Biologia Serbica, 2021, 43(1): 50-56

DOI 10.5281/zenodo.5512518

Review

Blood-retinal barrier breakdown in diabetic retinopathy - the protective role of melatonin

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Accepted: 25 August 2021 / Published online: 20 September 2021

Summary. Diabetes mellitus and its complications represent a global socio-economic burden with more than 450 million people affected worldwide. Diabetic retinopathy is present in over one-third of people living with diabetes and represents a leading cause of visual impairment among the working-age population. The integrity of the blood-retinal barrier (BRB) is essential for retinal neuronal health. Barrier breakdown results in fluid accumulation in the retina, macular edema, neuronal death, and vision loss. BRB breakdown may result from a disruption of the tight junctions, an up-regulation of vesicular transport across the inner or outer BRB, or by degenerative changes to the endothelial cells, the pericytes, and glia. The present review aims to discuss mediators of BRB dysfunction and molecular mechanisms of BRB breakdown in diabetes mellitus and the emerging evidence that patients with diabetic retinopathy might benefit from melatonin treatment. The data suggest that melatonin might protect ocular tissues by decreasing the production of reactive oxygen species (ROS) and pro-inflammatory mediators implicated in BRB breakdown, such as vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). Therefore, melatonin might be considered for treatment of ocular diseases characterized by BRB, although, the topic remains under investigation.

Keywords: blood-retinal barrier, diabetic retinopathy, melatonin.

INTRODUCTION

With more than 450 million people living with diabetes worldwide, it represents a global socio-economic burden. These numbers are expected to increase to 693 million by 2045 (Cho et al. 2018). Diabetic Retinopathy (DR) is a specific neurovascular complication of both type 1 and 2 diabetes, whose progression strongly correlates with the duration of diabetes and glycemic control (Solomon et al. 2017). DR is present in over one-third of people living with diabetes and represents the leading cause of visual impairment and preventable vision loss among the working-age population (Cheung et al. 2010; Yau et al. 2012). The incidence of vision-threatening stages of DR is highest in low and middle-income countries, and the number of diabetes mellitus cases are expected to increase 69% by 2030 (Shaw et al. 2010). Vision loss associated with DR is usually due to macular edema and neovascularization (Roy et al. 2017).

The retina has the highest oxygen consumption and metabolic activity in the human body, supported by unique dual circulation (Klaassen et al. 2013). The blood-retinal barrier (BRB) represents a selective physiological barrier that maintains homeostasis in the neural retina and restricts the entry of molecules and cells found in the blood. BRB has an inner and an outer component. The inner BRB surrounds all retinal blood vessels and is formed by tight junctions that seal the intercellular spaces between the retinal endothelial cells, while the outer component is formed by tight junctions between the retinal pigmented epithelial cells (Hildebrand and Fielder 2011). Small lipophilic molecules can, however, diffuse across the barriers.

The integrity of BRB is essential for retinal neuronal

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health. The term neurovascular unit refers to the functional relationship between neurons, glial cells, and blood vessels in the retina. These cells work in coordination to integrate retinal blood flow with metabolic activity (Gardner and Davila 2017). In case of barrier breakdown, excess fluid accumulates in the retina resulting in macular edema, neuronal death and vision loss. BRB breakdown (Fig. 1) may result from a disruption of the tight junctions, an up-regulation of vesicular transport across the inner or outer BRB, or by degenerative changes to the endothelial cells, the pericytes, and glia (Klaassen et al. 2013).

In recent years, increasing insight in the molecular mechanisms of BRB breakdown led to the development of new innovative treatments for DR (Cheung et al. 2014; Dehdashtian et al. 2018; Djordjevic et al. 2018; Scuderi et al. 2019).

The present review aims to discuss mediators of BRB dysfunction, the molecular mechanisms of BRB breakdown in diabetes mellitus and the emerging evidence that patients with diabetic retinopathy might benefit from melatonin treatment.

SEARCH FOR RELEVANT ARTICLES

Relevant articles included in this review were identified by searching the PubMed database using the search terms: diabetic retinopathy AND blood-retinal barrier breakdown AND mechanism/diabetic retinopathy AND melatonin). Only studies published in English in the last 10 years were included. The reference lists of relevant articles were also reviewed in order to identify additional appropriate articles. The abstracts of all derived papers were evaluated by two reviewers (B. Djordjevic and J. Milenkovic), and papers considered not to be relevant to the review's aim were excluded. Studies and reviews related to the mediators and mechanism of blood-retinal barrier breakdown were included if they dealt with the effect of vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α) and interelukin-1β (IL-1β). Most of the included studies evaluated the effects of melatonin in animal models or *in vitro*. There was no restriction to any dosage, duration, or administration route of melatonin. Observational studies in humans were included since randomized trials were not present in the search results.

The search retrieved 170 (130+40) records following

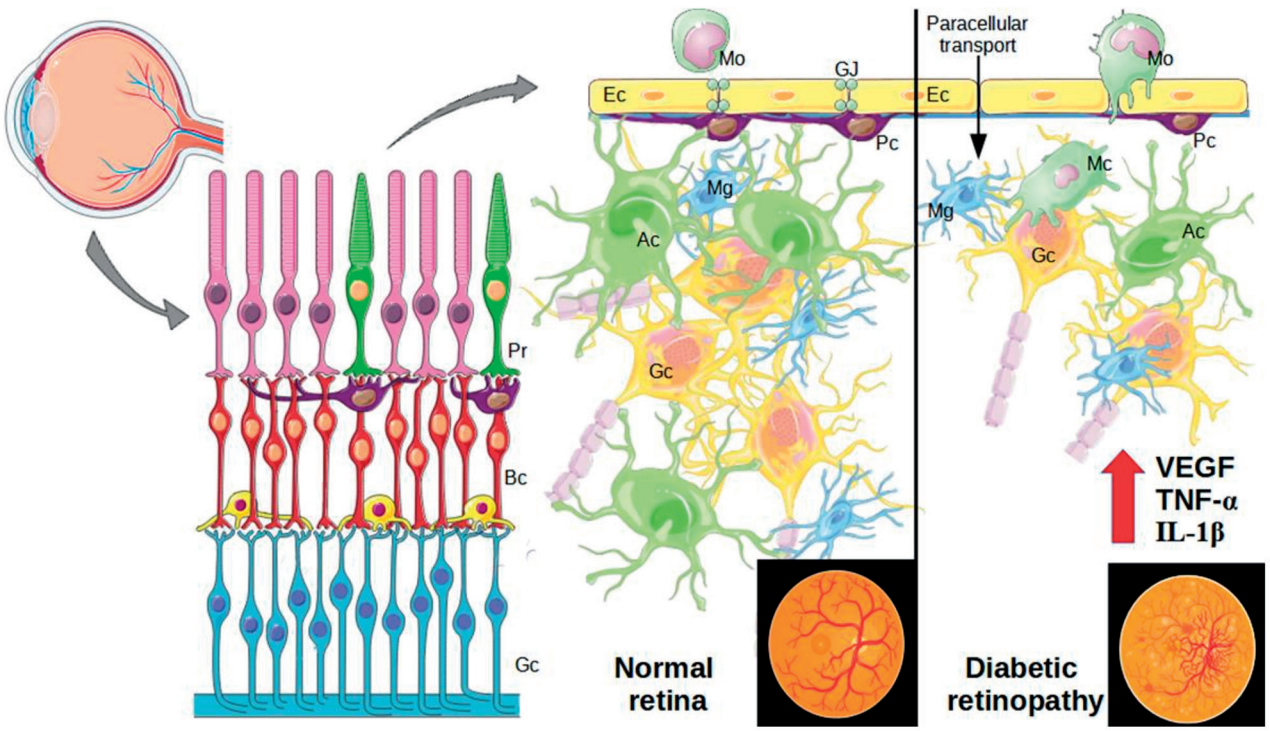

Fig. 1. Degradation of neurovascular unit and BRB breakdown in the diabetic retinopathy. Legend: Mo, monocyte; GJ, gap junctions; Ec, endothelial cell; Pc, pericyte; Mg, microglia; AC, astrocyte; Gc, retinal ganglion cell; Pr, photoreceptors; Bc, bipolar cells; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β. This figure was drawn using the vector image bank of Servier Medical Art (http://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

both criteria. We considered 36 (19+17) eligible to include in the discussion. Additional searches retrieved 22 records that were also included in the discussion.

DISCUSSION

Mediators of BRB dysfunction and increased permeability in diabetes mellitus

BRB breakdown is a complex process mediated by multiple interactions among factors operating through different receptors and signalling pathways. Still, hyperglycemia, hypoxia, oxidative stress, and inflammation are considered to be crucial underlying processes involved in BRB dysfunction.

Several metabolic pathways have been implicated in hyperglycemia-related cellular damage, including the formation of stable advanced glycation end products (AGEs), activation of protein kinase C (PKC), and the polyol pathway (Klaassen et al. 2013; Gui et al. 2020). The unifying mechanism behind these hyperglycemia-induced metabolic changes appears to be increased production of reactive oxygen species (ROS) in mitochondria that result in oxidative stress (Brownlee 2005). ROS can cause damage to macromolecules and cellular structures and promote inflammation in the diabetic retina (Kowluru and Chan 2007; Gui et al. 2020).

Changes in the circulating and local concentrations of pro-inflammatory mediators are a hallmark of inflammation. Their increased levels are related to the changes in the expression of pro-inflammatory transcription factors such as nuclear factor kappa B (NF-κB) (Tang and Kern 2011; Tarr et al. 2013). The increased adhesion of leukocytes to endothelial cells in the retina appears to correlate with an increase in vascular permeability. Leukocytes adhere to blood vessel walls after binding to vascular (VCAM-1) or intercellular adhesion molecules 1 (ICAM-1) on the surface of endothelial cells (Tarr et al. 2013). In addition to the physical occlusion of capillaries, leukocytes release cytokines and growth factors, and generate ROS that all contribute to capillary degeneration and BRB dysfunction (Tarr et al. 2013). A number of molecules have been identified as playing a role in BRB breakdown (Fig.1), however vascular endothelial growth factor (VEGF), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are considered to be among the most potent inducers of BRB breakdown (Gui et al. 2020).

VEGF is a key regulator of vascular permeability and angiogenesis in the retina (Gupta et al. 2013; Gui et al. 2020). It can be synthesized and released by Müller cells, retinal pigmented epithelium, retinal ganglion cells, pericytes, and endothelial cells in response to hypoxia, hyperglycemia, or oxidative stress (Sone et al. 1996; Tarr et al. 2013; Marazita et al. 2016). In addition, matrix metalloproteinase 9 (MMP-9) up-regulates VEGF and increases its bio-availability.

TNF-α is a pro-inflammatory cytokine that can be synthesized by Müller cells, activated macrophages, glial cells, retinal ganglion cells, and neurons (Tarr et al. 2013; Gui et al. 2020). This molecule is involved in leukostasis and modulation of BRB permeability through its effects on PKCζ/NF-κB, especially in late BRB breakdown (Aveleira et al. 2010; Huang et al. 2011).

IL-1β is an important mediator of innate immunity, whose synthesis is closely related to the activation of NLRP3 (NOD [nucleotide oligomerization domain]-, LRR [leucine-rich repeat]-, and PYD [pyrin domain]-containing protein 3) inflammasome (Grebe et al. 2018). It can be produced by glial cells, Müller cells, and astrocytes (Gui et al. 2020). NLRP3 inflammasome and IL-1β are considered mediators of pyroptosis, inflammation and angiogenesis in retinal age related diseases (Wooff et al. 2019).

Elevated levels of pro-inflammatory cytokines, including TNF-α, VEGF, and IL-1β, have been found in the blood and aqueous humour of diabetics who developed retinopathy (Wang et al. 2014; Das 2017; Feng et al. 2018; Yao et al. 2018; Wooff et al. 2019). The concentration of TNF-α in the circulation was found to correlate with the severity of DR (Nalini. et al. 2017). Additionally, high levels of VEGF in the aqueous humor are considered a marker of proliferative DR and may have prognostic significance (Wang et al. 2014).

Molecular mechanisms of BRB breakdown and increased permeability

Diabetes leads to the disintegration of the retinal neurovascular unit, BRB dysfunction, and increased vascular permeability (Fig. 1). Increased permeability of BRB may arise from tight junction disruptions, up-regulation of vesicular transport, or by degenerative changes to neurovascular-unit-forming cells including pericytes and glia (Klaassen et al. 2013).

Tight junctions or zonula occludens control the paracellular permeability of BRB. Junctional complexes contain occludin, claudins, and zonula occludens proteins 1, 2, and 3 (ZO-1, -2, and -3). Alterations in junctional protein content or phosphorylation might result in BRB dysfunction and increased permeability (Klaassen et al. 2013). Additionally, high molecular weight molecules and fluids are transported via a transcellular route across the BRB in pinocytotic vesicles or caveolae. Trans-cellular permeability might also be affected by pro-inflammatory cytokine levels.

TNF-α decreases the expression and synthesis of tight junction proteins ZO-1 and claudin-5, and alters the cellular localization of these proteins acting through the PKCζ/NF-kB signalling pathway (Aveleira et al. 2010; Lin et al. 2018). Alone, or in combination with IL-1β and VEGF,

TNF-α induces permeability of the BRB *in vitro* by a mechanism mediated by cAMP (van der Wijk et al. 2017). Additionally, TNF-α stimulates leukocyte adhesion by inducing ICAM-1 expression (Lee et al. 2015).

VEGF is considered to be the main factor responsible for neovascularization and the increased permeability of BRB (Gupta et al. 2013; Tarr et al. 2013; El Rami et al. 2017). VEGF-induced breakdown of the BRB is mediated through PKC β activation (Murakami et al., 2012). VEGF-mediated occludin phosphorylation is followed by ubiquitination of tight junction proteins and a subsequent increase in vascular permeability (Murakami et al. 2009). Additionally, VEGF regulates the expression of plasmalemma vesicle-associated protein (PLVAP), which is involved in trans-endothelial transport in BRB only in pathological conditions. (Hofman et al. 2001; Klaassen et al. 2009; Wisniewska-Kruk et al. 2014; Bosma et al. 2018).

NLRP3 inflammasome activation and consequent secretion of IL-1β plays a major role in DR pathogenesis, especially in inflammation-mediated cell death that leads to the hallmarks of neurovascular unit degeneration, pericyte dropout and the formation of acellular capillaries (Wooff et al. 2019).

Therapeutic potential of melatonin in the treatment of diabetic retinopathy

Melatonin is a neurohormone and an antioxidant that is synthesized mainly in the pineal gland and retina in the absence of light. This hormone synchronizes many physiological functions, including metabolism, adapting them to circadian rhythms. Melatonin synthesis in the retina appears to be reduced in diabetic rats, which is probably caused by a reduction in arylalkylamine N-acetyl transferase (AANAT) activity (do Carmo Buonfiglio et al. 2011). Plasma melatonin concentration is found to be decreased in patients with DR (Wan et al. 2021).

This small molecule is both lipophilic and hydrophilic, so it crosses biological barriers easily (Pourhanifeh et al. 2020). It accumulates within the mitochondria, where it exerts potent antioxidant activity and increases the stability of the respiratory chain (Reiter et al. 2016). In addition, melatonin metabolites generated via reaction with ROS act as antioxidants as well, thus forming a melatonin anti-oxidative cascade (Tan et al. 2014). In addition, melatonin stimulates antioxidant defences through a NRF2/ARE mediated mechanism (Wang et al. 2012; Vriend and Reiter 2015; Ahmadi and Ashrafizadeh 2020). The anti-inflammatory effects of melatonin (Fig. 2A) are mediated through silent information

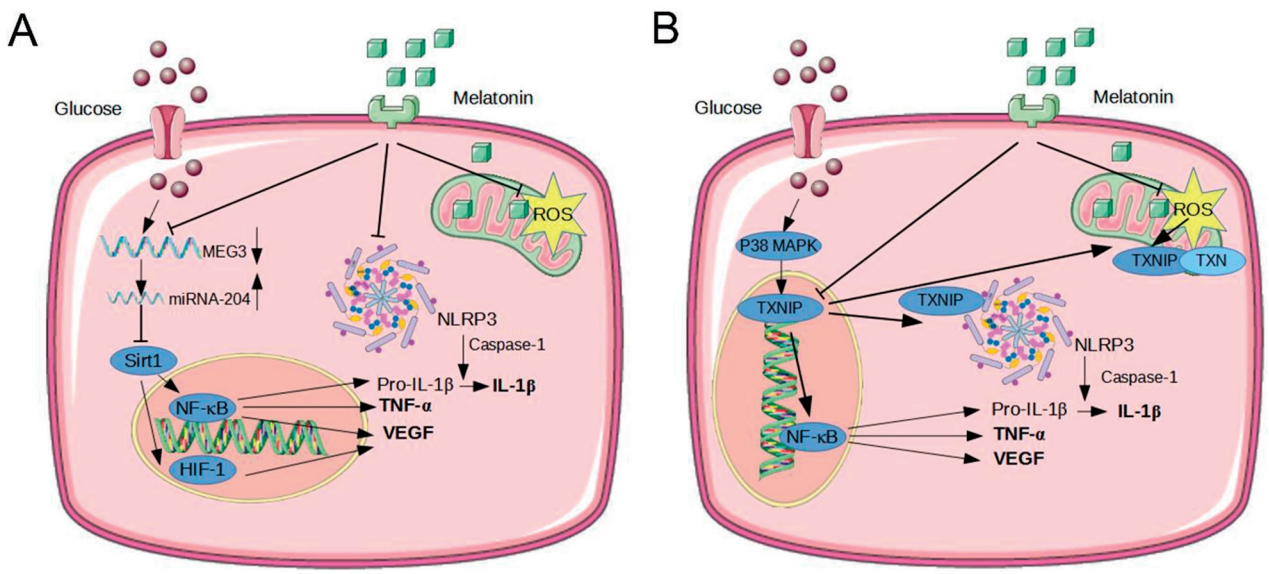

Fig. 2. The mechanism of melatonin action in diabetic retinopathy: the MEG3/Sirt1/NF-κB (A) and the p38 MAPK/TXNIP/NF-κB (B) pathway. Legend: ROS, reactive oxygen species; MEG3, lncRNA, maternally expressed gene 3; miRNA-204, microRNA-204; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; Sirt1, silent mating type information regulation 2 homolog 1; NF-κB, nuclear factor-κB; HIF-1, hypoxia-inducible factor; VEGF vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; P38 MAPK, p38 mitogen-activated protein kinase; TXN, thioredoxin; TXNIP, thioredoxin interacting protein. This figure was drawn using the vector image bank of Servier Medical Art (http://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

regulator T1 (Sirt1), NF-κB pathway, and NLRP3 inflammasome activity modulation (Favero et al. 2017; Che et al. 2020; Tu et al. 2020; Tang et al. 2021). The effect of melatonin on Sirt1 is mediated through the long non-coding RNA maternally expressed gene 3 (MEG3)/micro RNA-204 (miR-204) axis (Tu et al. 2020).

Melatonin administration to diabetic animals leads to a reduction in the concentration of oxidative damage biomarkers and pro-inflammatory cytokines in the retina (Salido et al. 2013; Özdemir et al. 2014; Jiang et al. 2016; Mehrzadi et al. 2018; Djordjevic et al. 2018; Ferreira de Melo et al. 2020). In addition, melatonin maintains the integrity of the inner BRB by upregulating the expression of tight junction proteins via inhibiting p38 mitogen-activated protein kinase (p38 MAPK)/thioredoxin interacting protein (TXNIP)/NF-κB pathway (Fig. 2B) and decreasing the production of pro-inflammatory factors such as VEGF, TNF-α and IL-1β in an animal model of diabetic retinopathy, as well as in cell culture (Tang et al. 2021). The effects of melatonin on VEGF concentrations is probably mediated by the inhibitory effect of melatonin on HIF1 synthesis in the retina (Xu et al. 2018), mediated by a Sirt1 dependent mechanism (Tu et al. 2020). Additionally, melatonin inhibited neuronal pyroptosis by decreasing levels of NLRP3 and IL-1β *both in vivo* and *in vitro* (Che et al. 2020).

CONCLUSION

Exploring efficient treatments for diabetic complications is an important topic, due to the rapid increase in diabetes cases worldwide. To date, intravitreal corticosteroids and anti-VEGF injections, as well as photocoagulation, have been used for the treatment of diabetic retinopathy. Preclinical studies have shown that melatonin modulates several signalling pathways and demonstrated its therapeutic or protective effects in the therapy of age-related ocular diseases, such as DR. To date, data suggest that melatonin may protect ocular tissues by decreasing the production of ROS and pro-inflammatory mediators, such as VEGF, TNF-α, and IL-1β. Based on currently available data, melatonin may be considered for clinical trials as a treatment for ocular diseases characterized by BRB, although, the topic remains under investigation.

ACKNOWLEDGMENTS

This study was founded by the Ministry of Science, Education and Technical Development of the Republic of Serbia (Grant № 451-03-9/2021-14/200113).

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