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The role of inflammation in diabetic retinopathy: a review

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Abstract. – OBJECTIVE: Diabetic retinopathy and diabetes represent serious health conditions, being considered among the main causes of blindness. In recent years, anti-VEGF therapies have been of great help in the treatment of retinal pathology and, until now, they represent the primary choice therapy for diabetic retinopathy. Nevertheless, many patients do not experience significant benefits of vision after an anti-VEGF monotherapy. For this reason, several researchers recently focused their attention on the mechanisms that play a central role in the development and progression of diabetic retinopathy.

RESULTS: Available scientific evidence confirms that diabetic retinopathy requires other molecules capable of modifying the mechanisms that, together with angiogenesis, contribute to the development of the condition, such as vascular and neuroinflammation.

CONCLUSIONS: This review summarizes the current knowledge of the pathological changes that occur in diabetic retinopathy and that might contribute to identify possible new strategies for the treatment of this condition.

Key Words:
Diabetic retinopathy, Diabetes, Anti-VEGF therapy, Neuroinflammation.

Introduction

Diabetic retinopathy (DR) affects nearly 40% of patients with diabetes mellitus, represents the main cause of blindness in subjects under the age of 50 years[1] and, similar to other conditions, has a significant impact on quality of life since patients with DR experience many socioemotional issues in addition to vision-related activity limitations[2-7]. It is expected that DR incidence will increase in the next decades due to the future increase of diabetes in the world population, affecting approximately 191 million people worldwide by 2030[8]. In the recent years, different therapeutic approaches have been developed for the treatment of ophthalmologic diseases previously considered difficult to manage[9-12]. Part of this success is due to the identification of the role played by the inflammatory process in these conditions[13].

Chronic inflammation is extensively involved in the development of DR and its complications[14-17], as well as in many other ocular diseases, such as conjunctivitis, keratitis, uveitis, glaucoma, age-related macular degeneration and diabetic retinopathy[18-20]. In immune-dependent pathologies, the inflammatory process induces a complex cascade of biological, molecular and cellular signals that alter the physiological responses of the affected eye tissues. Inflammatory stimulus (oxygen radicals, diabetes, and infections) may disrupt the natural balance of the eye tissues, thus producing an "inflamed" phenotype. The result of these processes is the increased expression of inflammatory cytokines (IL-1 and TNF), chemotactic proteins (MCP-1), growth factors (TGF-β and VEGF) and apoptotic phenomena which, taken together, contribute to the onset of different eye diseases.

To date, the molecular mechanisms that determine the development of ocular pathologies are not fully clarified and there is no therapy capable of preventing eye damage for people with diabe-

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tes[21-26]. Understanding the cellular and molecular mechanisms that lead to eye damage could be useful for the development of new anti-inflammatory agents and new therapeutic approaches, thus avoiding invasive surgical treatments.

Diabetic Retinopathy

DR is the most important ocular complication of diabetes mellitus. The main risk factors associated with the early onset and rapid evolution of diabetic retinopathy include the duration of diabetes, poor glycemic control and concomitant hypertension.

Glycemic control is undoubtedly the most important modifiable risk factor[27]. DR is classified into two forms, one early and less severe (non-proliferating) and one advanced (proliferating). The first, if not recognized and treated promptly, evolves towards the highly disabling proliferating form. In non-proliferative DR, the identification of advanced non-proliferating retinopathy is important as it develops, if left untreated, in proliferating DR in 40% of cases within 12 months.

Hyperglycemia damages the structure of the blood vessels predisposing to the formation of microaneurysms, microhaemorrhages and anomalies of the retinal vascular caliber. These anomalies may lead to the passage, through the damaged walls of the vessels, of some components of the blood, or to reduced perfusion of the retinal tissue up to a complete ischemia, which is initially manifested by the presence of cottony exudates. The occlusion of the retinal capillaries and the consequent formation of ischemic retinal areas represent the stimulus for the formation of retinal neovessels, which characterize the proliferating form[28].

Clinical Presentation

DR usually begins with the appearance of retinal microaneurysms and the presence of spot haemorrhages[11] (Figure 1). Subsequently, the disease evolves into the most severe form of proliferative DR. During this phase, neovascular changes occur with consequent deposition of fibrotic tissue, retinal detachments, vitreous haemorrhages and diabetic macular edema[11,29] (Figure 2). The retinal tissues of diabetic patients show increased thickening of the basement membrane of the capillaries[30]. Normally the retinal capillaries are made up of continuous internal layers of endothelial cells surrounded by the discontinuous processes of intramural pericytes. The basal membrane (BM) consists of a thin internal sub-endothelial membrane (IBM), interposed between the endothelial cells and the pericytes, and an external basal membrane (EBM) placed between the pericytes and the Müller glial cells. Under pathological conditions, a significant thickening of the external basal membrane is observed. The thickening of the basement membrane seems to be capable to involve mainly the diabetic capillaries located in the layer of nerve fibers near the internal limiting membrane. Furthermore, in diabetic retinas, the microvascular subcomponents of the extracellular matrix (ECM) are altered and there is a noticeable loss of pericytes[30]. To date, it is not clear whether pericytes are directly involved in the thickening of the EBM.

Changes in the Diabetic Retina

In conditions of hyperglycemia, there is the activation of the microglia which secretes cytokines and other pro-inflammatory molecules involved in phagocytosis and in the destruction of damaged cells, as well as in the initiation of repair processes that lead to the formation of glial scars.

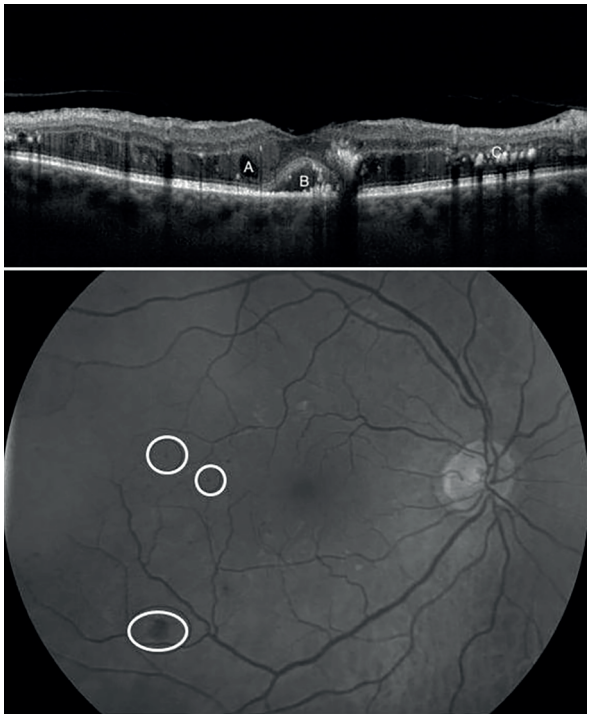

Figure 1. Upper panel: Optical coherence tomography (OCT) image showing several signs of advanced diabetic retinopathy: intraretinal edema **(A)** pigmented epithelium detachment **(B)** and hard exudates **(C).** Lower panel: Retinography of a patient with early stages of diabetic retinopathy; the white circle shows intraretinal haemorrhage.

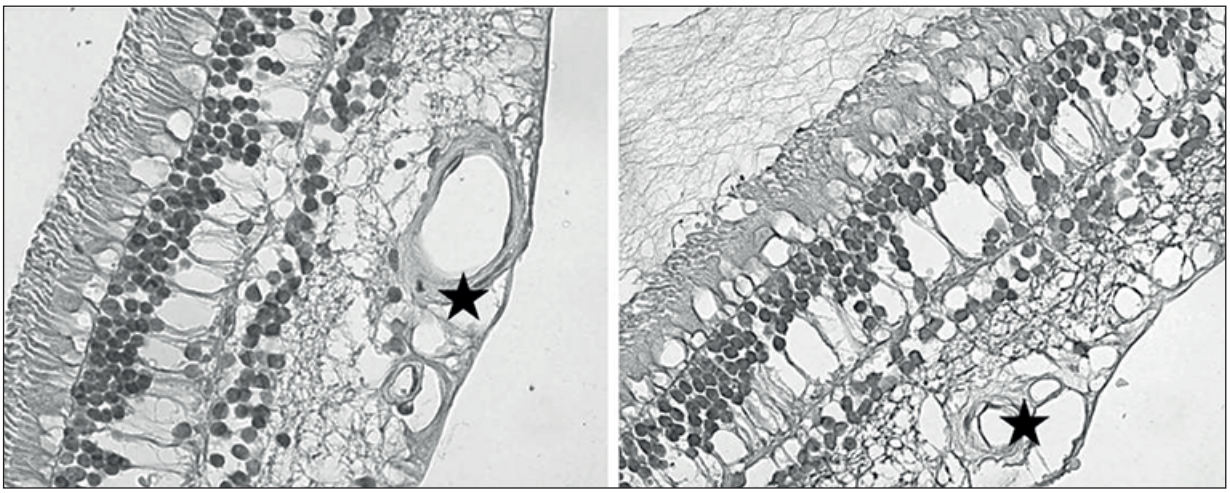

Figure 2. Light microscopy. Panels show human retina of patients affected by advanced diabetic retinopathy with ischemic areas (stars). H&E stain (×40).

However, if microglia remain in an activated state, cytokines may damage neighboring cells, particularly neuronal cells, inducing the appearance of other retinal pathologies, such as retinal degeneration and glaucoma[31]. Many ocular tissues are sensitive to the fibrotic process. This process intervenes in the pathogenesis of different ocular pathologies such as glaucoma, cataract, age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR)[32]. Pathologies of the posterior segment of the eye involving the retina and choroid are sensitive to the fibrotic process. In fact, they present vitreoretinal or subretinal fibrosis that often causes detachment of the retina and, in severe cases, blindness[33,34].

During the preclinical phase of diabetic retinopathy (PCDR), the increase in permeability of the blood-retinal barrier produces a hypoxic environment that determines the accumulation of numerous pro-angiogenic and inflammatory cytokines. According to this thesis, numerous histopathological studies performed on animals and humans have highlighted the activation of microglial cells, as well as the presence of various inflammatory molecules secreted by microglial cells[35]. This condition induces the secretion of TNF-α and the localized secretion of other proinflammatory cytokines, growth factors and bioactive molecules that play an important role in the onset and progression of diabetic retinopathy[36].

Liou et al[37] confirmed that the activation of the microglia occurs already at the beginning of the diabetic pathology, producing the secretion of a wide range of proinflammatory cytokines, such as IL-1β, IL-3, IL-6, TNF-α and other mediators of the inflammation such as ROS, glutamate, VEGF, metalloproteinases and NO. These proinflammatory mediators induce the expression of adhesion molecules (I-CAM and V-CAM), cell apoptosis, leukocytes infiltration and weakening of the blood-retinal barrier[37]. Langmann et al[38] also found that microglial cells are responsible for an increase in retinal production of iNOS, IL-1β, MIP-1α, IL-6 and M-CSF. Shelton et al[39] showed an increase in IL-1β, IL-6, IL-8, IL-13, IP-10, ICAM-1 and NO in Müller cells and in endothelial cells in the course of diabetes, confirming their participation in the inflammatory process. In addition, it is well known that many other mediators are expressed in retinal tissue during diabetes, since it is now clear that inflammation plays an essential role in DR[40].

The thickening of the basement membrane is one of the most studied morphological changes that occur in the microvascular system during diabetes. It is not clear which of the different factors primarily contributes to the development of the vascular diabetic anomalies. The occurrence of capillary thickening of the basement membrane suggests that this change may be related to a deterioration of some tissue functions during the development of the disease. Vascular abnormalities are represented by reduced contacts between pericytes and endothelial cells, in combination with an increase in the level of VEGF expression. Angiogenesis is an important physiological process that induces differentiation, tissue growth and damage

repair. Pathological angiogenesis, also known as neoangiogenesis, very commonly develops during ischemia, inflammation and neoplastic diseases. VEGF has been recognized as the main neo-angiogenic factor responsible for both sub-retinal (DMLE, pathological myopia, uveitis) and retinal (diabetic retinopathy, retinal vein occlusion, retinopathy of the premature newborns) conditions[41].

Diabetes is therefore considered a metabolic disease that accelerates the development of vascular thickening of the basement membrane in retinal capillaries, a process that normally occurs with aging.

Diabetic Retinopathy and Inflammation

Inflammation may play a key role in DR mediating harmful effects on neuronal and vascular components of the retina.

Cellular and molecular alterations typical of inflammatory processes are present in the retina of diabetic patients. DR is the manifestation of a low-level chronic inflammation state in which different effectors such as cytokines and leukocytes are responsible for damage to the retina. This pathology shows many similarities with chronic inflammatory diseases: increased vascular permeability, edema, infiltration of inflammatory cells, destruction of tissues, neovascularization and expression of angiogenic factors. In the retina of diabetic patients, significant inflammatory involvement is also evident in the early stages of the disease. In addition, leukocytes including monocytes-macrophages, microglia, neutrophils and a number of lymphocytes adhere to the vascular endothelium; this phase temporally coincides with the impairment of the blood-retinal barrier, with capillary occlusion and endothelial cell death[42]. These alterations induce the secretion of TNF-α and the localized secretion of pro-inflammatory cytokines, growth factors and other bioactive molecules that play an important role in the onset and progression of diabetic retinopathy.

Diabetic retinopathy was originally considered a purely micro-vascular disease. Currently it is instead considered as a chronic inflammatory disease that leads to changes in the retinal microcirculation. Generally, glial cells respond to hyperglycemia by producing pro-inflammatory cytokines. In fact, diabetes determines the activation of caspase-1 and the consequent production of IL-1β, which leads to the death of Müller cells[42]. Retinal glial cells, including Müller cells, can also be the initiators of neural inflammation in the diabetic retina acting as cytokine producers. IL-6 may also play a direct role in the infiltration of monocytes and T lymphocytes. Chronic inflammation induces the activation of myofibroblasts, that originate from fibroblast cells and determine the excessive deposition of ECM with consequent activation of the fibrotic process which induces laceration and retinal detachment[32,42,43].

Role of Inflammatory Cytokines in Diabetic eye Disease

There is a close correlation between inflammation and DR[44-46] demonstrated by the presence of high levels of growth factors and inflammatory cytokines in the eye fluids of patients with diabetic retinopathy[44-46]. In the retina, the pro-inflammatory state is related to the activation of NF-kB, as a consequence of an increase in oxidative stress which induces high levels of pro-inflammatory cytokines, such as IL-1b and TNF-a, chemokines and adhesion molecules (E-selectin, Intercellular Adhesion Molecule 1 - ICAM-1-)[47-52]. The activation of NF-kB due to hyperglycemia and the activation of the inflammatory process causes loss of pericytes, increase in vascular permeability and the appearance of microaneurysms[52], contributing to the development of retinopathy. During diabetes, we assist to the activation of macrophages located in the adipose tissue or in the pancreatic islets; this activation determines the secretion of cytokines such as interleukin (IL-1b and others)[53-55]. In fact, Catano Canizales et al[56] observed an increase in the levels of secretion of IL-1b by monocytes obtained from diabetic patients compared to healthy controls. A recent study on mice, whose β-pancreatic cells were treated with some inflammatory cytokines (IL-1 b, IFN-c, TNF-a), showed that TNF-a acted by inhibiting insulin secretion induced by glucose. These results demonstrated that oxidative stress and activation of the inflammatory process generate cell death and dysfunction of B cells, contributing to the development of diabetes and other related diseases[15,57].

The retina uses high quantities of glucose and oxygen to generate energy (ATP) and to guarantee the visual function by using the mitochondrial electron transport chain (ETC) in the internal membranes[58]. During this process, the electrons escape from ETC, which are recruited from molecular oxygen to form reactive oxygen species (ROS), thus creating harmful lipids, proteins and mtDNA of the mitochondrial membrane. Mitochondrial ROS and oxidized mtDNA are released into the cytosol and recognized as

damage associated molecular profiles (DAMP) by the receptors for the recognition of cytosolic patterns (PRR), including the toll-like receptors TLR4, TLR9 and NLRP3. These inflammatory receptors induce the production and activation of pro-IL-1β and pro-caspase-1[58].

Diabetic Retinopathy and Loss of Pericytes

Diabetic retinopathy is characterized by microvascular alterations of the capillary endothelium and loss of pericytes that lead to the formation of microaneurysms, as well as to the loss of the blood-retinal barrier (BRB) and the formation of new blood vessels (neovascularization)[59,60]. In DR, there are numerous dysfunctions at the level of the microvessels that induce subsequent alteration of the neurovascular component and degeneration of the blood-retinal barrier such as thickening of the basement membrane, formation of microaneurysms, loss of pericytes, and vasoregression. As already reported in a previous study from our group[20], the first vascular change observed in the retina is the thickening of the basement membrane of the retinal capillaries. This condition is related to the loss of pericytes and subsequent dysregulation of vascular tone. The thickening of the basement membrane causes serious functional damage, allowing the passage of proteins and inflammatory molecules in the interstitial space[61,62]. In recent years, studies have focused on the induced consequences of the loss of pericytes found in the retinal capillaries[63].

The pericytes, which are wrapped around the capillaries, are believed to be responsible for the structural integrity of the vessel walls. In fact, they adhere to the abluminal surface of the endothelium and are necessary for the stabilization of the microvascular network. In the course of DR, persistent hyperglycemia produces the loss of retinal pericytes and the uncontrolled proliferation of capillary endothelial cells. Depletion of pericytes in diabetic retinas induces a significant reduction of branching points in the retinal capillaries, together with a reduced endothelial germination, while the number of endothelial cells in the peripheral plexuses increases. In addition, the loss of pericytes leads to an increase in the size of the capillaries and the degeneration of BRB which increases vascular permeability until the appearance of edema in the macular area[64]. This condition results in a high blood concentration of inflammatory molecules and growth factors also found in intraocular fluids of diabetic patients[65,66]. Furthermore, activation of glial cells and degeneration of neuronal cells are connected to alterations of retinal capillaries in DR.

Activation of microglia as a consequence of high glucose levels causes the production and the local release of inflammatory cytokines. In fact, the activated microglia migrate from the internal-external retinal layers and induces release of TNF-α, IL-6, MCP-1 and VEGF. Astrocytes may also participate in the inflammatory cytokine secretion[67,68]. Damage to neuronal cells of the retina is one of the first processes that occur at the beginning of DR. In fact, the first cells to be damaged are the ganglion cells of the retina[69]. Pericytes also may play an active role in the progression of the inflammatory process in the retina, determining the attraction of immune cells towards the inflammation site[70]. The understanding of different mechanisms believed to be responsible for retinal damage is essential in the finding of new therapeutic strategies.

Discussion

To date, the exact mechanisms through which neuroinflammation and vascular inflammation occur in DR are still partially unknown.

Vascular inflammation is an inflammatory process that also affects endothelial cells, pericytes and immune cells located in the inner layer of blood vessels. During mitochondrial oxidative stress, the endothelial cells of the capillaries react following the activation of an inflammatory process that induces vascular inflammation and consequent vascular dysfunction[71]. In many tissues subjected to stress conditions, macrophages play a central role in the inflammatory response[72]. In the brain, microglia are made up of specialized macrophages capable of carrying out phagocytosis to protect neurons of the central nervous system. They constitute a network of cells that protects neurons from the surrounding environment. Therefore, neuroinflammation could depend on the continuous activation of glial cells through the action exerted by macrophages[73,74]. During the activation of the microglial infiltration specific pro-inflammatory molecules, ROS and toxic molecules[75] are locally released. In type 1 and type 2 diabetes, chronic hyperglycemia leads to the accumulation of advanced end glycation products (AEGP) with consequent endothelial dysfunction and vascular inflammation[76,77].

Vascular inflammation of the retinal tissues is one of the main factors contributing to the development of the disease[78]. Upregulation of pathological retinal tissue inflammatory genes has been observed in previous studies[79-81]. Furthermore, changes in morphology, number and position of microglial cells have been reported in the aged retinas[82]. DR usually begins with the appearance of retinal microaneurysms and spot haemorrhages[11], followed by degeneration and loss of function of retinal neurons, especially retinal ganglion cells (RGC)[83,84]. Notwithstanding this mechanism, neuronal depletion cannot be considered alone as the main cause of blindness; subsequently, the disease develops in the most severe form, the proliferative DR. During this terminal phase, neovascularization changes occur, and consequent deposition of fibrotic tissue, retinal detachments, vitreous hemorrhage and diabetic macular edema are observed[11,29].

Neuronal damage develops before vascular damage, thus attributing a neuropathic origin to the disease[83,84]. This observation also highlights the crosstalk between neuronal, glial and vascular cells, which may be responsible for the retinal neurovascular unit damage[85]. Damage to endothelial cells could induce activation of microglial cells[77]. Analyzing data obtained by the literature, DR could be caused by activation of the inflammatory response both at vascular and neuronal level. Inflammation is quite a defensive response that is triggered by stimuli and harmful conditions, such as an infection or a tissue damage. In the site where the inflammatory event begins, the involved cells produce a series of cytokines and chemokines that act on the local vascular endothelium, causing dilation of blood vessels, leakage of fluids and recruitment of neutrophils and monocytes from the blood into the tissue[86]. Initial response by resident macrophages produces the release of a variety of inflammatory mediators, including chemokines, cytokines (TNF-α and IL-1β), vasoactive amines and prostaglandins[87]. Consequently, local inflammatory exudate is formed. Plasma proteins and leukocytes (neutrophils and monocytes) exit the circulation and adhere to the tissues at the site of infection/damage. Once arrived in the damaged tissues, monocytes and neutrophils are activated (either through direct contact with the pathogenic material or through the assistance of cytokines secreted by resident cells) and, in an attempt to react against the onset of the process, release cytotoxic substances (reactive oxygen and nitrogen species, proteases, elastases, collagenases). These factors, undiscriminating between possible microbial targets and host tissues, cause tissue damage as a side effect of the defense activity. Despite this essential event for the defense of the integrity of the organism from external attacks, the inflammatory response requires a strict control of its activation but, above all, it has to circumscribe the effects of the harmful agent that triggered it, thus avoiding a significant damage to the human organism itself. In fact, the high levels of inflammatory cytokines and adhesion molecules may determine accumulation of leukocytes and formation of retinal neo-capillaries. Some studies have shown an altered expression of inflammatory cytokines not only at the vascular level, but also at the glial cells level. Therefore, it is ascertained that Müller cells and astrocytes can express inflammatory cytokines in conditions of hyperglycaemia[88].

Since both glial cells and vascular endothelial cells are found in close association, Barber et al[89] suggested that the reactivity of the former is a direct consequence of the infiltration of glucose and inflammatory agents into the nervous parenchyma and that the increase in vascular permeability is supported by the release of some glial factors with a consequent loss of integrity of the blood-retinal barrier. The release of cytokines and proinflammatory molecules, such as TNF-α, IL-1β, nitric oxide and VEGF, causes the spread of the inflammatory process throughout the entire retina, increasing vascular permeability and neuronal damage and thus creating a vicious circle[35,90]. Müller cells and astrocytes become active and produce proinflammatory cytokines and growth factors within retinal tissue during the chronic evolution of DR. The persistent inflammatory response then leads to death or cell damage.

IL-1β has proven to be the main cytokine capable of triggering the neuro-inflammatory cascade. This cytokine could have a crucial role in the amplification of inflammation itself. In fact, the secretion of IL-1β is widely expressed at the vascular endothelium level as a direct consequence of chronic hyperglycemia. The latter stimulates endothelial cells and microglial cells that respond not only by activation signals, but also by strengthening of the synthesis of IL-1β, thus enhancing the inflammatory process itself[91]. In a previous investigation[92] we have shown that VEGF may be secreted by different types of retinal cells such as EPR cells, pericytes, astrocytes,

Müller cells and endothelial cells. VEGF stimulates endothelial cells to degrade their basement membrane and migrate with concomitant release of MMPs and integrins. Studies carried out in the last decade have led to a better understanding of the fundamental role that VEGF exerts in the development of pathological angiogenesis in some retinal diseases characterized by intraocular neovascularization and in the pathogenesis of endothelial hyperpermeability associated with the accumulation of intra- and sub-retinal fluid. This typical condition may be observed in retinal vascular diseases, characterized by edema and exudation, which often affect the macular region, and which involve a global reduction of the central visual functions. However, the pro-inflammatory response induced by neovascular proliferation also involves the chemotactic migration of macrophages that secrete TNF-α, a cytokine that induces angiogenesis "*in vivo*" through an increase of the expression of VEGF receptors (VEGFR-2) on the endothelial membrane[93,94].

Several research groups support the idea that at retinal level the neurodegenerative process may be triggered by vascular inflammation. In fact, the same process occurs in neurodegenerative diseases of the brain[95-97]. Available evidence shows that the use of antioxidants and the modulation of the inflammatory response in the early stages of the disease may be considered useful in preventing the onset of ocular complications[10,98-102].

Conclusions

The discovery of the involvement of inflammatory process in the onset and progression of DR has led to the development of new pharmacological treatments. Currently, anti-inflammatory drugs and inhibitors of inflammatory molecules may be used alone or in association with VEGF inhibitors in the treatment of DR. Although anti-VEGF therapy remains the first-choice treatment for DR, it may not be able to satisfactorily control the inflammatory component that causes damage to the retinal tissues. This assumption explains the lack of a satisfactory clinical response found in most patients. For this reason, it has been postulated that the development of combined pharmacological treatments, which involve the use of anti-VEGF in association with anti-inflammatory drugs, may be a fruitful therapeutic option. Faricimab (formerly RG7716), is a bispecific anti-Ang-2/anti-VEGF monoclonal antibody used in the treatment of DR[103]. Currently, several drugs targeting cytokines and inflammatory molecules are under evaluation. The combined use of these new drugs in association to anti-VEGF drugs might be more effective and have less harmful effects than steroidal drugs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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Authors' Contribution

ST, MR, LZ and AM designed the study. MN, AG, MA, GA, MG and AMP consulted literature and collected data, ST and MR wrote the paper. LZ and AM reviewed and edited the manuscript. All authors read and approved the manuscript.

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