Protection by vitamin D against high-glucose-induced damage in retinal pigment epithelial cells

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1 **Protection by vitamin D against high-glucose-induced damage in retinal pigment epithelial cells**

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Abstract

Diabetic retinopathy (DR) is a diabetes-associated complication characterized by irreversible deterioration of the microvessels within the retina, leading subsequently to severe retinal damage and vision loss. Vitamin D (VITD), a steroid hormone, plays multiple physiological functions in cellular homeostasis. Deficiency of VITD has been suggested to be associated with DR. To study the potential protective function of VITD in DR, high-glucose-treated ARPE-19 cells and STZ-induced diabetic mice were used as *in vitro* and *in vivo* models. The protective effects of VITD were assessed based on the changes of expression of antioxidant enzymes and cytokines in high-glucose-treated RPE cells and in the retina and retinal pigment epithelium (RPE) of diabetic and VITD-treated diabetic mice. The present study demonstrated that exposure to a high level of glucose caused upregulation of pro-inflammatory cytokines and a decrease in anti-oxidant enzyme expression in both *in vitro* and *in vivo* models. VITD treatment increased cell viability, reduced reactive oxygen species (ROS) production and caspase-3/7 activities in high-glucose-treated RPE cells. Our data suggest that VITD can protect the retina and RPE from high-glucose-induced oxidative damage and inflammation.

Keywords diabetic retinopathy; vitamin D; oxidative stress; inflammation; protection

1. Introduction

Diabetic retinopathy (DR) is a microvascular consequence of diabetes mellitus. DR is defined as the progressive irreversible deterioration of the tiny blood vessels within the retina as a result of chronic hyperglycemia, leading to severe damage of the retinal microvasculature and associated vision problems including blindness [1]. DR can develop in both type 1 and type 2 diabetes mellitus. The incidence of DR is believed to be influenced by factors such as type and duration of diabetes, age, ethnicity and genetic susceptibility. DR can occur after the onset of diabetes and the development of DR is strongly associated with the duration and the management of diabetes. Pooled epidemiologic studies have explored the global incidence of DR and have concluded that there has been a significant increase of DR among type 1 and type 2 diabetes patients worldwide, estimated to range from 8% at 3 years to 100% at 15-20 years after diabetes onset [2,3].

Glucose is the main source of energy at the cellular level. Insulin is the principal regulator of glucose metabolism. Insulin deficiency or resistance can cause the significant disturbance in glucose metabolism that is the main characteristic of diabetes. Prolonged high plasma glucose concentration can cause severe diabetic complications, including DR. Although DR is recognized as a multifactorial disease, impairment of glucose metabolism is its primary cause. Previous studies have investigated molecular mechanisms of DR caused by hyperglycemia [1,4,5]. The retina has a high metabolic activity and oxygen consumption. Thus, chronic hyperglycemia and insulin deficit or resistance can alter glucose metabolism from normal glycolysis to other metabolic pathways and can produce reactive biochemical molecules that are capable of inducing irreversible damage in the retina. Several studies have shown that diabetes can trigger DR by increasing the production of free radicals from diverted glucose metabolism pathways including the polylol pathway, nonenzymatic glycosylation, hexosamine pathway, activation of PKC and poly (ADP-riobose) polymerase pathways [1,4,5]. A number of antioxidants have demonstrated protective effects in DR animal models, offering therapeutic potential for DR patients [6,7].

It is well established that inflammation contributes to DR development and progression [7-12]. Recent computational analyses of microarray datasets have demonstrated that the

82 pathogenesis of DR is linked to inflammation and fibrosis [8]. Increased production of

83 proinflammatory cytokines, such as IL-1, TNF-α and VEGF, have been reported in the vitreous of

84 DR patients and in the retinas of DR animal models [7,9]. High-glucose-induced upregulation of

85 proinflammatory cytokines can cause breakdown of the blood-retinal barrier (BRB), cell death and

86 angiogenesis [7,10]. VEGF-A is the major angiogenic factor and contributes to the pathogenesis of

87 diabetic macular edema [7]. VEGF-A production induced by high glucose is mediated by a variety

88 of signalling pathways, such as PKC, ERK and HIF pathways (6,7,11). Recently Platania et al.

89 demonstrated that miRNAs also mediate VEGF expression [12].   Currently anti-VEGF strategy is

90 effective in treating diabetic macular edema. However, other therapeutic strategies are urgently

91 needed for preventing and/or slowing the progression of DR [7].

92   Vitamin D (VITD), a secosteroid hormone, is found in many types of food and is also

93 endogenously produced in humans. VITD is found in two forms in biological systems: the inactive

94 form 25(OH)2D3 and the active form 1,25(OH)2D3. The active form of VITD is the principal

95 regulator of calcium and phosphate ions; however, it is also involved in regulating gene expression

96 in targeted cells and organs via genomic and non-genomic mechanisms [13]. VITD deficiency has

97 been associated with ageing and with a range of diseases [14]. VITD deficiency is also linked with

98 higher risk of retinopathy in type1 and 2 diabetic patients [15-17]. Lower serum VITD

99 concentration is associated with increased severity of retinopathy in patients with proliferative DR

100 [18,19]. Previous studies also reported that polymorphisms (*Apal*, *Bsml*, *Fokl* and *Taq I*) in the

101 vitamin D receptor (VDR) gene are associated with increased risk of retinopathy in diabetic

102 patients [20-22]. However, the mechanisms of VITD involvement in DR are not fully understood.

103   In the present study we have examined the protective role of VITD against oxidative stress

104 and inflammation in retinal pigment epithelial (RPE) cells under high glucose condition. We found

105 that VITD increased antioxidant capacity and inhibited inflammation *in vitro* and *in vivo*.

106 **2. Materials and methods**

107 *2.1. Cell viability*

108   ARPE-19 cells were cultured till confluence in a T-25 cm^2 flask containing 5 ml DMEM/F12

109   medium (Cat. BE12-719F, Lonza). The cells were detached using 0.5% trypsin-EDTA and seeded in

110   96-well plates at a density of 50,000 cells/well in DMEM containing normal glucose (5 mM and

111   0.1% ethanol) or high glucose (25 mM and 0.1% ethanol). Cells were treated with or without VITD

112   (50 nM) for 6 hours or 24 hours. VITD (Cat. D9257, Sigma, UK) was dissolved in ethanol and a dose

113   of 50 nM VITD was chosen based on our previous studies [23,24]. Cell viability was measured using

114   the MTT assay (Sigma, UK) according the manufacture's guidance.

115   *2.2. Biochemical assays*

116   Caspase 3/7 activity was measured with a kit (Cat. G8090) from Promega according to the

117   manufacturer's instructions; activities of catalase (Cat. STA-341) and superoxide oxidative

118   dismutase (SOD, Cat. STA-340) were measured using kits from Cambridge Bioscience following the

119   manufacturer's guidance. The levels of glutathione (GSH, Cat. STA-312) and malondialdehyde

120   (MDA, Cat. STA-330) were assessed according to our previous description [24].

121   *2.3. Enzyme-linked immunosorbent assay (ELISA)*

122   ELISA was performed to detect human IL-1β (Cat. 900-TM95), IL-8 (Cat. 900-M18), IL-33 (Cat.

123   900-M398), TNF-α (Cat. 900-M25) and VEGF-A (Cat. 900-M10) in cultured media from ARPE-19

124   cells cultured 12-well plates and to detect mouse IL-33 (Cat. 14-8332-80) and VEGF-A (Cat.

125   900-M99) in the lysates from mouse retinas and retinal pigment epithelial cells. The ELISA kit for

126   mouse IL-33 was purchased from eBioscience; all other ELISA kits were from PeproTech. The

127   detection of individual cytokines was performed according to the manufacturers' guidance.

128   *2.4. Quantification of reactive oxygen species (ROS)*

129   ARPE-19 cells were seeded in 96-well plates (25,000 cells/well) in DMEM-F12 medium for 24

130   hours. Cells were then treated with high glucose (25 mM and 0.1% ethanol) or a mixture of high

131   glucose and VITD in DMEM for 6 or 24 hours, while control cells were treated with 0.1% ethanol

132   only in DMEM containing normal glucose (5 mM). Cells were washed with PBS and incubated with

133   10 μM DCFH-DA (6-Carboxy-2′,7′-Dichlorofluorescin diacetate) for one hour. ROS production was

134   measured according to previous description [24].

135 *2.5 Quantitative real-time polymerase chain reaction (qRT-PCR)*

136 Total RNAs were extracted from cultured cells or mouse tissues using TRIzol™ Reagent (Thermo

137 Fish Scientific, UK) following the manufacturer's protocol. cDNA was set up using using the

138 High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, UK) according to the

139 manufacturer's guidance. Gene expression was detected using Platinum SYBR Green reagents

140 follwing the manufacturer's protocol. Primers used for qRT-PCR are listed in Table S1.

141 *2.6. Western blotting*

142   Control and treated cells were collected and lysed using lysis buffer (T-PER, Thermo Scientific,

143 UK) containing proteinase inhibitor. The concentration of the extracted protein was determined

144 using a Bradford protein assay (DCTM protein assay kit, Bio-Rad, UK) in a 96-well microtitre plate.

145 Individual samples (50μg/each) were loaded to the pre-cast protein gel (Bio-Rad, No. 4569034,

146 Mini PROTEAN TGX Gel) then electrophoresed for 45 minutes at 200V in the running buffer.

147 Separated proteins in the pre-cast gel were transferred into nitrocellular membrane, following

148 which the membrane was blocked with milk/TBST buffer containing 5% milk powder (w/v),

149 tris-buffered saline and 0.1% Tween 20 for one hour at room temperature. The blocked membrane

150 was incubated with anti-NRF2 (1:1000, Cat. LS-C31637, LifeSpan BioSciences, USA) or with

151 anti-GAPDH antibody (1:1000, Cat. 60004-1-Ig, Protentech, UK) in milk/TBST buffer overnight at

152 4°C. The primary antibody solution was discarded and the membrane was washed three times

153 with TBST (15 minutes each wash). The membrane was then incubated at room temperature for

154 1h with the secondary antibody solution (goat anti-rabbit antibody, Cat. No. 926-32211 or donkey

155 anti-mouse antibody, Cat. No. 926-68072 (1:10000 diluted in milk/TBST solution and then washed

156 five times (20 minutes each wash) using TBST. The membrane was subjected to a final wash with

157 PBS for 10 minutes and scanned using the LI-COR Odyssey FC Imaging System. The signal intensity

158 of NRF2 or GAPDH was analyzed with the Image Studio™ Lite analysis software (LI-COR

159 Biosciences, USA).

160 *2.7. Assessment of VITD toxicity, induction of type 1 diabetic (T1D) mouse model and vitamin D*

161 *treatment*

The potential toxicity of VITD was evaluated by administering three intraperitoneal (IP) injections per week of 2.5 µg/kg of VITD or equivalent volume of carrier solution to six C57BL/6 mice (three mice were treated with VITD while the other three received only carrier solution). The chosen dose of VITD was based on previous *in vivo* studies in mice [25-27]. VITD was dissolved in ethanol (10µg/ml) and diluted in corn oil for IP injection. Prior to injection, animal weight and blood glucose were recorded. All animals received twelve IP injections of either VITD or equivalent volume of corn oil (control group) over a 4-week treatment course. The toxicity of VITD was assessed on the basis of daily monitoring of each animal's survival and weight. At the end of this experiment, weight and blood glucose were recorded and all mice were sacrificed using a Schedule One method.

Type I diabetes (T1D) was induced in eighteen mice (6 weeks old) by three consecutive IP injections of Streptozotocin (STZ, 55 mg/kg, dissolved in the citrate buffer, 0.1M citric acid, pH 4.5) according to protocols described by Feilt-Leichman et al. (2005) and Talahalli et al. (2009) [28, 29]. The control group (8 mice) received IP injections of equivalent volume of the citrate buffer. Development of diabetes was defined as a random blood glucose concentration greater than 13.5 mM. The weight and blood glucose level of each animal were monitored every two weeks over a period of 8 months for all animals. The STZ-induced T1D mice were supplied with 10% sucrose (w/v, every 2-3 days) to avoid sudden hypoglycaemia.

After 8 months of T1D, eight diabetic mice were given 12 intraperitoneal injections with VITD (2.5 µg/kg) over a 4-week treatment course; eight diabetic mice received 12 intraperitoneal injections with corn oil over the same time course (control group). All mice were housed in the same conditions for another 4 weeks. The weight and blood glucose of each animal were recorded every two weeks. At the end of this experiment, weight and blood glucose were recorded. The average animal weight was 34.12 g ± 1.27, while the average blood glucose concentration was 19.30 mM ± 3.03. All mice were sacrificed by a Schedule One method. Blood and eye samples were collected

187   for experiments. To dissect retinas and RPE cells, individual eyes were placed in a 35 mm petri dish

188   and merged with 1X PBS. Muscles, optic nerves and connective tissues were removed using two

189   pairs of sharp-angled forceps. With the cornea facing upward, a small puncture was created using

190   sharp forceps in the center of the eye. The eye was incised around the cornea, the cornea was

191   removed and the lens was exposed. The retina was gently peeled off from the lens. Similarly, the

192   retinal pigment epithelium (RPE) was isolated by removing any loose remnants of the iris.

193   All animal experiments were performed in accordance with the UK Home Office Animals

194   (Scientific Procedures) Act 1986 (Project licence P8C815DC9).

195   *2.8. Data analysis*

196   Experimental data were statistically analysed by non-parametric Kruskalis-Wallis followed by

197   Dunn's multiple comparison test using GraphPad Prism version 6 software. *p* <0.05 was recognized

198   as significance. Data were presented as mean ± standard deviation.

199   **3. Results**

200   *3.1. Vitamin D increased cell viability and reduced ROS production in human RPE cells*

201   ARPE-19 cells treated with 25 mM glucose showed a significant decrease in cell viability at

202   both 6 and 24 hours compared to cells cultured in physiological conditions (5 mM glucose and

203   0.1% ethanol). VITD treatment (50 nM) significantly improved cell viability in normal glycemic

204   conditions at 24 hours compared to untreated cells (0.1% ethanol); in cells treated with a

205   combination of 25 mM glucose and 50 nM VITD there was a significant increase in cell viability at

206   both 6 and 24 hours compared to cells treated with 25 mM glucose alone (Figure 1A).

207   High glucose has been shown to cause increased intracellular ROS production [30]. We also

208   found a significant increase in ROS production at both 6 and 24 hours in ARPE-19 cells treated with

209   25 mM glucose compared to cells treated with 5 mM glucose. ROS production was significantly

210   reduced in cells treated for both 6 and 24 hours with a combination of 25 mM glucose and 50 nM

211   VITD compared to cells treated with 25 mM glucose alone (Figure 1B).

212   Increased ROS can promote apoptosis [31]. We examined Caspase 3/7 activity, an indicator of

213   apoptosis, and found a significant increase in Caspase 3/7 activity in ARPE-19 cells treated for both

214   6 and 24 hours with 25 mM glucose compared to cells treated with 5 mM glucose (0.1% ethanol).

215   Co-treatment with VITD and 25 mM glucose significantly reduced Caspase 3/7 activity compared

216   to cells treated with 25 mM glucose alone for both 6 and 24 hours (Figure 1C).

217   *3.2. Vitamin D enhanced expression of antioxidant genes in human RPE cells*

218   Given that ARPE-19 cells cultured in high glucose conditions had increased ROS production,

219   we examined the expression of antioxidant genes and found that catalase (CAT) was significantly

220   reduced in cells treated with 25 mM glucose compared to cells treated with 5 mM glucose (0.1%

221   ethanol) for both 6 and 24 hours; expression of CAT was significantly increased in cells treated

222   with a combination of 25 mM glucose and VITD compared to cells treated with 25 mM glucose

223   alone for both 6 and 24 hours (Figure 2A).

224   A significant reduction of SOD1 expression was observed in ARPE-19 cells treated with 25 mM

225   glucose compared to cells treated with 5 mM glucose (0.1% ethanol); expression of SOD1 was

226   significantly increased in cells co-treated with 25 mM glucose and VITD compared to cells treated

227   with 25 mM glucose alone for both 6 and 24 hours (Figure 2B). Similarly, expression of SOD2 was

228   significantly reduced in cells treated with 25 mM glucose compared to cells treated with 5 mM

229   glucose (0.1% ethanol), while expression of SOD2 was notably increased in cells co-exposure to 25

230   mM glucose and VITD compared to cells treated with 25 mM glucose alone for both 6 and 24

231   hours (Figure 2C).

232   GPX1 expression in ARPE-19 cells was not significantly changed following treatment for 6 or

233   24 hours with 5 mM glucose (0.1% ethanol), 25 mM glucose or a combination of 25 mM glucose

234   and VITD (Figure 2D). Expression of GPX2 was significantly reduced in cells treated with 25 mM

235   glucose (0.1% ethanol) compared to cells treated with 5 mM glucose (0.1% ethanol) for both 6 and

236   24 hours; expression of GPX2 was significantly increased in cells co-incubated with 25 mM glucose

237   and VITD compared to cells treated with 25 mM glucose alone for 24 hours (Figure 2E). Finally,

238   expression of GPX3 was also significantly decreased in cells treated with 25 mM glucose (0.1%

239   ethanol) compared to cells treated with 5 mM glucose (0.1% ethanol), while expression of GPX3

240   was notably increased in cells co-incubated with 25 mM glucose and VITD compared with cells

241   treated with 25 mM glucose (0.1% ethanol) alone for 24 hours (Figure 2F).

242   We also measured SOD and CAT activities and found that cells cultured in 25 mM glucose

243   (0.1% ethanol) had significantly decreased activities of these enzymes compared to cells cultured

244   in 5 mM glucose (0.1% ethanol); co-treatment with VITD and 25 mM glucose significantly

245   increased the activities of SOD and CAT compared to treatment with 25 mM glucose (0.1%

246   ethanol) alone (Figures 3A, B). Treatment with 25 mM glucose (0.1% ethanol) also led to

247   significantly decreased GSH and increased MDA when compared to levels recorded to treatment

248   with 5 mM glucose (0.1% ethanol); co-exposure to VITD significantly counteracted the effects of

249   treatment with 25 mM glucose (Figure 3C,D).

250   NRF2, a redox-sensitive transcription factor, can regulate expression of antioxidant genes

251   under oxidative stress. Cells treated with 25 mM glucose (0.1% ethanol) had significantly lower

252   NRF2 compared to cells treated with 5 mM glucose (0.1% ethanol). Co-treatment with VITD and 25

253   mM glucose resulted in significantly increased NRF2 compared to cells treated with 25 mM glucose

254   (0.1% ethanol) only (Figure 3E,F).

255   *3.3. Vitamin D mediated cytokine production in human RPE cells*

256   The expression of IL-1β was significantly increased in cells exposed to 25 mM glucose (0.1%

257   ethanol) compared to cells treated with 5 mM glucose (0.1% ethanol) for both 6 and 24 hours,

258   whereas the expression of IL-1β was significantly reduced in cells co-treated with 25 mM glucose

259   and VITD compared to cells treated with 25 mM glucose alone for both 6 and 24 hours (Figure 4A).

260   Expression of IL-8 was significantly increased in cells exposed to 25 mM glucose (0.1% ethanol)

261   compared to cells treated with 5 mM glucose (0.1% ethanol) for both 6 and 24 hours, whereas the

262   expression of IL-8 was significantly reduced in RPE cells co-treated with 25 mM glucose and VITD

263   compared to cells treated with 25 mM glucose (0.1% ethanol) alone for both 6 and 24 hours

264   (Figure 4B). Similarly, the expression of TNF-α was notably increased in RPE cells treated with 25

265   mM glucose (0.1% ethanol) compared to cells treated with 5 mM glucose (0.1% ethanol) for both 6

266 and 24 hours, while the expression of TNF-α was notably decreased in cells co-exposed to 25 mM

267 glucose and VITD compared to cells exposed to 25 mM glucose (0.1% ethanol) alone for both 6

268 and 24 hours (Figure 4C).   Expression of VEGF-A was significantly increased in RPE cells treated

269 with 25 mM glucose (0.1% ethanol) compared to cells treated with 5 mM glucose (0.1% ethanol)

270 for both 6 and 24 hours, whereas VEGF-A expression was significantly decreased by in cells

271 co-treated with 25 mM glucose and 50 nM VITD compared to cells treated with 25 mM glucose

272 (0.1% ethanol) alone for 24 hours (Figure 4D). We also measured protein levels of secreted IL-1β,

273 IL-8, TNF-α and VEGF-A by ELISA and found that RPE cells cultured in media with 25 mM glucose

274 (0.1% ethanol) for 24 hours had significantly increased levels of the above four cytokines

275 compared to cells cultured in media with 5 mM glucose (0.1% ethanol) for the same period of

276 time. The levels of IL-1, IL-8, TNF- and VEGFA were significantly reduced in cells co-treated with 25

277 mM glucose and 50 nM VITD for 24 hours compared to cells treated with 25 mM glucose (0.1%

278 ethanol) alone for 24 hours (Figure 4E-H).

279   IL-33, a cytokine of the IL-1 family, is believed to have regulatory functions in inflammation

280 and is link to metabolic diseases such as cardiovascular and diabetes [32]. Expression of IL-33 at

281 mRNA level was significantly reduced in ARPE-19 cells exposed to 25 mM glucose (0.1% ethanol)

282 compared to cells exposed to 5 mM glucose (0.1% ethanol) for both 6 and 24 hours (Figure 5A).

283 Likewise, secreted IL-33 protein level was significantly decreased in cells exposed to 25 mM

284 glucose (0.1% ethanol) compared to cells exposed to 5.0 mM glucose (0.1% ethanol) for 24 hours

285 (Figure 5B). VITD treatment reversed high-glucose caused effects on IL-33 expression (Figure 5A,B)

286 *3.4. Vitamin D upregulated expression of antioxidant genes in diabetic mouse retina and RPE*

287   Initially the toxicity of VITD was assessed by treating C57BL/6 black mice with 2.5 μg/kg of

288 VITD or equivalent volume of corn oil (control group). The VITD toxicity assessment was based on

289 the animal's behavior and weight change. There was no abnormal motion, activity, changes in

290 food and water consumption or aggressive behavior observed during the period of treatment.

291 There was no significant difference ($P = 0.48$) in weight between the VITD-treated group and the

292 control group (Supplementary data Figure S1), indicating that there was no

293 VITD-associated-toxicity with the dose of 2.5 µg/kg.

294   The diabetic mouse retina had significantly decreased expression of catalase, Sod1, Sod2,

295 Gpx1, Gpx2 and Gpx3 compared to control mice; VITD treatment led to a significant increase in

296 expression of these antioxidant genes compared to untreated diabetic mice. The diabetic mouse

297 RPE also had significantly decreased expression of catalase, Sod1, Sod2, Gpx1 and Gpx3 (though

298 not Gpx2) when compared to the control mouse RPE; in VITD-treated diabetic mice the RPE also

299 had a significant increase in the expression of catalase, Sod1, Sod2, Gpx1 and Gpx3 (though not

300 Gpx2) compared to untreated diabetic mice (Figure 6).

301 *3.5. Vitamin D regulated cytokine expression in diabetic mouse retina and RPE*

302   Diabetic mouse retina and RPE had significantly increased mRNA levels of IL-1, IL-8 and TNF-

303 and VEGF-A compared to control mice. VITD treatment resulted in significantly reduced expression

304 of these cytokine genes in both retina and RPE when compared to untreated diabetic mice (Figure

305 7A-D). We also measured VEGF-A protein level by ELISA and found it was significantly increased in

306 diabetic retinas and RPE; VITD-treated retinas and RPE had lower VEGF-A compared to untreated

307 diabetic mice (Figure 7E). We further examined IL-33 expression in control, VITD-treated and

308 untreated diabetic mouse retinas and RPE: we found that diabetic retinas and RPE had significantly

309 decreased IL-33 at mRNA and protein levels compared to control mice; however, IL-33 expression

310 was significantly increased in VITD-treated retinas and RPE compared to untreated diabetic mice

311 (Figure 7F,G).

312 **4. Discussion**

313   It has been suggested that VITD plays a regulatory role in different types of ocular disease

314 [33,34]. An earlier study demonstrated that VITD deficiency was associated with DR in type 1

315 diabetes [17], while a recent meta-analysis found that VITD deficiency is also a risk factor for DR in

316 type 2 diabetes [35,36]. The underlying mechanisms of VITD's association with DR are poorly

317 understood. Our present study demonstrated that VITD treatment decreased ROS production and

318 lipid peroxidation, enhanced antioxidant gene expression and suppressed inflammation in

319 high-glucose-treated cells and in the diabetic mouse model. These effects may be associated with

320 activation of the NRF2 signal pathway, given that NRF2 expression was upregulated in

321 VITD-treated cells and animals.

322   Hyperglycemia is one of the causes of increased oxidative stress in mitochondria, resulting in

323 high production of oxygen free radicals in DR [30]. BRB dysfunction represents one of the most

324 significant changes occurring during diabetic retinopathy. Inner and outer BRBs are formed by

325 tight junctions between adjacent endothelial and RPE cells, respectively. The outer BRB plays many

326 essential roles in the maintenance of normal physiological processes in the retina. Oxidative stress

327 significantly contributes to the alteration of the outer BRB [37]. We found that

328 high-glucose-treated RPE cells had significantly increased ROS production and lipid peroxidation,

329 downregulated expression of antioxidant genes, decreased activities of SOD and catalase, and a

330 lower level of GSH. VITD exposure counteracted these high-glucose-induced effects (Figures 1B, 2

331 and 3). Similarly, diabetic mice had a lower expression of antioxidant genes, including Catalase,

332 Sod1, Sod2, Gpx1, 2 and 3, in retinas and in the RPE; VITD treatment significantly promoted

333 expression of these genes (Figure 6). It has previously been reported that 3T3L1 adipocytes

334 exposed to high glucose (25 mM) had significantly increased ROS production and NOX4 expression

335 [38]. VITD supplementation resulted in a marked decrease in ROS production and NOX4

336 expression. High glucose also induced ROS generation and TXNIP expression in human retinal

337 microvascular endothelial cells (HRMECs), while VITD treatment reversed these changes. VITD has

338 also been shown to inhibit high-glucose-induced cell death in HRMECs and in diabetic rat retinas

339 [39]. In the current study we detected higher Caspase 3/7 activities in RPE cells, suggesting

340 increased cell death; again, VITD treatment reversed these effects (Figure 1C).

341   NRF2 is known to be the principal mediator of expression of many antioxidant genes [40].

342 Under physiological conditions, NRF2 activity is inhibited by binding to its substrate adapter

343 protein, Kelch-like ECH-associated protein-1 (Keap-1), in the cytoplasm; however, in a stressed

344 environment NRF2 is dissociated from Keap-1 via Keap-1 oxidation and translocated to the nucleus

345 where it upregulates antioxidant gene expression by binding to the antioxidant response element

346 (ARE) [14]. Previous studies have demonstrated that high glucose level regulates NRF2 expression

347 in different cell types, such as renal tubular epithelial cells and 3T3L1 adipocytes [38,41]. In the

348 current study we found that exposure to a high glucose level decreased NRF2 expression (Figure

349 3E,F). VITD has been identified as a potent upregulator of NRF2 [14]. It has previously been shown

350 that VITD ameliorated diabetic nephropathy in a rat model by activating the NRF2 signalling

351 pathway [42]; recent work also showed that VITD functions as an anti-aging factor in rodents via

352 activation of the same pathway [43]. In a previous study we found that VITD protected RPE cells

353 from H_2O_2-induced damage by upregulation of NRF2 expression [24], while VITD has been shown

354 to reverse downregulation of NRF2 expression caused by high glucose in 3T3L1 adipocytes [38]. In

355 our current study we also found that VITD counteracted the high-glucose-induced effect on NRF2

356 expression (Figure 3E,F).

357 Inflammation as a result of hyperglycemia is another regulated process in DR. The

358 inflammatory process in DR is modulated by augmented gene expression and the production of

359 many proinflammatory cytokines, chemokines, complement factors and adhesion molecules. Thus,

360 DR is regarded as an inflammatory disease [44-46]. IL-1β has been identified as a critical

361 determinant in the development and progression of DR [47]. It has been reported that

362 hyperglycemia increased the gene expression and production of IL- 1β compared to the

363 physiological state [47]. Furthermore, there is evidence that IL-8 (also known as neutrophil

364 chemotactic factor or chemokine 8, CXCL8) is also upregulated in the retina of diabetic patients

365 and diabetic rabbits [48,49]. IL-8 mediates immune signaling through interaction with its two

366 receptors CXCR-1 and CXCR-2 with high binding affinity [49], and induces the activation of p38,

367 ERK1/2 and cJNK MAKP pathways [48]. TNF-α is another key cytokine involved in the pathogenesis

368 of DR. It is produced by activated macrophages and its gene expression is regulated by NF-kB [34].

369 TNF-α modulates the inflammatory response through binding to its two receptors, TNFR-1 and

370 TNFR-2, on target cells and triggering of the p38 MAPK and cJNK pathways [50]. Previous studies

371 have shown that IL-1β, IL-8 and TNF-α upregulate the expression and production of VEGF in

372 diabetic animal models, resulting in a substantial decrease of retinal pericytes, marked capillary

deterioration and breakdown of the blood-retinal barrier [44]. The expression and activity of VEGF have also been reported to be upregulated in diabetic patients' samples, high-glucose-treated cells and in STZ-induced diabetic animal models [51,52]. Our current data from *in vitro* and *in vivo* experiments have shown that the expression of IL-1β, IL-8, TNF-α, and VEGF was substantially increased in ARPE-19 cells treated with high glucose and in the retinas of STZ-induced diabetic mice compared to control groups; furthermore, the gene expression of these cytokines in both experimental models was notably repressed following VITD treatment (Figures 4, 7).

There is evidence to suggest that IL-33 can play a protective role in obesity, cardiovascular diseases and diabetes [53,54]. Recent studies showed that IL-33 treatment slowed down disease progression of type 1 diabetes in mice, mainly via participation of $CD4^+Foxp3^+$ regulatory T cells (Tregs) [55,56]. IL-33 expression has also been reported to be downregulated in diabetic patients and in STZ-induced diabetic mice [57-59]. Previous reports have demonstrated that IL-33 is expressed in RPE cells and in mouse brain and retina [24,60]. Our present data demonstrated that IL-33 was notably decreased at mRNA and protein levels in ARPE-19 cells incubated with high glucose and in the retinas and RPE of STZ-induced diabetic mice; however, IL-33 expression was notably increased following VITD treatment in both experimental models (Figures 5 and 7F,G).

In summary, our present study demonstrates that VITD has a protective role against high-glucose-induced oxidative damage and inflammation in retina and RPE, suggesting VITD as a potential therapeutic agent for diabetic retinopathy.

Author Contributions X.S. developed the concept. A.M.T., M.A., R.H.A., L.B., X.Z. performed the experiments. A.M.T. and X.S. analyzed the data. J.R., Z.Z. and X.S. drafted the manuscript.

Conflicts of Interest: No conflict of interest needs to be declared.

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Figures and legends

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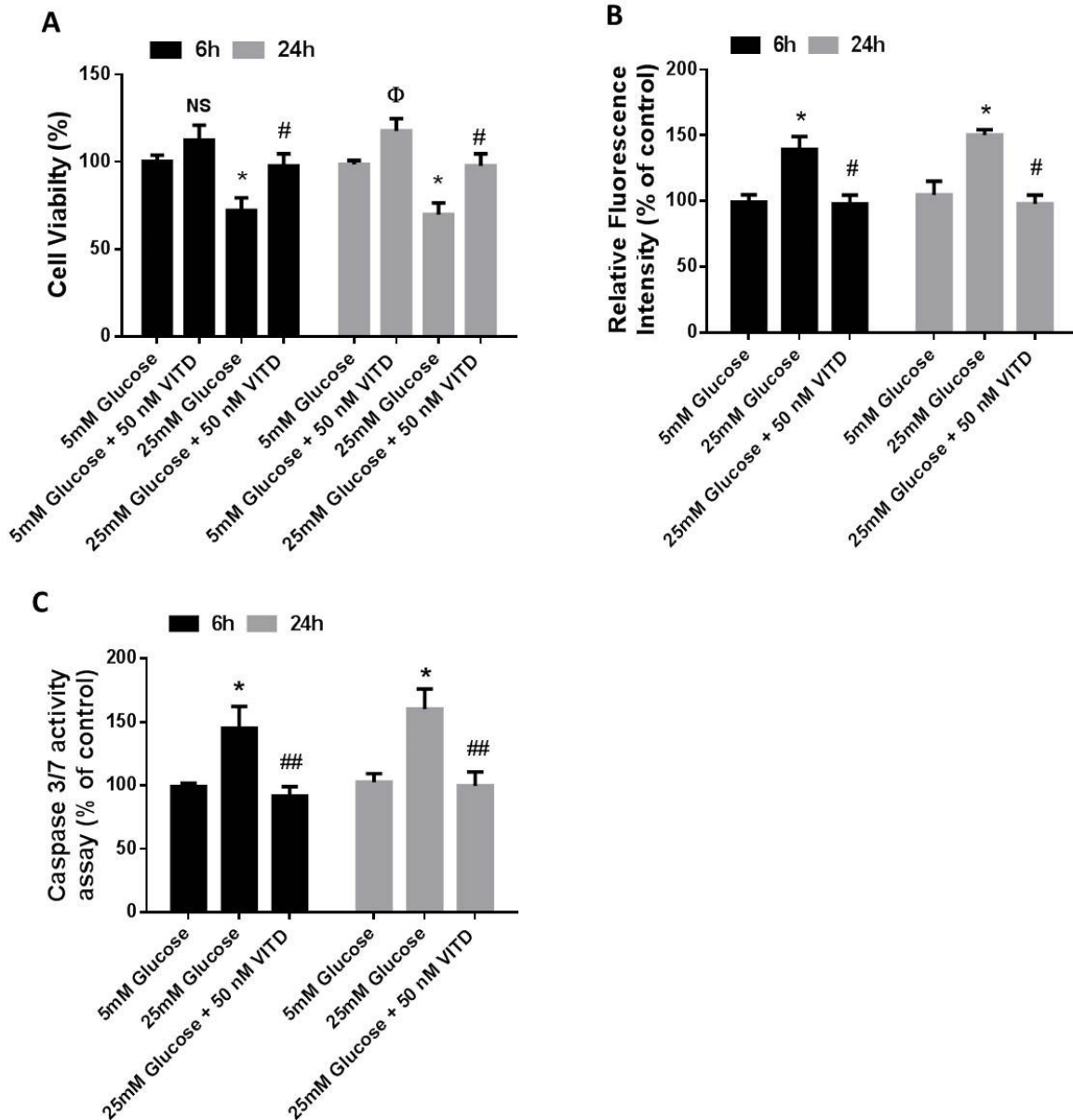
Figure 1. (A) ARPE-19 cell viability. Cells treated with 5mM glucose, 25 mM glucose or a

combination of 25mM glucose and VITD (50 nM) for 6 and 24 hours. Cell viability was measured

using the MTT assay described. (B) VITD treatment can protect against ROS production in cells

exposed to a high level of glucose. Cells were treated with or without VITD (50 nM) for 6 and 24

hours, ROS was measured. (C) VITD treatment can protect against apoptosis in cells treated with a

high concentration of glucose. Cells were treated with or without VITD (50 nM) and activities of

Caspase 3/7, a biomarker for apoptosis, were measured. Data were analysed with non-parametric

614   Kruskal-wallis followed by Dunn's multiple comparison test (n=5). Φ p <0.05, 5 mM Glucose +

615   VITD vs.5 mM Glucose; *p <0.05, 25 mM Glucose vs. 5 mM Glucose; #p <0.05, 25mM Glucose +

616   VITD vs. 25 mM glucose; ##p <0.01, 25mM Glucose+VITD vs. 25 mM glucose. NS, 5 mM glucose +

617   VITD vs. 5 mM glucose.

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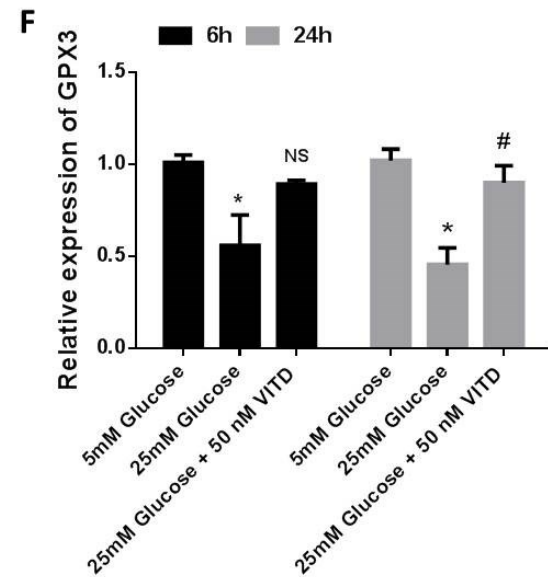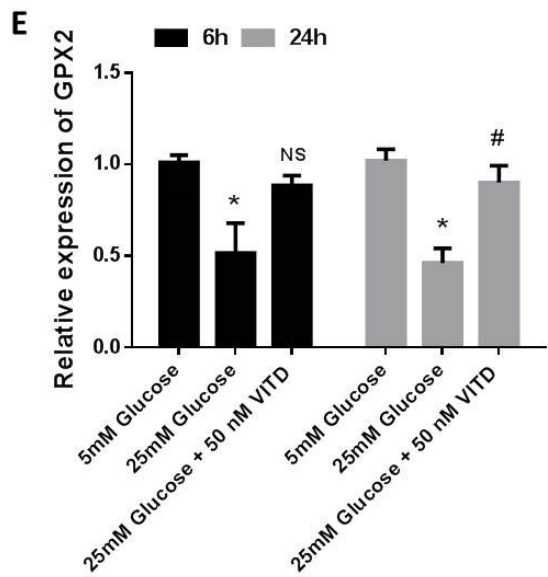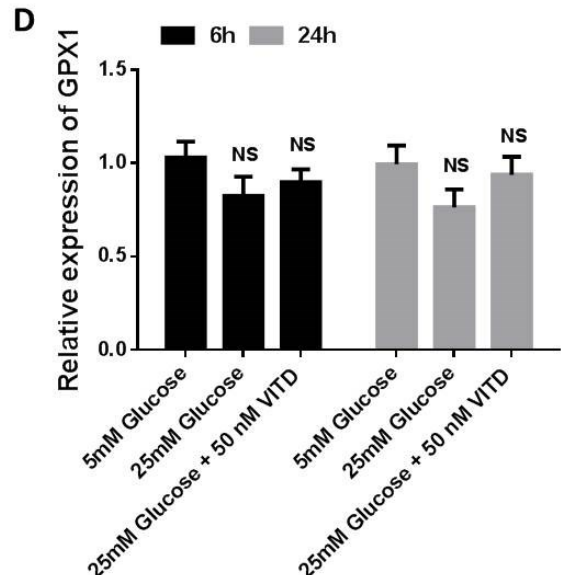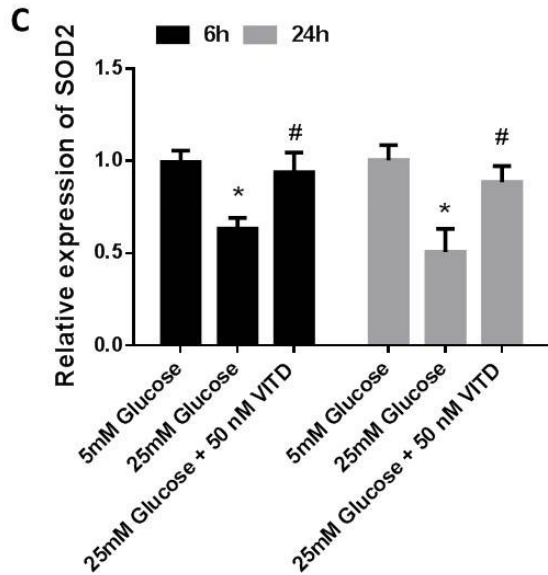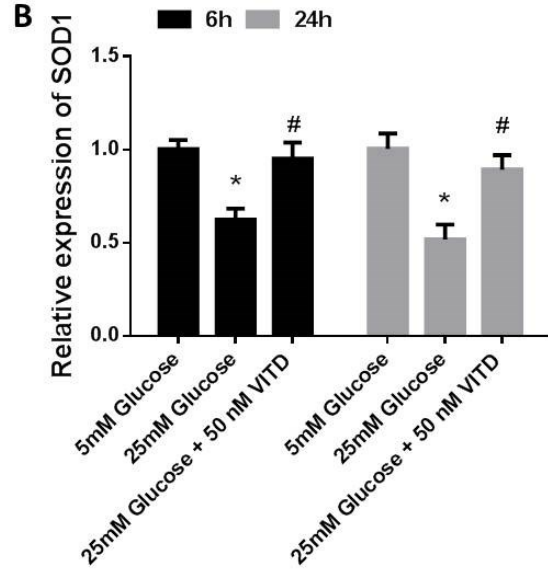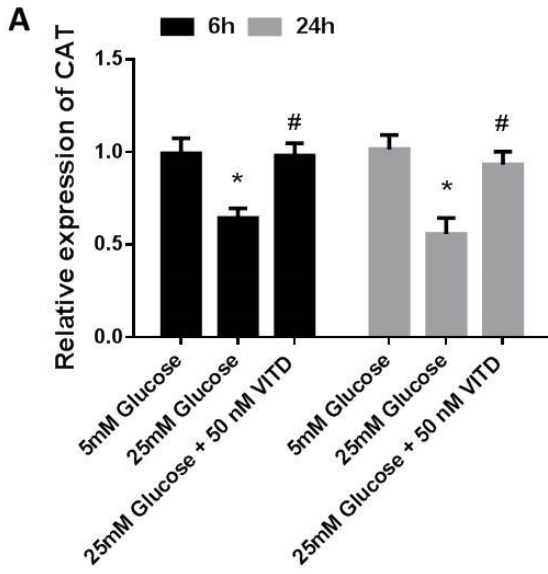
619   **Figure 2.** VITD treatment can enhance the expression of antioxidants genes in ARPE-19 cells

620   challenged with a high level of glucose. Cells were treated with or without VITD (50 nM) for 6 and

621   24 hours. the expression of the antioxidant genes catalase (A), SOD1 (B), SOD2 (C), GPX1 (D), GPX2

622   (E) and GPX3 (F) was measured using qRT-PCR assay. Data were analysed with non-parametric

623   Kruskal-wallis followed by Dunn's multiple comparison test (n=5). *p <0.05, 25 mM Glucose vs.

624   5 mM Glucose; #p <0.05, 25mM Glucose + VITD vs. 25 mM glucose. NS in Figure 2D, 25

625   mM Glucose vs. 5 mM Glucose or 25mM Glucose + VITD vs. 25 mM glucose; NS in Figure

626   2E and F, 25mM Glucose + VITD vs. 25 mM glucose.

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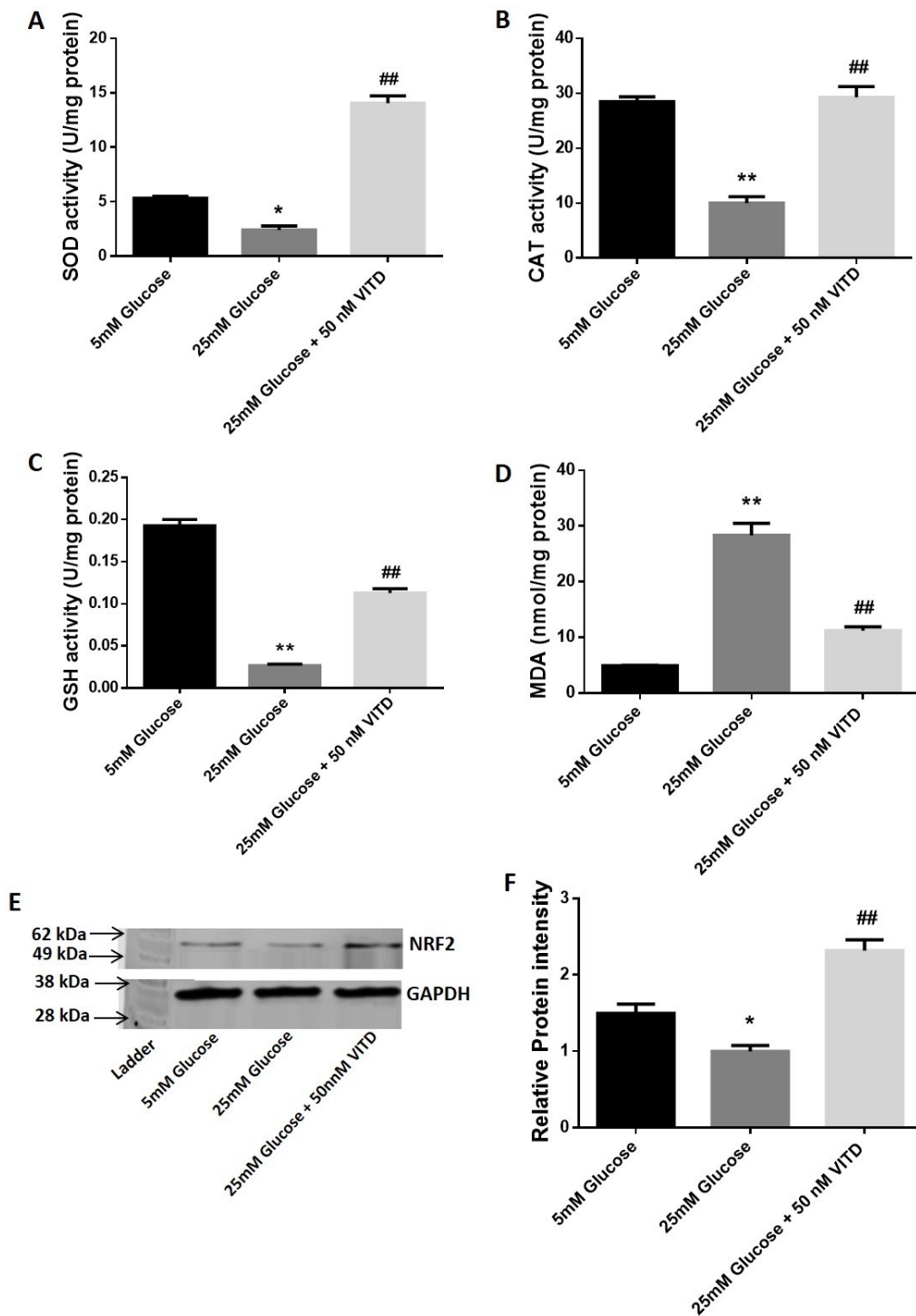
**Figure 3.** VITD treatment increased antioxidant capacity. Cells were treated with or without

VITD (50 nM) for 24 hours. Activities of superoxide dismutase (SOD) (A) and catalase (CAT) (B)

and levels of glutathione (GSH) (C) and malondialdehyde (MDA) (D) were measured using

commercial kits. NRF2 protein detected in control and treated cells was examined by Western

blotting (E). NRF2 protein levels were quantified by normalizing with GAPDH protein (F). Data

635   were analysed with non-parametric Kruskal-wallis followed by Dunn's multiple

636   comparison test (n=4). $*p$ <0.05, 25 mM Glucose vs. 5 mM Glucose; $**p$ <0.01, 25 mM

637   Glucose vs. 5 mM Glucose; ##p <0.01, 25mM Glucose+VITD vs. 25 mM glucose.
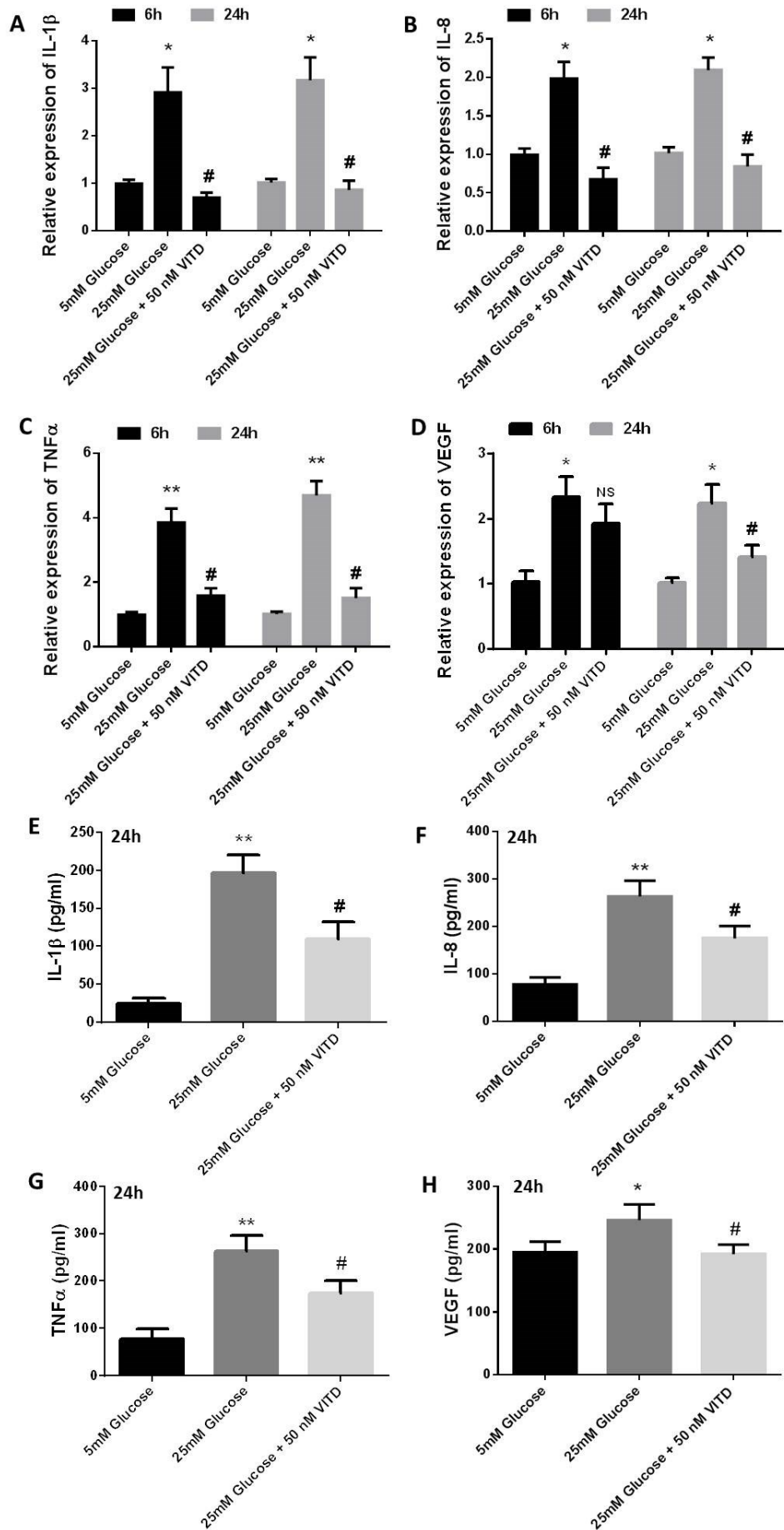
639   **Figure 4.** VITD regulated the expression of pro-inflammatory cytokines in ARPE-19 cells

640   treated with high glucose or co-treated with high glucose and VITD (50 nM) for 6 and 24

641   hours. The relative expression of IL-1β (A), IL-8 (B), TNF-α (C) and VEGF (D) was determined by

642   qRT-PCR assay. Secreted protein levels of IL-1β (E), IL-8 (F), TNF-α (G) and VEGF (H) were

643   measured by ELISA. Data were analysed with non-parametric Kruskal-wallis followed by

644   Dunn's multiple comparison test (n=4). *p <0.05, 25 mM Glucose vs. 5 mM Glucose; **p

645   <0.01, 25 mM Glucose vs. 5 mM Glucose; #p <0.05, 25mM Glucose+VITD vs. 25 mM glucose.

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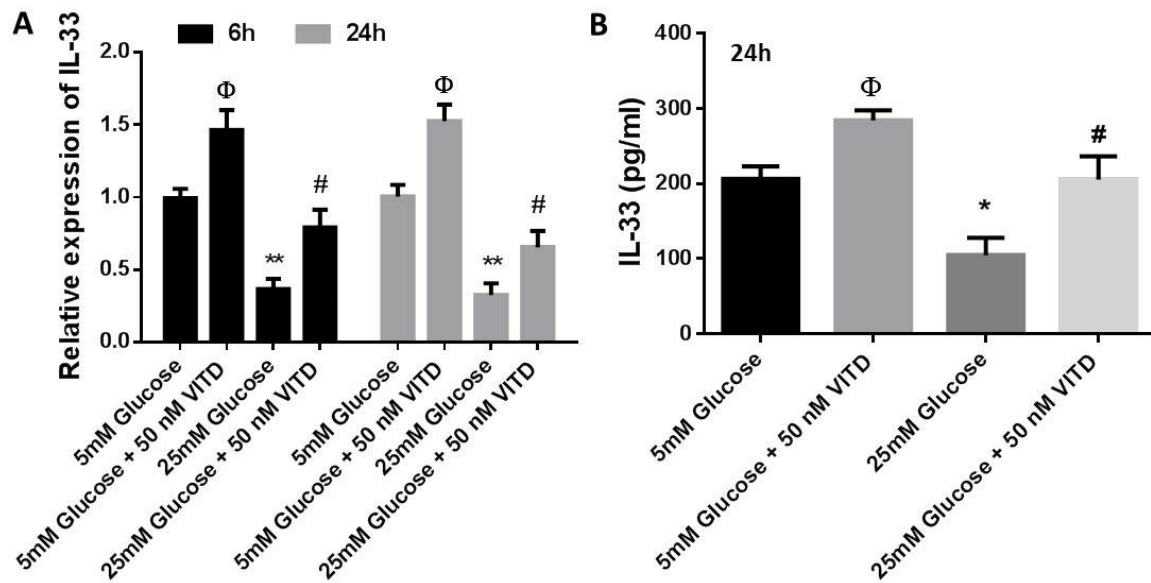
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Figure 5. The expression of IL-33 in stressed ARPE-19 cells. (A) The relative expression of IL-33 as determined by qRT-PCR assay in ARPE-19 cells treated for 6 and 24 hours with 5 mM glucose, with 25 mM glucose, or co-treated with 25 mM glucose and VITD (50 nM). (B) Secreted IL-33 protein levels in media of cells treated with or without VITD (50 nM) for 24 hours were measured by ELISA. Data were analysed with non-parametric Kruskal-wallis followed by Dunn's multiple comparison test (n=4). $\Phi$ p <0.05, 5 mM Glucose + VITD vs.5 mM Glucose; *p <0.05, 25 mM Glucose vs. 5 mM Glucose; **p <0.01, 25mM Glucose vs. 5 mM glucose. #p <0.05, 25mM Glucose + VITD vs. 25 mM glucose.

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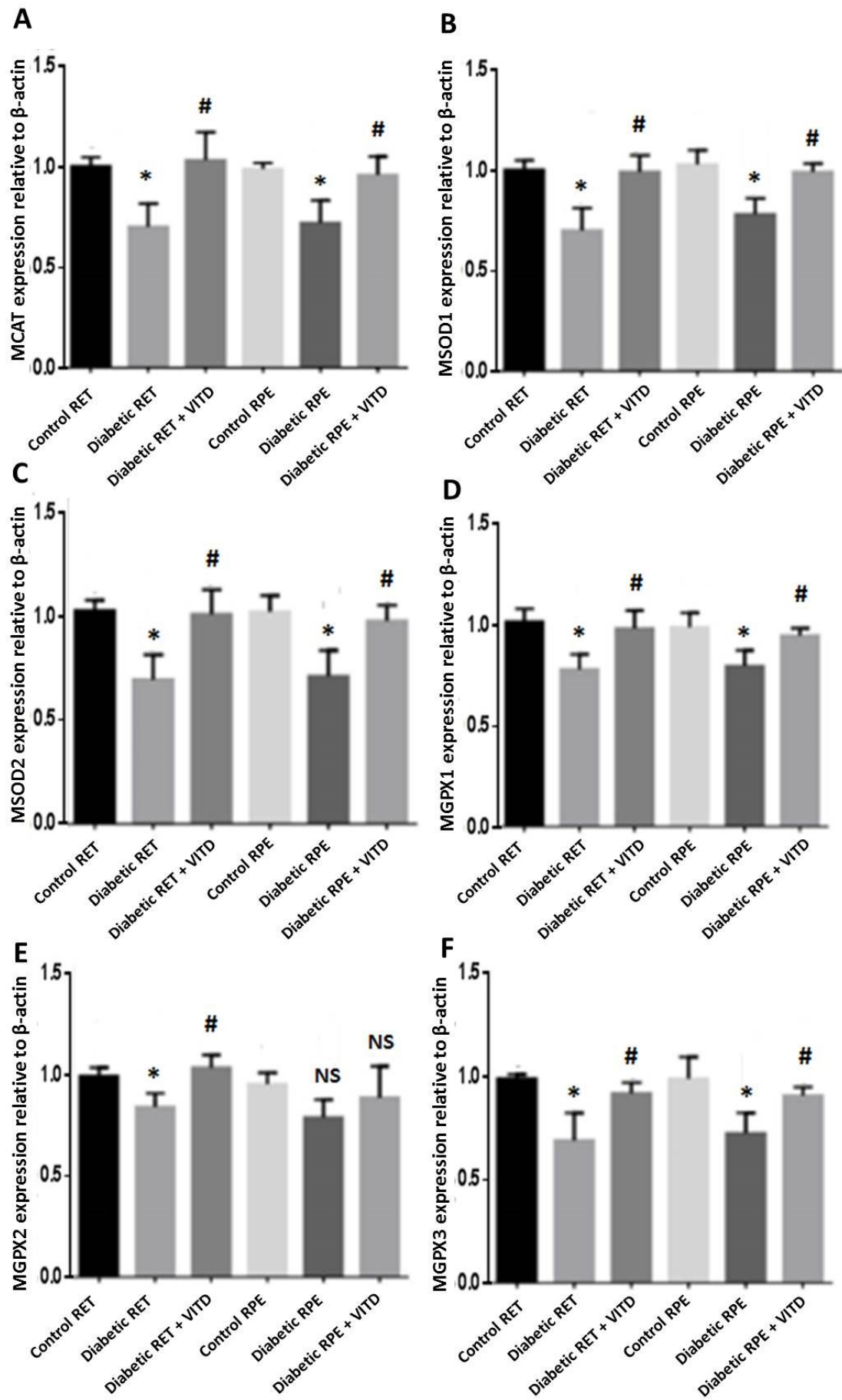
677   **Figure 6.** Effect of VITD on antioxidant gene expression in diabetic retinas and RPE. Retinas

678   and RPE were dissected from control, diabetic and VITD-treated mice (described in section

679   2.7). RNA was extracted and cDNA was synthesized. Relative expression of antioxidant genes:

680   catalase (MCAT) (A), SOD1 (B), SOD2 (C), GPX1 (D), GPX2 (E) and GPX3 (F) in the retina (RET)

681   and RPE was examined by qRT-PCR. Data were analysed with non-parametric

682   Kruskal-wallis followed by Dunn's multiple comparison test (n=6 samples in each group). *p

683   <0.05, diabetic vs. control; #p <0.05, diabetic + VITD vs. diabetic. NS, diabetic vs. control or

684   diabetic + VITD vs. diabetic.

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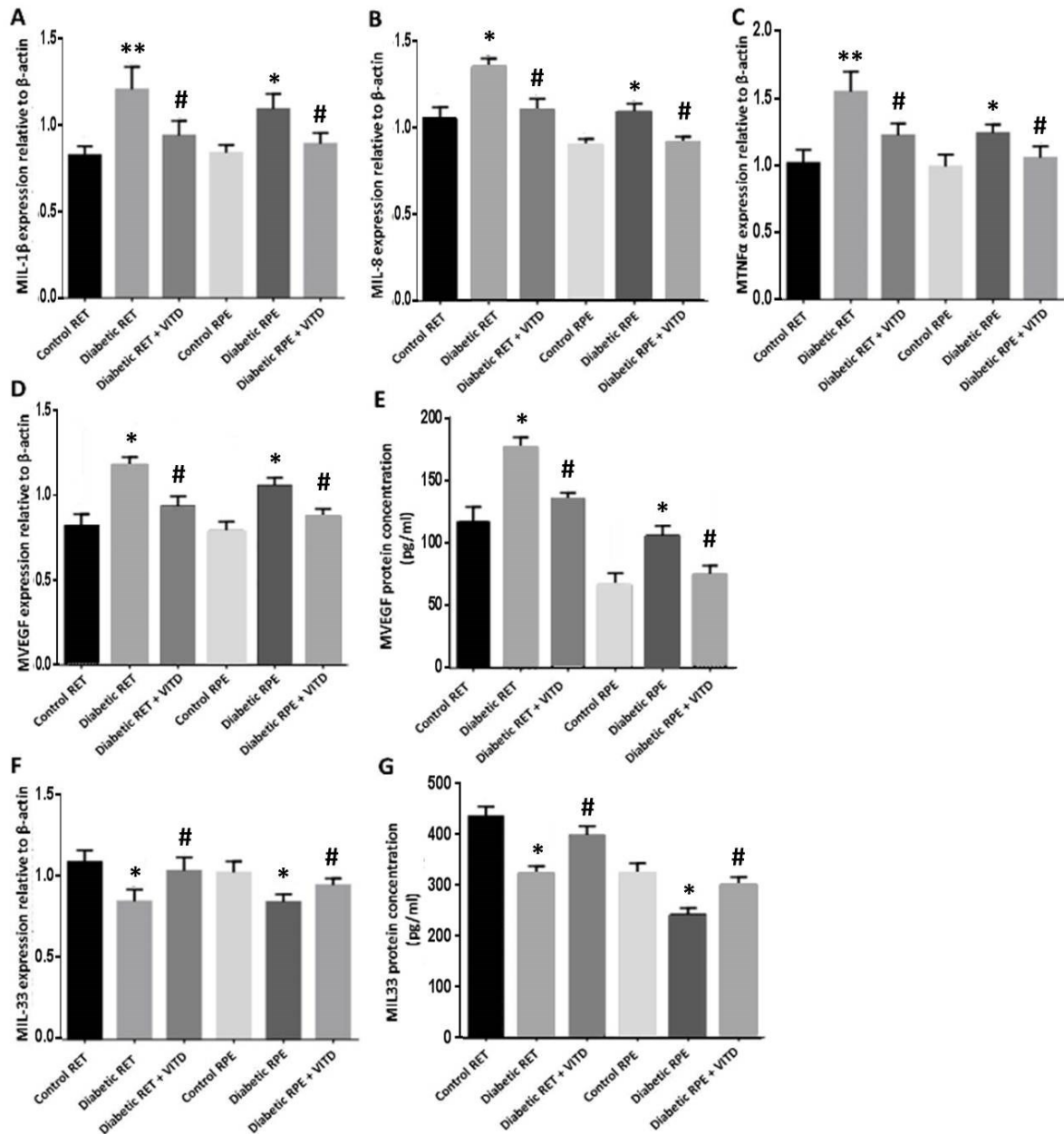
Figure 7. Effect of VITD on proinflammatory gene expression in diabetic retinas and RPE.
Retinas and RPE were dissected from control, diabetic and VITD-treated mice (described in
section 2.7). RNA was extracted and cDNA was synthesized. Relative expression of
inflammatory cytokines IL-1β (A), IL-8 (B), TNFα(C), VEGF (D) and IL-33 (F) in retina (RET) and
RPE was examined by qRT-PCR. Protein levels of VEGF (E) and IL-33 (G) were measured by
ELISA. Data were analysed with non-parametric Kruskal-wallis followed by Dunn's multiple
comparison test (n=6 samples in each group). *p <0.05, diabetic vs. control; **p
<0.01, diabetic vs. control; #p <0.05, diabetic + VITD vs. diabetic.