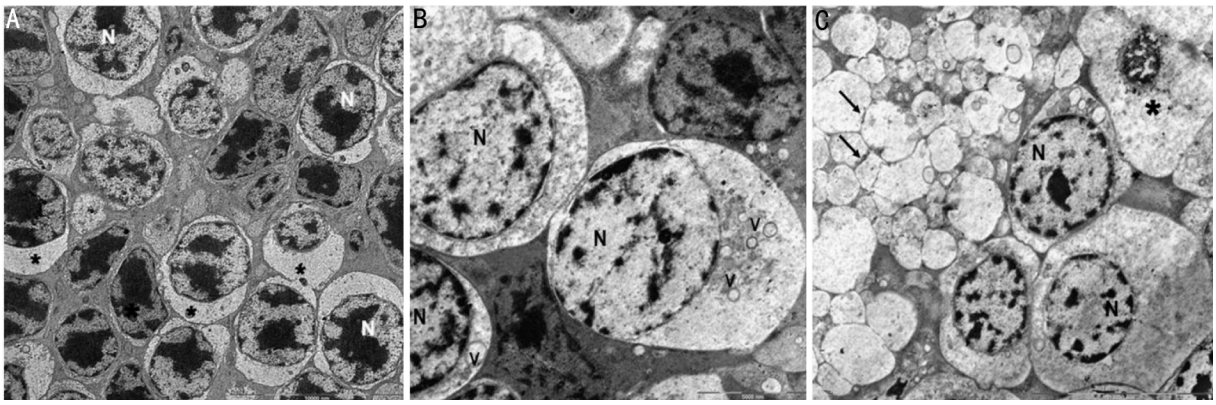

Figure 2 The fine structural view of the control group neural retina The fine structure of outer nuclear layer (A), inner nuclear layer (B) and inner nuclear-inner plexiform layers (C) of the neural retina in the control group. Nuclei (N), cytoplasmic vacuoles (V), synaptic areas (arrows) among the extensions of the inner plexiform layer and degenerative cells with dens picnotic nuclei and large cytoplasm in the inner nuclear layer (asterix). Bar: A and C: 10 000nm. B: 5 000nm

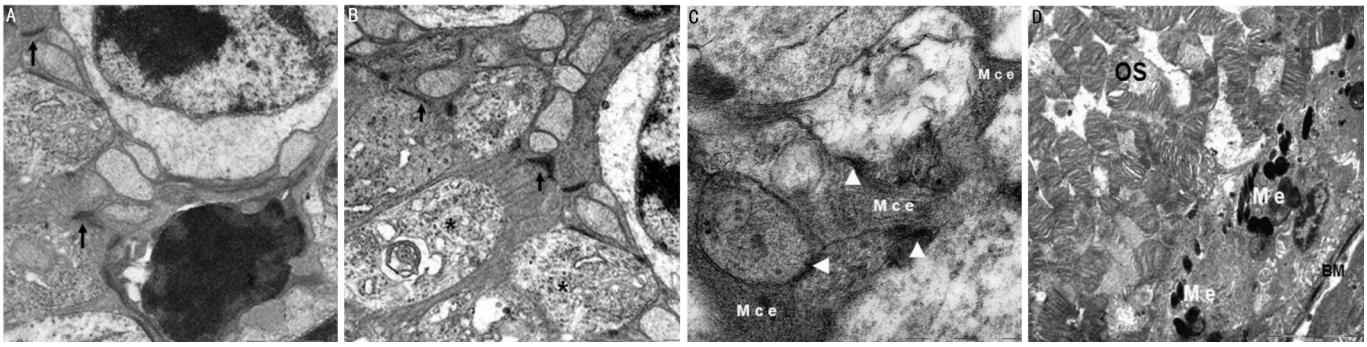

Figure 3 The fine structure of the retina in the control group A: Zonula adherens type junctional complexes (arrows) between photoreceptors and the extensions of Müller cells. Bar: 2 000nm; B: Junctional complexes (arrows) and rod cell spherules (asterix). Bar: 2 000nm; C: Synaptic areas (arrow heads) and Muller cell extensions (Mce) in the inner plexiform layer. Bar: 2 000nm; D: Melanin granules (Me), basement membrane (BM) and outer segments (OS) of photoreceptors in pigmented retinal cells with heterochromatic nuclei (N). Bar: 5 000nm.

(Figure 2A). The inner cell layer nuclei were larger and more euchromatic than the outer layer cell nuclei in which the cytoplasmic regions were more abundant. Heterochromatin was located as thin strips beneath the nuclear membrane (Figure 2B). Small cytoplasmic vacuoles were also evident in the inner cell layer (Figure 2B, 2C). The inner plexiform and the inner nuclear layers were in close contact with each other and the inner nuclear layer which was in normal morphology had also degenerative cells with heterochromatic, picnotic nuclei and a lucid cytoplasm (Figure 2C). Synaptic regions with dens appearance in the inner plexiform layer of neural retina were also observed (Figure 2C). Discontinuous outer limiting membrane established a border between the photoreceptor layer and the outer nuclear layer (Figure 3A). Zonula adherens type junctional complexes were evident between photoreceptors and Müllerian cells as observed in electron microscope (Figure 3A, 3B). The fine structure of rod cell spherules conserved their normal appearance (Figure 3B). While synaptic regions between the inner plexiform layer cells were observed as dens areas, Müllerian cell protrusions were also present among them (Figure 3C). Oval shaped retina pigment cells with melanin granules and the outer segments of photoreceptors were observed as normal morphology (Figure 3D).

The Diot Laser Photocoagulation Application Group Histopathologically retinal cell losses were observed throughout the whole retinal thickness. The most affected area seemed to be the outer nuclear layer, and cells of this particular layer were as totally diminished. However, cells in the neighbouring regions preserved their fine structures. The second degree affected areas were the inner nuclear layer and layers containing the inner and outer segments of photoreceptors. The outer plexiform and the inner nuclear layer cells seemed to fill the injury area that was formed by the laser shot. Those inner nuclear layer cells, had distinctive euchromatic nuclei. The continuity of retinal epithelial cells in the damaged region also vanished. Retina pigment epithelial cells in this region seemed to move to the upper areas. In this region, choroidal blood vessels were dilated (Figure 4A). Local histopathological alterations were observed in choroid and in the neural retina in the semi-thin sections of this group. The most distinct finding was the protrution of the burned region towards the surface area, increasing its thickness. The most affected area by the diot

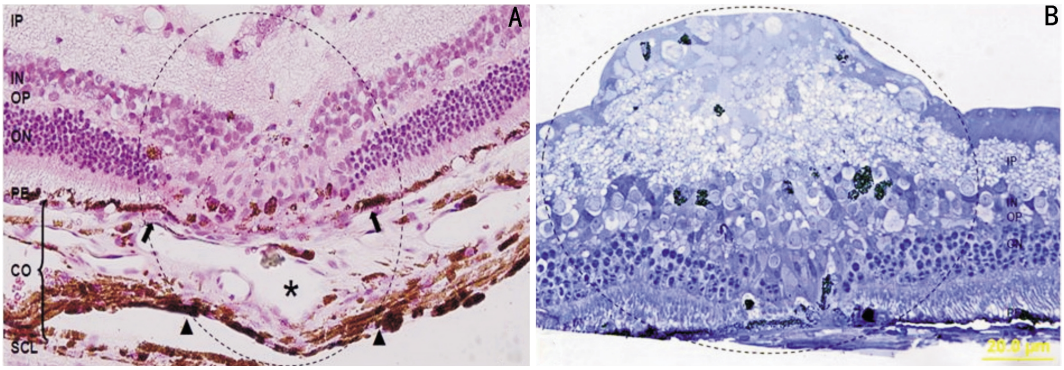

Figure 4 The diot laser photocoagulation application group Evagination towards the vitrous (arrow), invagination towards the choroid layer (arrow heads), dilatation in the choroidal capillaries (asterix) along with destruction and nuclear loss in the outer nuclear layer (ON) and discontinuity in the retinal pigmented epithelial layer (PE) in the damaged area (dashed line) of the diot laser photocoagulatin application group paraffin (A) and semi-thin resin (B) sections. Sclera (SCL), choroid (CO), pigmented epithelium (PE), outer nuclear (ON), outer plexiform (OP), inner nuclear (IN) and inner plexiform (IP) layers. A: H&E, ×40, B: Toluidin blue-O, Bar: 20μm.

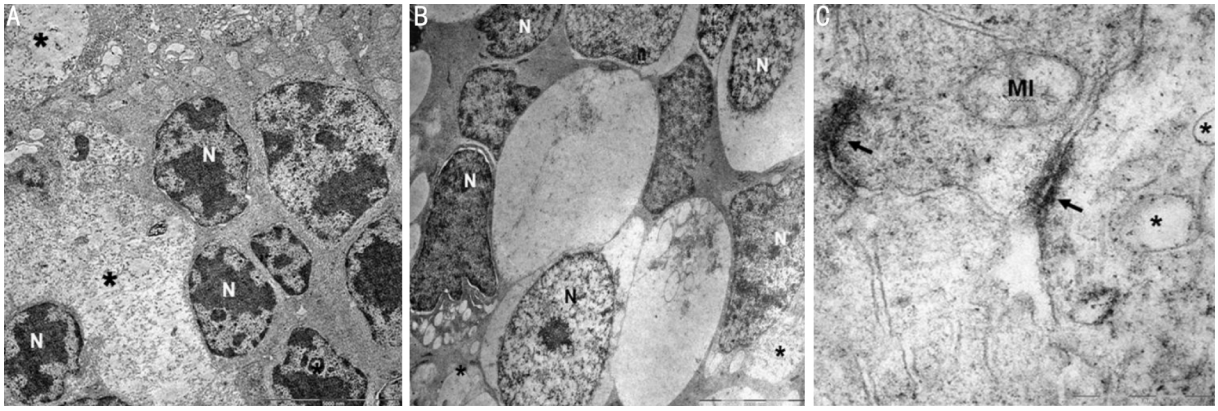

Figure 5 The fine structure of the damaged area ten days after the diot laser application A: Edematous fields (asterix) are seen among the outer nuclear layer cells.Bar: 5 000nm; B: Cells of the outer nuclear layer with heterochromatic nuclei (N) among the cells of the inner nuclear layer cells with euchromatic nuclei along with edematous areas (asterix). Bar: 5 000nm; C: Synaptic areas (arrows) among the ganglionic cells, bipolar cells and amacrine cells of the inner plexiform layer, mitochondria (MI) and synaptic vesicles (Sv) in the synaptic fields. Bar: 5 000nm.

laser shots seemed to be the outer nuclear layer. Nuclear loss in the central portion of the lesion region and an obvious destruction in the outer nuclear layer were quite evident. While the thickness of the outer nuclear layer seemed to be decreased, the inner nuclear layer invaginated towards the choroidal region. In addition, the retina pigment epithelium had also lost its continuity (Figure 4B). Transmission electron microscopical examination revealed that the outer nuclear layer cells had dens heterochromatic nuclei with irregular nuclear contours along with an evident cytoplasmic organelle loss (Figure 5A). Cells migrating from the inner nuclear layer had large euchromatic nuclei and distinctive nucleoli (Figure 5B). Extensive edematous areas were also evident among the inner nuclear layer cells of the damaged region (Figure 5A, 5B). Synapses between the inner plexiform layer ganglionic cells, bipolar cells and amacrine cells preserved their structure and synaptic regions had synaptic vesicles along with mitochondria (Figure 5C).

bFGF Administration Group Following Photocoagulation by Diot Laser The histological findings of this group have shown that the outer nuclear layer totally disappeared whereas cells of the inner nuclear layer proliferated and invaginated towards the choroid layer and came into contact with the retina pigment epithelium and finally filled the injury area. While the retina pigment epithelial cells paved the basis of the injury area, spherical, large and pigmented cells left the epithelium, moved towards the surface and localized among the inner nuclear layer cells (Figure 6A). While the retina pigment epithelium seemed to be continious in the injury area, extensive edematous fields were also observed just above the pigment epithelium (Figure 6A, 6B). On the other hand, other layer near to the injury area were found to preserve their normal morphology (Figure 6A, 6B). In some retinal samples, layers in the defect area were regularly lined up along with evaginations and invaginations. Retina had normal thickness and a succesful regeneration in the injury area was also achieved (Figsure 6A, 6B). In those samples, the most reactive layer was found to be the continiously progressing pigmented retinal epithelium (Figsure 6B, 6C). In addition, cellular proliferation in the

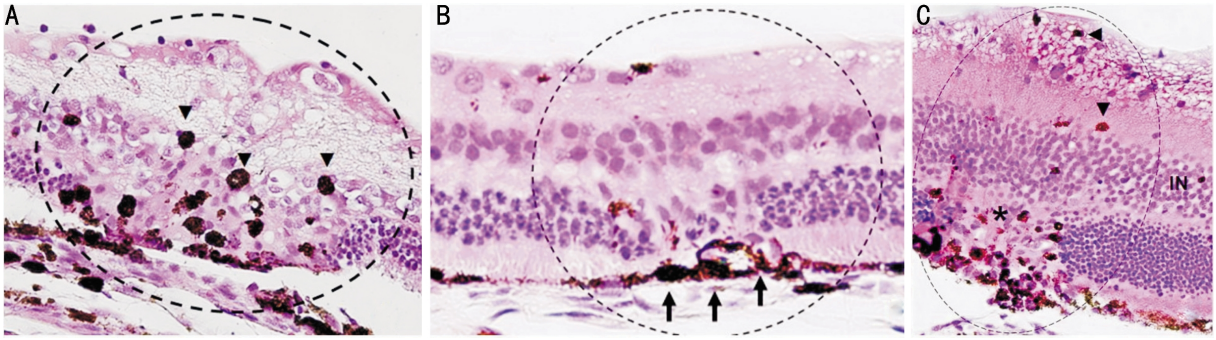

Figure 6 The view of the damage area (dashed line) in the bFGF administration group following diot laser photocoagulation A: Pigmented cells (arrow heads) gone into the inner nuclear layer. H&E, ×40; B: Continuous pigmented epithelium (arrows) in the damage area in which the repairing process was completed. H&E, ×40; C: Thickening in the inner nuclear layer (IN), advancement of the proliferating pigmented retinal layer cells towards the topmost layers (arrows heads) and invagination in the outer plexiform layer (asterix) could be seen. H&E, ×40.

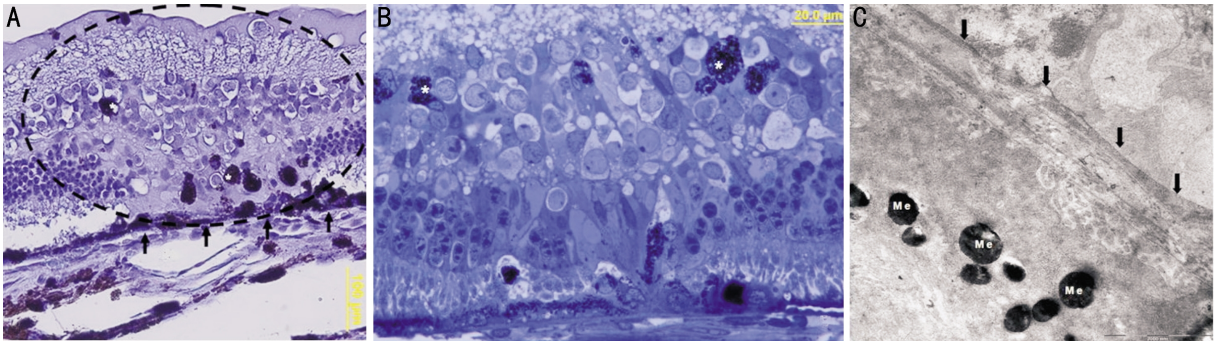

Figure 7 Findings of the bFGF administration group following diot laser photocoagulation A and B: Advancement of the decreasing number of pigmented retinal epithelial cells (asterix) towards the surface and the continuous Bruch membrane (arrow) beneath the pigmented epithelial layer could be seen. Toluidin blue-O, Bars A: 100μm, B: 20μm; C: The fine structure of the melanin granules (Me) in the pigmented retinal epithelial layer and continuous Bruch membrane (arrow). Bar: 2 000nm.

inner nuclear layer rather increased and it had an invaginated form towards the subjacent retinal epithelial layer (Figure 6C). The examination of semi-thin and ultra-thin sections analyzed under the electron microcope, revealed that the cells of inner nuclear layer increased their size along with euchromatic nuclei and periferally located nucleoli (Figure 7A, 7B). On the other hand, the bruch membrane and the retinal pigmented epithelium preserved their integrity (Figure 7A, 7B, 7C). Dilated choroidal capillary vessels were also observed during the repair process in this group (Figure 7A). The retina pigment epithelium cells were abundantly located in the lost outer nuclear layer whereas a small number of those cells reached the ganglionic cell layer following the diot laser photocoagulation (Figure 7A, 7B). The number of layers in the inner nuclear cell layer of the injury area, seemed to decrease following diot laser application when compared to the diot laser application group. In addition, the adjacent regions to the injury area seemed to be filled by the outer plexiform layer (Figure 7A, 7B). The ultrastructure of retina pigment cells progressing towards the surface demonstrated large, euchromatic nuclei with irregular contours and couple of nucleoli along with oval or spherical shaped pigment granules (Figure 7B). Ultrastructural examination of the retina pigment epithelium revealed that this layer preserved its integrity and continuouty, and cuboidal cells of this layer had spherical shaped melanin granules (Figure 7C).

DISCUSSION

In the present study, the effects of diot laser on retinal tissue and the healing effects of exogenous bFGF application on those laser injuries have been evaluated in a rabbit model. Even though the rabbit retina has no fovea, it almost has the same characteristics as a human retina. Since diot laser device was developed for human applications and thus small laboratory animals could not be used for diot laser studies, rabbit models might be used for such purposes. Since the burns caused by diot laser application were rather difficult to evaluate in albino rabbits' eyes, pigmented chinchilla rabbits were used in the present study. On the other hand, only male rabbits were used since the sexual differences were not studied in the present study. Corticosteroids along with neuroprotective agents used for the retinal injury therapy are commonly used for the neuroal tissue injury treatments [6,9,10,14-16]. Glial cells supporting the neurons could be found in all regions of the nervous system. Trauma, neurodegeneration, inflammatory diseases or central nervous system haemostazis disorders are caused by exitotoxicity activate glial cells and they undergo reactive gliosis[17-21]. This

process is characterized by changes in gene expression and cellular proliferation which are crucial mechanisms for neuronal protection and repair [17,19,21-23]. It has also been suggested that along with the benefits of glial-neuronal interaction, glial cell activation might have adverse effects thus might induce the progression of diseases[22].

The vertebrate retina is differentiated from the multipotent retinal progenitor cells. Therefore retinal stem cells were identified in the non-pigmented cilliar edge in the adult mammals' retina. In several *in vitro* studies, those stem cells were demonstrated to have proliferation and differentition abilities as multipotent retinal progenitor cells[22-24]. Therefore endogenous progenitors could be living sources for neurogenesis. Adult mammals' retina demonstrate neurogenic alterations when get injured. Vertebrate retina has a specific type of glial Müller cells that are not found in other parts of the central nervous system. It has been suggested that Müller cells could behave like stem cells following retinal injuries[19,24]. Müller cells in fish and birds dedifferentiate, proliferate and form new neurons as a response to retinal injuries. On the other hand, Müller cells in rodents could proliferate and form rod photoreceptors with bipolar cells following toxic damage[25]. These data clearly indicate that Müller cells could be a potential source for the proliferation of adult mammals retinal regeneration. Müller cells like the other central nervous system glial cells, might undergo reactive gliosis following acute retinal damage or chronic neuronal stress [24,26,27]. Therefore Müller cell gliosis could be characterized by cellular proliferation, changes in cell shape because of changes in the production of intermediate filaments, changes in ion transport properties and the secretion of signal molecules such as VEGF [25,26,28,29]. However, adult mammals' retina could take part in regenerative processes by demonstrating neurogenic alterations. In this process, Müller cells play a basic role as neural stem cells. For instance, they can renew themselves and have potentials of forming retinal neurons both *in vivo* and *in vitro* [30].

Gliosis is essential for the repair and the preservation of retinal neurons. However, some pathologies such as diabetic retinopathies might get worse by reactive gliosis [25,28,29]. Changes in Müller cell shape by the rapid regulation of glial fibrillar acidic protein and Müller glial cell proliferation following acute retinal damage were the first findings of reactive gliosis [26,31]. The role of reactive gliosis in several neuropathological situations in the central nervous system has been well studied[32,33].

bFGF is a versatile factor in the central nervous system related to the cellular events such as proliferation, regeneration and survivaling of the cell[34,35]. Increase in bFGF levels in the damaged region might well indicate that the extra- and intra-cellular secretion and/or activation of bFGF could play crucial regulatory roles in this process [11,15,36-39]. Previous studies have shown that the exogenous bFGF application, not only mimics the damage-dependent results, but also helps in surviving of neurons, induces the proliferation of non-neuronal retinal cells and increases the expression of intermediate filament proteins in retinal glial cells[40-42]. bFGF also prevents or delays the photoreceptor cell loss and degeneration in the light damaged retina and in the retinal distrophic rats [39,43-45]. It could lead to the renewal of ganglionic cell axons following the cut down of the optic nevre which stimulates the whole renewal of the retina in chick embryos undergone retinal excision [46,47]. It has been demonstrated that bFGF application to some regions of the central nervous system might decrease the neuronal death degree, following axonal injuries. Since bGF is present in the neural retina and in the retinal pigment epithelium in some forms of retinal degenerations which could take part in the retinal regeneration like seen in the central nervous system[8]. bFGF binds both to the high affinity cellular surface tyrozine kinase receptors and to the low affinity extracellular matrix receptors. It has been suggested that the bFGF binding to heparan sulfate containing molecules was essential for the functional interaction with the high affinity receptors [48]. The receptor-ligand complex is then taken into the cell like seen in several other growth factors [49]. The immunolocalization and *in situ* hibridization studies in the retina containing high concentration of bFGF, indicate the presence of bFGF in several cells and in extracellular fields [39,50-53]. When anti-bFGF was applied to the retinal sections, binding was observed in the inner and outer plexiform layers as well as in the retinal basement membrane [53]. Default high affinity bFGF receptors were determined in many retinal cell types such as photoreceptors, Müller cells, the retina pigment epithelial cells and ganglionic cells [49,53,56-60]. Exogenous bFGF application is a stimulus for the non-neuronal retinal cell proliferation in damages like retinal decollement.

The focal laser application to rabbit eyes induces local and widely distributed alterations both in rods and in cone-mediated retinal functions. Focal laser photocoagulation blocks the inner retinal signal transduction by PKC-alfa mediated inhibitory regulation [62]. In the present study, the focal diot laser application to the rabbit retina caused morphological alterations both regionally and in the neighbouring areas. The dimisenishing of the outer nuclear layer in the damaged area, intraretinal migration of the retina pigment epithelial cells, the continuous contact of the damaged area and the neighbouring regions, all of which were the findings of the present study; were all consistent with the findings of a previous study by Wallentén and co-workers [62]. Dilated choroidal capillaries following focal diot laser photocoagulation could indicate an increased blood flow during the repair process. Since the rabbit retina has no blood vessels, retinal blood flow is supplied by choroidal capillaries.

The adverse effects caused by diot laser application on retina pigment epithelium, were more distinct, when bFGF was not applied just after the laser application, The interruption or totally dissapearing of retinal pigmented epithelium and Bruch membrane could be the result of the absorption of radiation by chromofors like melanin.

In conclusion, even though the diot laser has an intense use for clinical purposes, damages in the application area, in the neighbouring regions and reversal of those adverse effects in the retinal layer and cellular morphologies by bFGF application could be suggested by the findings of the present study.

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