The impairment of the blood retinal barrier (BRB) represents one of the main features of diabetic retinopathy, a secondary microvascular complication of diabetes. Hyperglycemia is a triggering factor of vascular cells damage in diabetic retinopathy. The aim of this study was to assess the effects of vitamin D3 on BRB protection, and to investigate its regulatory role on inflammatory pathways. We challenged human retinal endothelial cells with high glucose (HG) levels. We found that vitamin D3 attenuates cell damage elicited by HG, maintaining cell viability and reducing the expression of inflammatory cytokines such as IL-1β and ICAM-1. Furthermore, we showed that vitamin D3 preserved the BRB integrity as demonstrated by trans-endothelial electrical resistance, permeability assay, and cell junction morphology and quantification (ZO-1 and VE-cadherin). In conclusion this in vitro study provided new insights on the retinal protective role of vitamin D3, particularly as regard as the early phase of diabetic retinopathy, characterized by BRB breakdown and inflammation.

KEYWORDS vitamin D3, blood retinal barrier, diabetic retinopathy, inflammation, angiogenesis, P2X7R

Introduction

The etiopathogenesis of diabetic retinopathy is still not fully elucidated and several pathways are involved in the exacerbation of this pathological condition. Oxidative stress, inflammation, and vascular dysfunction affect the integrity of inner blood retinal barrier (iBRB composed, among others, by pericytes, endothelial cells and Müller cells) and the outer blood retinal barrier (oBRB composed by retinal pigment epithelium RPE). Moreover, the upregulation of some proangiogenic factors such as vascular
endothelial growth factor-A (VEGF-A), leads to retinal ischemia and blood retinal barrier (BRB) impairment (Bucolo and Drago, 2004; Tarr et al., 2013; Duh et al., 2017; Lazzara, 2022; Shukla and Tripathy, 2022). The iBRB and oBRB modulate the transport of molecules regulating the permeability across the retinal endothelium and the pigmented epithelial cells, respectively. Tight junctions (TJs) and adherent junctions are multiple junctional protein complexes endowed of regulation of BRB integrity, which is strongly altered by high plasmatic levels of glucose. Hyperglycemia causes retinal microvascularopathy, inflammation, and retinal neurodegeneration (Gui et al., 2020). The activation of toll-like receptors 4 (TLR-4), which leads to the over expression of inflammatory markers, such as IL-1β (Cao et al., 2021; Bayan et al., 2022), is one of the diabetes-associated retinal alterations (Wang et al., 2015). It has been demonstrated that the upregulation of IL-1β in retinal endothelial cells is induced by hyperglycemia (Demircan et al., 2006; Liu et al., 2012; Wooff et al., 2019). Moreover, IL-1β is also a stronger inducer of other inflammatory cytokines through the activation of p38MAPK/NF-κB pathway (Liu et al., 2015). High glucose levels represent a strong stimulus that triggers the phosphorylation/activation of ERK proteins, in retinal endothelial cells (Liu et al., 2014; Liu et al., 2015; Lazzara et al., 2019). All these diabetic-related events are correlated to the up-regulation of ICAM-1, induced by both angiogenic (overexpression of VEGF-A) and inflammatory stimuli (up-regulation of inflammatory cytokines). In fact, retinal endothelial cells are the main producers of ICAM-1, which exacerbates the microvascular leukostasis, i.e., the adhesion and endothelial cells are the main producers of ICAM-1, which exacerbates the microvascular leukostasis, i.e., the adhesion and

### Methods

#### Cell culture

Human retinal endothelial cells (HRECs) were purchased from Innoprot® (Derio – Bizkaia, Spain). Cells were cultured at 37°C, in humidified atmosphere (5% CO2), in Endothelial Cell Medium (ECM) supplemented with 5% fetal bovine serum (FBS), 1% ECGS (Endothelial Cell Growth Supplement) and 100 U/ml penicillin 100 μg/ml streptomycin, in flask precoated with fibronectin (1 mg/ml) (Innoprot, Derio – Bizkaia, Spain) for 1 h at 37°C. After reaching confluence (approximately 70%), cells were used for experimental procedures. All the treatments were carried out in medium containing 2.5% FBS. Cells growth in medium containing 5 mM glucose (physiological glucose concentration) served as control group. HRECs were also exposed to medium containing 40 mM glucose (high glucose, HG) (Huang et al., 2016; Lazzara et al., 2019) obtained from the basal glucose concentration of medium (5 mM) with the addition of 35 mM of glucose on basis of used final volume. HRECs were pre-treated for 24 h with vitamin D3 (1 μM) and then were exposed to HG with or without vitamin D3 for 24, 48 and 72 h.

#### MTT

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT; Chemicon, Temecula, CA, United States) was used to assess cell viability after HG (40 mM) challenge and vitamin D3 (1 μM) treatment. Optimal cell density was obtained by seeding 1.5 × 10^4 cells/well in 96-well plates (Costar, Corning, NY, United States). After pretreatment with vitamin D3, HRECs were subjected to co-treatment in a fresh medium for 24 and 48 h with vitamin D3 (1 μM) and HG (40 mM). At the end of the treatment, HRECs were incubated at 37°C with MTT (0.5 mg/ml) for 3 h; then DMSO was added, and absorbance was measured at 570 nm in a plate reader (VariosKan, Thermo Fisher Scientific, Waltham, MA, United States). Graphs were built converting absorbance (abs) to viability (% of control) using the following equation (abs_n + abs_ki) × 100, where abs_n is absorbance in the x well, and abs_ki is the average absorbance of negative control cells (untreated cells).

#### Lactate dehydrogenase

Lactate dehydrogenase (LDH) cell release was measured using the Cytotoxicity Detection KitPLUS (LDH) (ROCHE, Mannheim, Germany). HRECs cells were seeded at 1.5 × 10^4 cells/well in 96-well plates (Costar, Corning, NY, United States). After pretreatment with vitamin D3, HRECs were subjected to co-treatment in a fresh medium for 24 and 48 h with vitamin D3 (1 μM) and HG (40 mM). After these time
points, according to manufacturer’s protocol, lysis solution was added to positive control wells (non-treated cells) for 15 min. After transferring 100 μl of medium in a new multi-well plate, 100 μl of working solution was added. After 10–15 min at room temperature, at last, 50 μl of stop solution was added. The absorbance values were measured at 490 nm using a plate reader (VarioSkan, Thermo Fisher Scientific, Waltham, MA, United States). LDH release is reported as LDH (% control) (absx + absctrl) × 100. In the equation, absx is absorbance in the x well and absctrl is the average absorbance of positive control cells (untreated lysed cells). Absorbance values were corrected by subtracting medium absorbance.

Blood retinal barrier integrity assessment

The effect vitamin D₃ and HG challenge on BRB integrity was evaluated by measurements of TEER, by using a Millicell-Electrical Resistance System (ERS2) (Merck, Millipore, Burlington, MA, United States) as previously described (Giurdanella et al., 2017; Fresta et al., 2020). To evaluate the modification of paracellular permeability under the above-mentioned conditions, the luminal-to-abluminal movements of Na-F, across endothelial cell monolayers, were measured by using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, United States) as previously described (Fresta et al., 2020).

Immunocytochemistry

ZO-1 immunodetection was carried out as follows. Glass chamber slides were coated with a fibronectin for 1 h at 37°C and washed with sterile water. HRECs (6 × 10⁴ cells/well) were seeded on 24-well fibronectin coated glass chamber slides. Cells were incubated for 4 days at 37°C in a 5% CO₂ humidified atmosphere. Cell adhesion and confluence was reached within 5 days and the medium was changed every 2 days. Cells were shifted for 24 h with vitamin D₃ and for 48 h to a medium containing 40 mM glucose (HG), with or without vitamin D₃. HRECs growth in medium with physiological glucose concentration (5 mM) served as control. After 48 h, cells were fixed with ice-cold acetone for 15 min and with ice-cold methanol for 20 min. Thereafter, cells were washed with cold phosphate buffered saline (PBS, pH 7.4) and blocked with 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS solution, for 30 min at room temperature. Cells were then incubated overnight at 4°C with primary antibody against ZO-1 (dilution 1:100, rabbit monoclonal; catalog n. 61-7300, Life Technology, Monza, Italy). After overnight incubation and primary antibody washout with PBS, the secondary anti-rabbit Alexa 488-conjugated antibody (dilution 1:200, Life Technology, Monza, Italy) was added for 1 h at room temperature in the dark. VE-cadherin immunodetection was carried out with a different protocol. HRECs (6 × 10⁴ cells/well) were seeded on 24-well fibronectin coated glass chamber slides pre-coated with fibronectin for 1 h at 37°C and then incubated for 4 days at 37°C in a 5% CO₂ humidified atmosphere. The medium was changed every 2 days. Thereafter, the cells were shifted to different medium, as described for ZO-1 staining. After 48 h of treatment the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with cold PBS and permeabilized with 0.3% Triton X-100 in PBS (pH 7.4) for 5 min at room temperature. After blocking with 1% bovine serum albumin (BSA) in PBS for 1 h, the cells were incubated with the rabbit anti-VE-cadherin antibody (1:100, Catalog n. 2500 Cell signaling, Technology, Danvers, MA, United States) in 1% BSA-PBS solution, overnight at 4°C. Then, the slides were washed three times with PBS and 1 h incubation was carried out with anti-rabbit Alexa 488-conjugated secondary antibody (1:200 dilution, Life Technologies, Monza, Italy), at room temperature in the dark. For p-NFκB p65 immunostaining HRECs were plated at a density of 4 × 10⁴ in 24-well glass chamber slides pre-coated with fibronectin for 1 h at 37°C and then incubated for 3 days at 37°C in a 5% CO₂ humidified atmosphere. Thereafter, the cells were pretreated for 24 h with vitamin D₃ and for 24 h with high glucose. Then, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with cold PBS and permeabilized with 0.2% Triton X-100 in PBS (pH 7.4) for 15 min at room temperature. After blocking with 5% NGS and 0.3% Triton X-100 in PBS solution, for 30 min at room temperature, the cells were incubated with the mouse-anti-phospho-NFκB p65 (Ser536; 1:200, Catalog n. 3036 Cell signaling, Technology, Danvers, MA, United States) in 1% NGS and 0.2% Triton X-100 in PBS solution overnight at 4°C. After overnight incubation, the slides were washed three times with PBS. Then, 1 h incubation was carried out with anti-mouse IgG H + L (Dylight 550) secondary antibody in 0.1% Triton X-100 in PBS (1:300 dilution, Abcam, Cambridge, United Kingdom), at room temperature in the dark. Nuclei staining was carried out for 10 min with 4′,6-diamidino-2-phenylindole (DAPI) (1:10.000; D1306, Life Technologies, Monza, Italy). Finally, the slides were mounted using mounting medium (Life Technologies, Monza, Italy). Images were acquired with a fluorescence microscope Zeiss Observer Z1 equipped with the Apotome.2 acquisition system connected to a digital camera (Carl Zeiss, Oberkochen, Germany). Images were acquired at 40x. Semi-quantitative evaluation of junction protein expression was carried out analyzing images from slides of each condition n = 4 (5 mM glucose, 40 mM glucose, 40 mM glucose + 1 μM vitamin D₃). The images (n = 4 per group) were analyzed by two investigators unaware of experimental design.
Extraction of total ribonucleic acid and cDNA synthesis

Extraction of total RNA, from HREC cells was performed, after 72 h of treatment, with a TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, United States). The A$_{260}$/A$_{280}$ ratio of optical density of RNA samples (measured with Multimode Reader Flash di Varioskan™) was 1.95–2.01; this RNA purity was confirmed with the electrophoresis in non-denaturing 1% agarose gel (in TAE). cDNA was synthesized from 2 µg RNA with a reverse transcription kit (SuperScript™ II Reverse transcriptase, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, United States).

qRT-PCR

Real-time PCR was carried out with the Rotor-Gene Q (Qiagen). The amplification reaction mix included the Master Mix Qiagen (10 µl) (Qiagen QuantitiNova SYBR Green Real-Time PCR Kit) and cDNA (1 µl, 100 ng). Forty-five amplification cycles were carried out for each sample. Results were analyzed with the 2$^{-\Delta\Delta C_t}$ method. Quantitative PCR experiments followed the MIQE guidelines (Bustin et al., 2009). Gene expression levels were normalized with levels of housekeeping gene (18S). Primers were purchased from Eurofins Genomics (Milan, Italy). Forward and reverse primer sequences are herein listed: IL-1β (forward: 5′-AGCTACGAAATCTTCCGACC-3′; reverse: 5′-CGTTATCCCATGTGTCTGAAGAA-3′), VEGF-A (forward 5′-AGGGCAAGATCATCGAAAG-3′; reverse 5′-ATCCGCATAATC TTCGATGTT-3′), 18S (forward 5′-AGTCCCTGCCCTTTG-3′; reverse 5′-GAGTAGGGGCATTT-3′), TLR-4 (forward 5′-CCCATGTGTCGAAGAA-3′; reverse 5′-AGGAGATTGAGTAGGGGCATTT-3′).

Western blot

HRECs were cultured in 60 mm Petri dishes (4 × 10³). Proteins of whole cell lysates were extracted with RIPA Buffer, including protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, United States). Total protein content, in each cell lysate sample, was determined by means of the BCA Assay Kit (Pierce™ BCA Protein Assay Kit, Invitrogen, Life Technologies, Carlsbad, United States). Extracted proteins (30 µg) were loaded on 4%–12% tris-glycine gel. After electrophoresis, proteins were transferred into a nitrocellulose membrane (Invitrogen, Life Technologies, Carlsbad, CA, United States). Membranes were blocked with milk, 5% Trisbuffered saline, and 0.2% Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated overnight (4°C) with appropriate primary phospho-p44/42 MAPK (Rabbit, phospho-Erk1/2, 1:500 dilution, Catalog n. 9101 Cell Signaling Technology, Danvers, MA, United States), p44/42 MAPK (Rabbit, Erk1/2, 1:500 dilution, Catalog n. 9102 Cell Signaling Technology, Danvers, MA, United States) and anti-GAPDH (Rabbit mAb, 1:500 dilution, Catalog n. 2118 Cell Signaling Technology, Danvers, MA, United States) antibodies. After overnight incubation, the membranes were then incubated with secondary chemiluminescent antibody (ECL anti-rabbit, 1:2000 dilution, NA934) for 1 h at room temperature. After secondary antibody, the membranes were incubated with ECL (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Carlsbad, CA, United States) and were detected through I-Bright™ 1500 (Invitrogen, Life Technologies, Carlsbad, CA, United States) by using chemiluminescence. Densitometry analyses of blots were performed at non-saturating exposures and analyzed using ImageJ software (NIH, Bethesda, MD). Values were normalized to GAPDH, which was also used as loading control.

In vitro tube formation assay

Tube formation assay was performed in vitro with Matrigel Basement Membrane Matrix system (BD, Bedford). The experimental protocol was run according to the manufacturer’s instructions. Gel solution was thawed at 4°C overnight, then 96-well plates were coated with 50 µl of Matrigel/well and allowed to solidify at 37°C for 2 h. HRECs were seeded at 15,000 cells per well in 50 µl assay medium, with or without HG and/or 1 µM vitamin D₃. Each condition was run in triplicate. After 8 h of incubation, tube-like structures were photographed by using an inverted microscope. The total tube length was quantified with the ImageJ software (NIH, Bethesda, MD).

Statistical analysis

Statistical analysis and graphs design were carried out with GraphPad Prism (GraphPad Software, La Jolla, CA, United States). Data are reported as mean ± SD. One-way ANOVA, followed by Tukey-Kramer post-hoc test, was carried out for multiple comparisons. Post-hoc test was carried out given an F with p < 0.05, and no significant variance inhomogeneity was found within groups. Differences between groups were considered significant at p < 0.05.
Results

Cell viability and lactate dehydrogenase release

After 24 and 48 h, high glucose induced a significant ($p < 0.05$) cell toxicity in terms of reduction of cell viability, in comparison to control (roughly 26% and 21% after 24 and 48 h, respectively) (Figure 1A). Pre-treatment with vitamin D$_3$ (1 µM) significantly ($p < 0.05$) attenuates cell toxicity after 24 and 48 h, compared to high glucose treated cells (roughly 16% and 32% after 24 and 48 h, respectively). The same profile was observed in terms of LDH release (Figure 1B).

Inner blood retinal barrier integrity

To evaluate vitamin D$_3$ effects on iBRB integrity, we measured trans endothelial electric resistance (TEER), a parameter of barrier permeability in cell cultures. We found TEER values significantly ($p < 0.05$) reduced (22%) after 48 h of high glucose damage, compared to control cells (Figure 2A). On the contrary, vitamin D$_3$ treated cells showed significant ($p < 0.05$) increased TEER values, superimposable with control group (Figure 2A). These data were supported by the measurement of apical-to-basolateral permeability of sodium fluorescein (Na-F), a spectrophotometric approach for the assessment of cell monolayer permeability. Treatment with vitamin D$_3$ (1 µM)
FIGURE 3
Vitamin D₃ re-establishes iBRB integrity through modulation of VE-cadherin and ZO-1. HRECs were pretreated with vitamin D₃ (1 µM) for 24 h and subsequently co-treated with HG (40 mM) for 48 h. Vitamin D₃ increased the expression of VE-cadherin and ZO-1 proteins, which were significantly reduced by HG. Representative images for VE-cadherin (A,B,C) and ZO-1 (E,F,G) expression in HRECs after treatment with HG and vitamin D₃. VE-cadherin and ZO-1 were labeled with FITC (green); nuclei were labeled with DAPI (blue). Images were acquired at × 40 magnification. Scale bar: 10 µm. Fluorescence semi-quantification of VE-cadherin (D) and ZO-1 (H) protein (mean grey levels). Values are reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *p < 0.05 vs. control; †p < 0.05 vs. HG.
was able to significantly (p < 0.05) preserve monolayer permeability (15’ and 30’) elicited by HG (Figure 2B).

Since BRB integrity is related to the expression and cell membrane localization of tight junction (TJ) proteins such as ZO-1 and adherens junction such as VE-cadherin (AJ), the expression of these proteins was analyzed by immunocytochemistry (Figure 3). High glucose damage significantly (p < 0.05) decreased the expression of both proteins in HRECs, compared to control cells (roughly 33% and 47% of VE-cadherin and ZO-1, respectively) (Figure 3). On the other hand, pre-treatment with vitamin D3 protected HRECs from HG-damage preserving the expression of ZO-1 and VE-cadherin after 48 h of exposure to HG and vitamin D3 (roughly 32% and 37% of VE-cadherin and ZO-1, respectively) (Figure 3).

Further, we analyzed at transcriptional level the modulation of VE-cadherin and ZO-1. After 48 h of HG exposure, ZO-1 and VE-cadherin mRNAs levels were significantly (p < 0.05) down-regulated in HRECs (roughly 0.6-fold and 0.5-fold for VE-cadherin and ZO-1, respectively), while pre-treatment with vitamin D3 reversed this effect (Figures 4A,B), maintaining levels of mRNA expression to control values.

**Inflammatory process modulation**

After 24 h, HG exposure elicited a significant (p < 0.05) increase of phosphorylated ERK protein, in comparison to control cells (Figures 5A,B). As shown in Figures 5A,B, vitamin D3 (p < 0.05) led to a significant (p < 0.05) reduction of ERK phosphorylation (0.5-fold of reduction compared to HG). Furthermore, we analyzed mRNA expression of inflammatory cytokines after 24 and 48 h of HG challenge, but although the trend was rising, data were not significant (data not shown). Instead, we found that after 72 h, HG challenge induced a significant (p < 0.05) up-regulation of ICAM-1 and IL-1β (Figures 5C,D), whose mRNA levels were significantly (p < 0.05) reduced by vitamin D3 treatment (0.4-fold compared to HG-treated cells). Further, TLR-4 mRNA levels were higher in HRECs challenged with HG, compared to control cells (Figure 5E), and vitamin D3 treatment restored TLR-4 mRNA to control cell levels (0.5-fold compared to HG) (Figure 5E).

Finally, we evaluated the effects of vitamin D3 on NFκB activation and nuclear translocation, to confirm the anti-inflammatory activity of vitamin D3 in HRECs after 24 h of HG exposure. HG induced the nuclear translocation of the phosphorylated p65 subunit of NFκB, as shown in Figure 6A. NFκB activation and translocation was significantly (p < 0.05) counteracted by the pre-treatment with vitamin D3, inhibiting the p65 nuclear translocation (Figures 6A,B).

**Anti-angiogenic activity**

After 72 h of HG challenge, retinal endothelial cells expressed significant (p < 0.05) higher levels of VEGF-A, compared to control cells (Figure 7A). The treatment with vitamin D3 significantly (p < 0.05) reduced VEGF-A mRNA levels, in comparison to cells exposed to HG (Figure 7A). Furthermore, to confirm the anti-angiogenic effect of vitamin D3, we carried...
FIGURE 5
Vitamin D₃ counteracts inflammation and angiogenesis in HREC after HG-induced damage. Vitamin D₃ effect on the inflammatory pathway activated by high glucose (HG) in HRECs. (A) Immunoblot analysis of ERK1/2 phosphorylation in lysates from HRECs, pre-treated for 24 h with vitamin D₃ (1 µM) and subsequently co-treated with HG (40 mM) for other 24 h. (B) Bar graphs show the densitometry analysis of each band, carried out with the Image J program, p-ERK densitometry has been normalized to total ERK values. The effect of HG and vitamin D₃ at mRNA levels was evaluated after 72 h of HG challenge. The treatment with vitamin D₃ reduced ICAM-1 (C), IL-1β (D), TLR4 (E) mRNA expression. The mRNA levels were evaluated by qPCR. Each bar represents the means ± SD (n = 4; each run in triplicate). *p < 0.05 vs. control; †p < 0.05 vs. HG.
out the tube-formation Matrigel assay (Figures 7B–H), as previously used for the evaluation of angiogenic potential of HRECs (Yadav et al., 2012; Giurdanella et al., 2017; Platania et al., 2020). Vitamin D₃ exerted a significant (p < 0.05) anti-angiogenic activity on HRECs treated with 80 ng/ml VEGF-A (Figures 7B–H). In particular, vitamin D₃ significantly (p < 0.05)
FIGURE 7
Effect of vitamin D₃ on angiogenesis. (A) Real-time PCR: VEGF-A mRNA expression. HRECs were pre-treated with vitamin D₃ (1 µM) for 24 h and then with or without HG (40 mM) for 72 h. Vitamin D₃ decreased mRNA levels of VEGF-A and exerted antiangiogenic activity. (B–G) Quantification of total tube length, nb nodes, nb junctions, nb master junctions, total master segments length and total segment length, was carried out using the Angiogenesis Analyzer tool for ImageJ software. HRECs were treated with 80 ng/ml VEGF-A in presence or absence of vitamin D₃ (1 µM). (H) Representative optical phase-contrast micrographs of tubelike structures (× 40 magnification) observed in the tube formation assays (Matrigel) after 8 h. Values are reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *p < 0.05 vs. control; †p < 0.05 vs. HG or VEGF-A.
decreased the number of branches point of new vessels and the tube length of new vessels in comparison to cells treated with exogenous VEGF-A (Figures 7B–H).

Discussion

Blood retinal barrier breakdown is a hallmark of diabetic retinopathy. The BRB is a tight and limiting barrier that manages the flux of ions, proteins, metabolic waste compounds, and water flow through the retina, and consists of two distinct regions, the inner BRB (iBRB) and outer BRB (oBRB). The iBRB is established by tight junctions between retinal capillary endothelial cells, surrounded by pericytes and supported by glial cells (Cunha-Vaz et al., 2011; Frey and Antonetti, 2011). The outer BRB (oBRB) is formed by retinal pigmented epithelial cells connected by tight junction proteins, which regulate transport between the choriocapillaris and the retina. Both iBRB and oBRB include tight junction proteins (TJs) (i.e., occludin, claudin family and zonula occludens proteins) and adherens junction proteins (i.e., VE-cadherin) (Cunha-Vaz et al., 2011; Frey and Antonetti, 2011). Hyperglycemia, oxidative stress and inflammation are detrimental events that compromise the stability and the expression of those proteins (Tien et al., 2013; Yuan et al., 2014; Platania et al., 2019). The protective effects of vitamin D3 have been studied in different pathological systems, including eye diseases (Jia et al., 2019; Gouni-Berthold and Berthold, 2021; Johansson et al., 2021; Plesa et al., 2021; Bakhshaee et al., 2022). Beyond the role of vitamin D3 in calcium and bone homeostasis, several evidence highlight the anti-inflammatory, antioxidant and anti-angiogenic activity of this natural compound (Saad El-Din et al., 2020; Ghanavatinejad et al., 2021). Recently, the attention has been focused on the correlation between vitamin D3 deficiency and diabetic retinopathy progression (Aksoy et al., 2000; Kaur et al., 2011; Lu et al., 2018), although the mechanism behind its effect on DR pathogenesis is not so clear. It has been hypothesized that vitamin D3 deficiency has a role in type 1 and type 2 diabetes pathogenesis; in particular, different studies highlighted the leading role of vitamin D receptor (VDR) in maintenance normoglycemia, and the alteration of VDR function has been linked to insulin resistance (Zeitz et al., 2003; Oh et al., 2015; Ni et al., 2016). Moreover, different allelic variations in vitamin D3 metabolism-related genes have been proposed as predictive markers of insulin imbalance and glucose intolerance (Ren et al., 2012; Yu et al., 2018; Shaat et al., 2020). Furthermore, vitamin D3 showed promising implications for diabetic retinopathy treatment, preventing inflammatory-related complications. Indeed, Lu et al. (2018) demonstrated that vitamin D3 inhibits the activation of inflammasome both in an in-vitro and in-vivo model of DR reducing the detrimental effects induced by high concentration of glucose. Interestingly, vitamin D3 also showed a relevant anti angiogenic activity in a mouse oxygen-induced ischemic retinopathy model (Albert et al., 2007). The mechanism underlying the protective effect of vitamin D3 in hyperglycemia-stimulated endothelial cells has not been fully elucidated. Different cytoplasmic and nuclear pathways are involved following VDR activation (Ryan et al., 2015). Incidentally, the anti-inflammatory effect of vitamin D3 could be related to calcium homeostasis and purinergic receptors (P2X7R) activation (Uekawa et al., 2018). On this regard, some studies demonstrated that vitamin D3 was able to reduce the calcium influx through P2X7R in resting human mononuclear cells and, as consequence, to down-regulate the expression of this receptor strongly linked to the exacerbation of inflammation in several diseases (Lajdova et al., 2008; Adinolfi et al., 2018). Based on this evidence the binding of vitamin D3 on P2X7 receptor, acting as allosteric modulator, cannot be rule out, and it is worthy of further investigations. Moreover, long-term vitamin D3 supplementation was shown to normalized intracellular Ca2+ levels in early-stage chronic kidney disease patients without any changes in intracellular calcium storage or cellular intake (Lajdova et al., 2009).

In the present study, vitamin D3 was able to counteract the effects mediated by inflammatory processes induced by high concentrations of glucose. In fact, we found a relevant rescue in the BRB integrity of retinal endothelial cells mediated by vitamin D3 in HG conditions with restored levels of junction proteins. As expected, the stimulation with HG significantly reduced TEER values after 48 h compared to control cells (Figure 2). The Na-F permeability test confirmed the BRB integrity impairment after HG treatment, thus mimicking the clinical features of DR patients (Fresta et al., 2020; Nian et al., 2021). Data reported in Figures 3, 4 indicate that vitamin D3 treatment reduced paracellular permeability in presence of hyperglycemia by preventing the HG-induced decrease of junction protein levels, ZO-1 and VE-cadherin, restoring their central role regarding the tight and the adherens junctions, respectively. Similarly, Won S. et al. demonstrated that vitamin D3 treatment was able to prevent hypoxia/reoxygenation-induced blood-brain barrier disruption through VDR-mediated NF-κB signaling pathways, in an in vitro model of blood brain barrier (Won et al., 2015). In our model, the protective effect of vitamin D3 against HG can be ascribed to its capability to block inflammatory processes that underlie the pathogenesis of diabetic retinopathy (Forrester et al., 2020). We evaluated the effects of vitamin D3 regarding the mRNA levels of inflammatory cytokines, ICAM-1, IL-1β, and TLR-4 in endothelial cells treated with HG. As previously reported, HG treatment led to a significant increase in the pro-inflammatory cytokines mRNA levels, as well as TLR-4 (Xie et al., 2014; Wang et al., 2018; Zhou et al., 2019; Giurdanella et al., 2021). It has been demonstrated that high glucose promotes the activation of TLR (2/4) and, through myeloid differentiation proteins (MyD88)-dependent and -independent signaling pathway, it stimulates the release of inflammatory mediators (Devaraj et al., 2008; Dasu and Jialal, 2013).
which are also significantly increased in the vitreous fluid of DR patients (Ross et al., 2017; Iyer et al., 2021; Wu et al., 2021). Our data (Figure 5) are in line with other evidence about the stimulation of TLR-4 pathway exerted by HG (Pahwa, Nallasamy and Jialal, 2016). Moreover, we have previously demonstrated that HG mediate cell damage through the activation of MAPK/NFκB axis through the phosphorylation of both these proteins (Giurdanella et al., 2017; Giurdanella et al., 2020; Lazzara et al., 2019). On these bases, here we tested the HG-induced cytokines mRNA up-regulation, as direct consequence of the activation of ERK/NFκB pathways; in our model, vitamin D₃ clearly reduced the phosphorylation of ERK protein and counteracted the nuclear translocation of phosphorylated p65 NFκB subunit and the cognate increase in cytokine mRNA levels (Figures 5, 6). Vitamin D₃ could exert a pleiotropic anti-inflammatory activity considering its capability to counteract different pathways; this point would certainly need further investigation. We cannot rule out the hypothesis that vitamin D₃ could interfere with the activation of ROS-related HMGB1–TLR4 signaling, described to induce endothelial dysfunction in presence of HG (Rao et al., 2017; Zhang et al., 2018; Fernandez-Robredo et al., 2020; Huang et al., 2020). Moreover, our results could be consistent with a putative contribution of vitamin D₃ in calcium homeostasis through the involvement of the purinergic system (P2X7R) that we found involved in high glucose-induced retinal endothelial damage (Platania et al., 2017). Our in vitro findings confirm the effect of vitamin D₃ as inhibitor of retinal neo-angiogenesis. It has well demonstrated, that vitamin D₃ hampered VEGF-induced endothelial cell sprouting and elongation (Mantell et al., 2000; Albert et al., 2007; Jamali et al., 2019). It has also been shown that vitamin D₃ treatment inhibited VEGF-induced activation of VEGFR-2, ERK and Akt pathway (Kim et al., 2017). Indeed, in our study we found that vitamin D₃ affects the pro-angiogenic activity of VEGF-A on HRECs, and significantly reduced VEGF-A mRNA levels elicited by high levels of glucose (Figure 7).

In conclusion, we provided new evidence on the role of vitamin D₃ in an in vitro model of DR using human retinal endothelial cells. The BRB integrity, significantly compromised by high glucose exposure, was restored by vitamin D₃ treatment. These data suggest that vitamin D₃ could be a good candidate to counteract inflammation in several retinal conditions and warranting further clinical evaluation of the efficacy profile.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Author contributions

FL, AL, and CB made substantial contributions to conception, design, and interpretation of data. FL, AL, GG, carried out formal analysis of data. FL, AL, GG, GL, CA, and CB wrote initial draft of the manuscript. FL, AL, GG, GL, CA, CBMP, SR, FD, and CB reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.

Funding

MUR grant PRIN 2020FR7TCL; PIACERI 2020/22 Linea2 NanoRet.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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