Effect of vitamin B$_{12}$ supplementation on retinal lesions in diabetic rats

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Purpose: Diabetic retinopathy (DR) is the most common complication of diabetes involving microvasculature and neuronal alterations in the retina. Previously, we reported that vitamin B$_{12}$ deficiency could be an independent risk factor for DR in humans. However, the effect of vitamin B$_{12}$ supplementation in experimental DR is unknown. Thus, in this study, we investigated the impact of dietary supplementation of vitamin B$_{12}$ on retinal changes in diabetic rats.
Methods: Diabetes was induced in 2-month-old Sprague-Dawley rats and maintained for 4 months. One group of diabetic rats were fed normal levels of vitamin B$_{12}$, and one group double the quantity of vitamin B$_{12}$ (50 µg/kg diet). Vitamin B$_{12}$ and homocysteine levels in the plasma were analyzed with radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC), respectively. At the end of 4 months of experimentation, the eyeballs were collected. Retinal changes were analyzed with hematoxylin and eosin (H&E) staining, immunoblotting, and immunofluorescence methods.
Results: Dietary supplementation of vitamin B$_{12}$ had no effect on food intake, bodyweight, fasting blood glucose, and plasma homocysteine levels in the diabetic rats. However, vitamin B$_{12}$ supplementation prevented loss of rhodopsin, and overexpression of VEGF, and completely prevented overexpression of HIF1α, GFAP, and endoplasmic reticulum (ER) stress markers (GRP78, ATF6α, XBP1, CHOP, and caspase 12) in the diabetic rat retina. Further, vitamin B$_{12}$ ameliorated apoptosis in the retina as shown with terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and prevented retinal thinning.
Conclusions: Vitamin B$_{12}$ supplementation of diabetic rats appeared to be beneficial by circumventing retinal hypoxia, VEGF overexpression, and ER stress-mediated cell death in the retina. The present study adds another potential therapeutic strategy of vitamin B$_{12}$ in diabetes.

Diabetes has become one of the most challenging health problems worldwide. Prolonged exposure to chronic hyperglycemia in diabetes can lead to various short- and long-term complications. Approximately 463 million adults globally live with diabetes, and by 2045, this will rise to 700 million International Diabetes Federation. With the increase in the incidence of diabetes, its complications have also increased accordingly, impairing quality of life and causing socioeconomic burdens. Poor glycemic control and long disease duration are significant risk factors of the microvascular and macrovascular complications that cause most of the morbidity and mortality associated with diabetes. Diabetic retinopathy (DR) is the most common microvascular complication of diabetes, and is the leading cause of blindness and visual impairment affecting one-third of people with diabetes worldwide [1]. DR leads to vision loss through two main mechanisms: leakage of fluid in the macula and growth of new blood vessels and mechanical damage to the retina. Persistent hyperglycemia disrupts the microvasculature and neuronal functioning in the retina leading to vision loss. DR is a common complication in both types of diabetes, and the symptoms include blurred vision, floaters, difficulty seeing colors, and even total loss of vision. The early stages of DR (non-proliferative DR, NPDR) are characterized by the presence of microaneurysms, dot and blot hemorrhages, cotton wool spots, and venous abnormalities, depriving blood supply to areas of the retina. In the advanced stage of DR known as proliferative DR (PDR), neovascularization is seen that is fragile and leaky causing further damage to the retina. The prevalence of PDR is 50% in type 1 and 15% in type 2 diabetics with 25 years of disease [2]. According to the World Health Organization (WHO), almost 32 million Indians have DR, and this number is estimated to increase to almost 80 million by 2030 [3], the highest number in any country.

Multiple factors are likely to be involved in the onset and progression of DR. Age, duration of diabetes, lower body mass index, higher fasting plasma glucose, and higher HbA1c levels have been identified as the risk factors most strongly associated with the development of DR [4-7]. Studies have reported ethnic differences in the prevalence and severity of DR even after controlling for systemic risk factors. A cross-sectional study conducted in the United Kingdom (UK) showed that DR was much more prevalent in people of Afro-Caribbean descent and South Asians compared to

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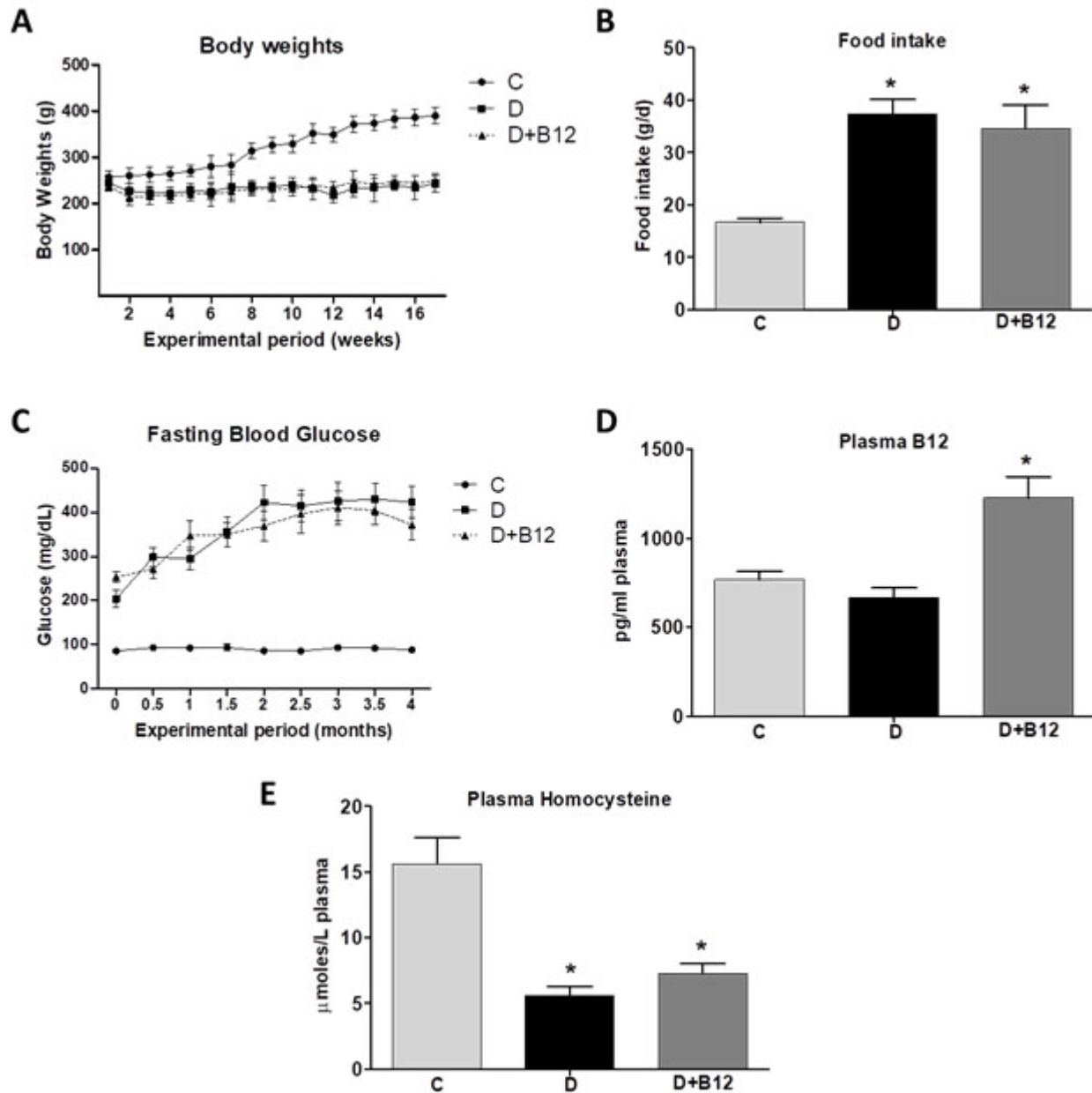
Figure 1. Animal general and biochemical characteristics. **A**: Bodyweight. **B**: Food intake. **C**: Fasting blood glucose levels. **D**: Plasma vitamin B_{12}. **E**: Plasma homocysteine levels in rats (n = 8). Data are mean ± standard error of the mean (SEM). C, control; D, diabetes, D+B12, diabetic rats treated with vitamin B_{12}. *Significant difference from the control group; #significant difference from the diabetes group ($p < 0.05$).

Caucasians, and they were at higher risk for sight-threatening complications [8]. Studies suggested that patients with diabetes are at higher risk for deficiency of micronutrients [9-12]. Recently, we reported a high prevalence of multiple subclinical micronutrient deficiencies, dietary inadequacies (along with hyperhomocysteinemia) in apparently healthy adults (30–70 years old), particularly B vitamins, including vitamin B_{12} [13-15]. However, to date, only a few studies

have evaluated the possible role of nutritional factors in the development of DR [11,12]. Most importantly, our previous studies suggested that vitamin B_{12} deficiency could be an independent risk factor for DR [11].

Vitamin B_{12} or cobalamin is a water-soluble vitamin that plays a fundamental role in DNA synthesis, optimal hemopoiesis, and neuronal and vascular functions. Several studies

[11,16-19] have shown vitamin B_{12} deficiency in diabetes. A meta-analysis showed that treatment with vitamin B_{12} improved nerve conduction velocity in patients with diabetic peripheral neuropathy [20]. Mizukami et al. reported that vitamin B_{12} supplementation improved nerve conduction velocity in diabetic rats by preventing impaired neural signaling of protein kinase C and oxidative stress-induced damage [21]. Another study concluded that exogenous vitamin B_{12} delayed the onset of diabetic peripheral neuropathy via upregulation of sciatic nerve IGF-1 gene expression [22]. Rathod et al. proved that fortification of foods with vitamin B_{12} helps improve brain development [23]. However, the effect of vitamin B_{12} supplementation in experimental DR is unknown. Therefore, in the present study, we investigated whether vitamin B_{12} supplementation could influence DR in diabetic rats.

METHODS

Animal experiments: Two-month-old male Sprague-Dawley rats with an average bodyweight of 200 g were obtained from the National Center for Laboratory Animal Sciences (Hyderabad, India). All animals were fed with the AIN-93 rodent diet obtained from Research Diets Inc. (New Brunswick, NJ). The rats were housed in individual cages with temperature (22 ± 2.0 °C), humidity ($55\pm5.0\%$), and light (12-h:12-h light-dark) controlled conditions. Diabetes was induced in rats with a single intraperitoneal injection of streptozotocin (STZ; 38 mg/kg) in citrate buffer (pH 4.5), whereas the control group of rats received vehicle alone (n = 8). The STZ-induced diabetic rat is the most widely used animal model to study DR [24]. Rats with fasting blood glucose ≥150 mg/dl were included in the study. Half of the animals (n = 8) were fed a vitamin B_{12}-supplemented diet, and the remaining half (n = 8) were fed a normal diet similar to that of the control group of rats. The vitamin B_{12} -supplemented diet consists of 50 µg/kg diet, double the vitamin B_{12} content compared
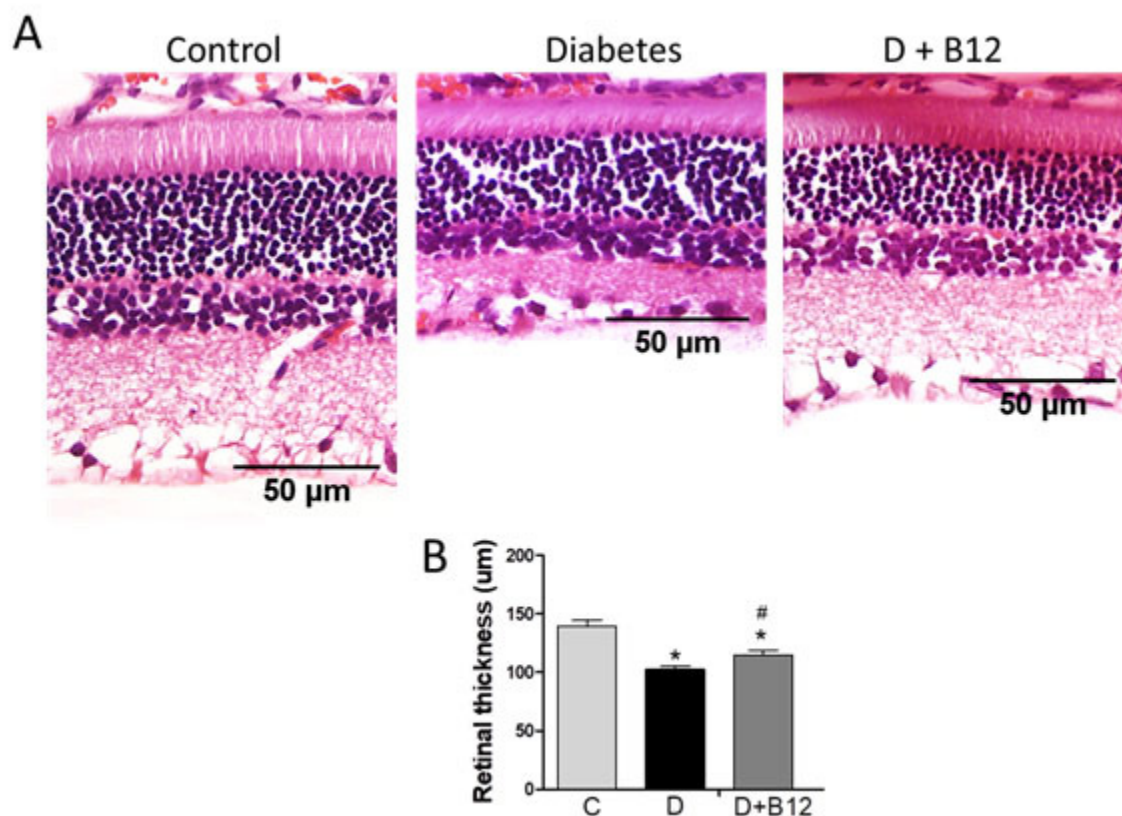

Figure 2. Retinal morphology and thickness. **A**: Representative images of hematoxylin and eosin (H&E) staining on the rat retinal sections after the 4-month experimental period. Magnification = 400X. Scale bar = 50 µm. **B**: Graphical representation of the retinal thickness of the rat (n = 4). Data are mean ± standard error of the mean (SEM). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B_{12}. *Significant difference from the control group; #significant difference from the diabetes group ($p<0.05$).
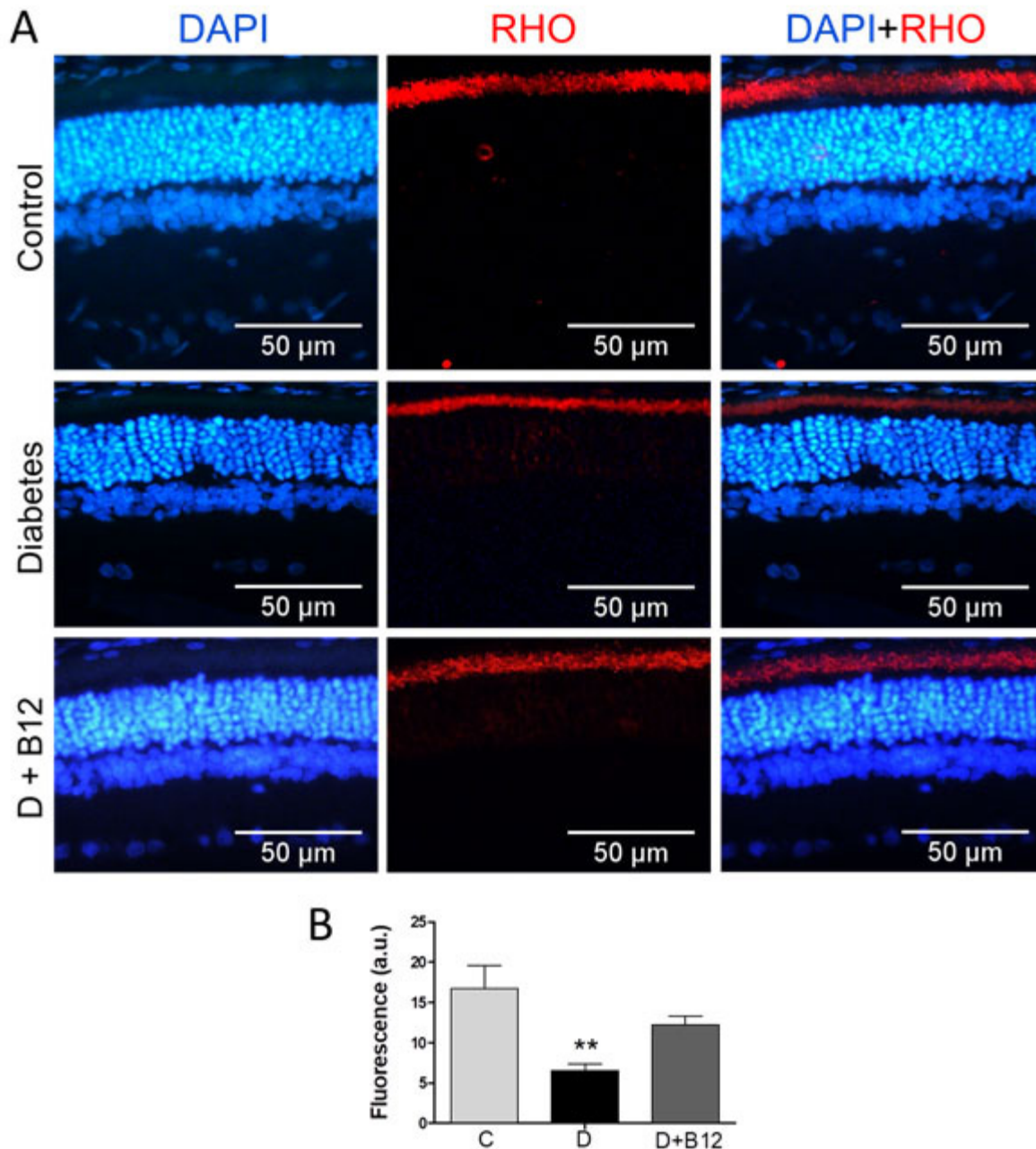
Figure 3. Immunofluorescence staining for rhodopsin in the rat retina. **A**: Representative images of immunofluorescence staining for rhodopsin (red), counterstained with 4′, 6-diamidino-2-phenylin-dole (DAPI; blue) for cellular nuclei. Magnification = 400X. Scale bar = 50 μm. **B**: Quantification of rhodopsin staining. Data are mean ± standard error of the mean (SEM, n=3). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B_{12}. **Significant difference from the control group (p<0.01).

to that of the normal diet (25 μg/kg diet). All rats had ad libitum access to water and food. Bodyweight and fasting blood glucose levels were measured weekly until the end of the 4-month experimentation period. Animal care protocols were in accordance with and approved by the Institutional Animal Ethics Committee (IAEC). The study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. At the end of the experimentation period, overnight fasted rats were euthanized by CO_2 inhalation to collect the eyeballs. Four eyeballs per group, each from a different animal, were fixed in 4% paraformaldehyde for immunohistochemical analysis, and the remaining were

dissected to collect the retinas, snap-frozen in liquid nitrogen, and stored at −80 °C for protein analysis.

Plasma analysis for vitamin B$_{12}$ and homocysteine: Plasma total vitamin B$_{12}$ levels were analyzed using a commercially available solid-phase radioimmunoassay kit (MP Biomedicals, Diagnostic Division, New York, NY) according to the

manufacturer's instructions. A gamma counter equipped with dual channels for determining the radioactivity of ^{57}Co simultaneously was used (Perkin Elmer, Waltham, MA; 3 wizard 1480). Plasma total homocysteine was determined by engaging a unique reversed phase column for separating the analytes, supplied in the commercially available reverse phase high-performance liquid chromatography (HPLC) kit
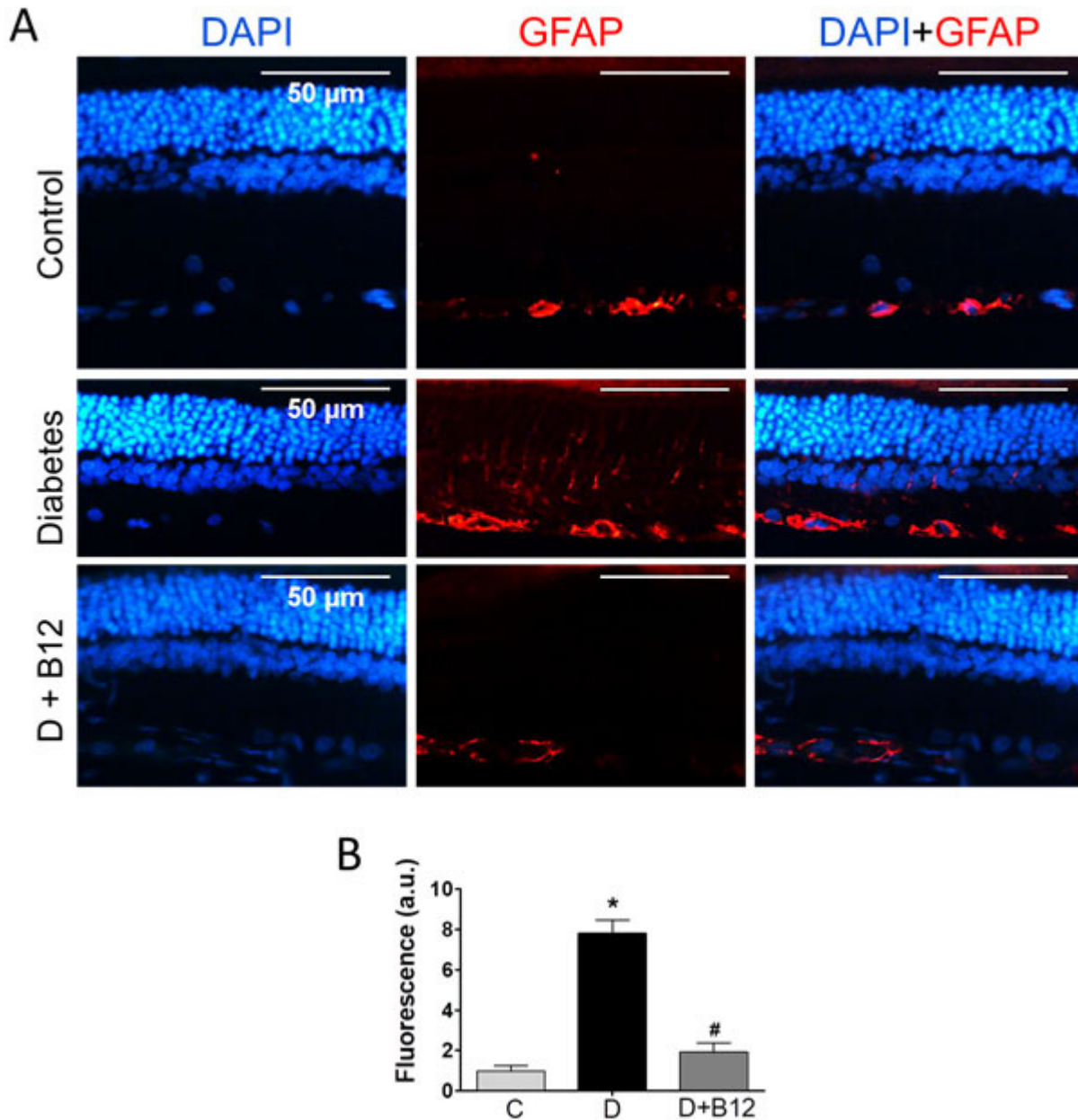

Figure 4. Immunofluorescence staining for GFAP in the rat retina. **A**: Representative images of immunofluorescence staining for GFAP (red), counterstained with 4′, 6-diamidino-2-phenylin-dole (DAPI; blue) for cellular nuclei. Magnification = 400X. Scale bar = 50 μm. **B**: Quantification of GFAP staining. Data are mean ± standard error of the mean (SEM, n=3). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B$_{12}$. **Significant difference from the control group; #significant difference from the diabetes group (p<0.05).

with fluorescence detection (Recipe Chemicals and Instruments. GmbH, Germany) [11].

Immunoblotting: Retinal cellular protein was extracted in Tris-lysis buffer (pH 7.5) with protease inhibitors (Sigma, St. Louis, MO), and the protein concentration was measured with the Lowry method. Equal amounts of protein from each group were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. The primary antibodies for HIF1α (ab187524, Abcam antibodies, Cambridge, UK), GRP78/Bip (PA5–19503, ThermoScientific, Waltham, MA), ATF6 α (MA5–16172, ThermoScientific), XBP1 (ab37152, Abcam), CHOP (2895s, Cell Signaling Technologies, Danvers, MA), caspase 12 (ab62484, Abcam), BAX (2772s, CST), VEGF (MA1–16629, tubulin (ThermoScientific), and the respective secondary antibodies were added sequentially, and the protein was detected with an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc., Berkeley, CA). Tubulin served as a loading control. Band intensity was measured with ImageJ software (National Institutes of Health [NIH], Bethesda, MD).

Immunohistochemistry: Eyeballs fixed in paraformaldehyde were oriented and embedded in paraffin. The eyeballs were sectioned sagitally, and sections passing through the optic nerve and center of the cornea (4 µm thickness) were stained with either hematoxylin and eosin (H&E) or immunofluorescence for Rho, GFAP, XBP1, and CHOP as reported previously [25]. Briefly, the ocular sections were dewaxed and rehydrated for heat-induced antigen retrieval and incubated overnight with primary antibodies for rhodopsin, GFAP, XBP1, and CHOP. The secondary antibody incubation with Alexa Fluor (488 and 568) was at room temperature

for 2 h. 4′, 6-Diamidino-2-phenylin-dole (DAPI) staining was performed to label the cell nuclei. Labeled sections were visualized in a Leica fluorescence microscope (Leica Microsystems, Germany) at 400X. Images were analyzed and measured using ImageJ software (NIH). The extent of fluorescence intensity was measured as the mean gray value for rhodopsin and GFAP (as the fluorescence is prominent across the section area), and as the number of positive cells per sectional area for XBP1 and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.

Retinal thickness: H&E-stained ocular sections were used to measure retinal thickness. LAS v4.8 software (Leica Microsystems, Wetzlar, Germany) was employed to measure retinal thickness on 400X magnified images, at either side of the optic nerve (three on each side), approximately at equal distance from the optic nerve, and the mean was calculated. Similarly, four different animals per group were measured, and the means of all groups were subjected to statistical analysis.

TUNEL labeling: TUNEL assay was performed on the ocular sections to investigate apoptosis in the retina. The In Situ Cell Death Detection Kit (Roche Diagnostics; Basel, Switzerland) was employed following the manufacturer's protocol [25]. After the antigens were retrieved, the sections were incubated with the TUNEL reaction mixture for 1 h at 37 °C. The cell nuclei were labeled with DAPI staining. The labeled sections were visualized in a Leica fluorescence microscope at 400X. TUNEL positive cells were analyzed and quantitated using ImageJ software (NIH).

Statistical analysis: Values are represented as the mean ± standard error of mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA)
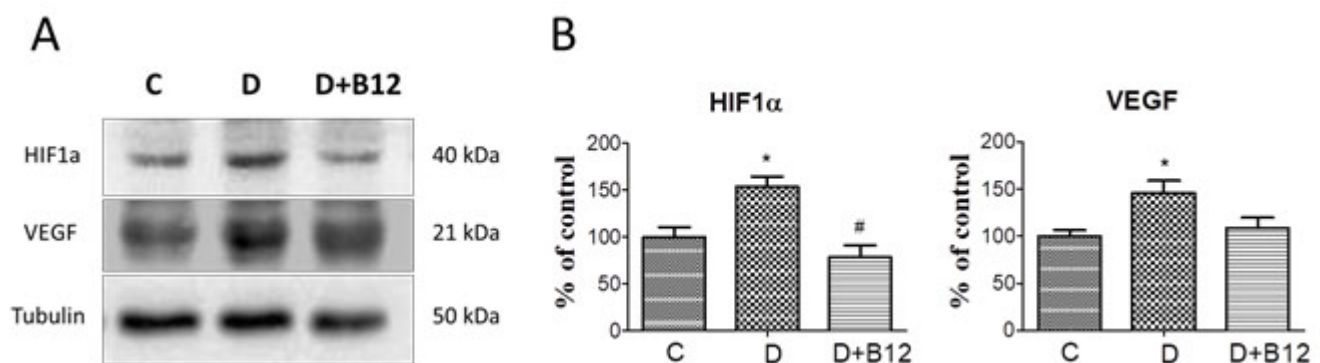

Figure 5. Immunoblotting for HIF1α and VEGF in the rat retina. **A**: Representative immunoblots for HIF1α and VEGF. **B**: Quantification of the corresponding densitometry data. The protein expression was normalized to the tubulin and was represented as %control. Data are mean ± standard error of the mean (SEM, n=3). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B$_{12}$. *Significant difference from the control group; #significant difference from the diabetes group (p<0.05).
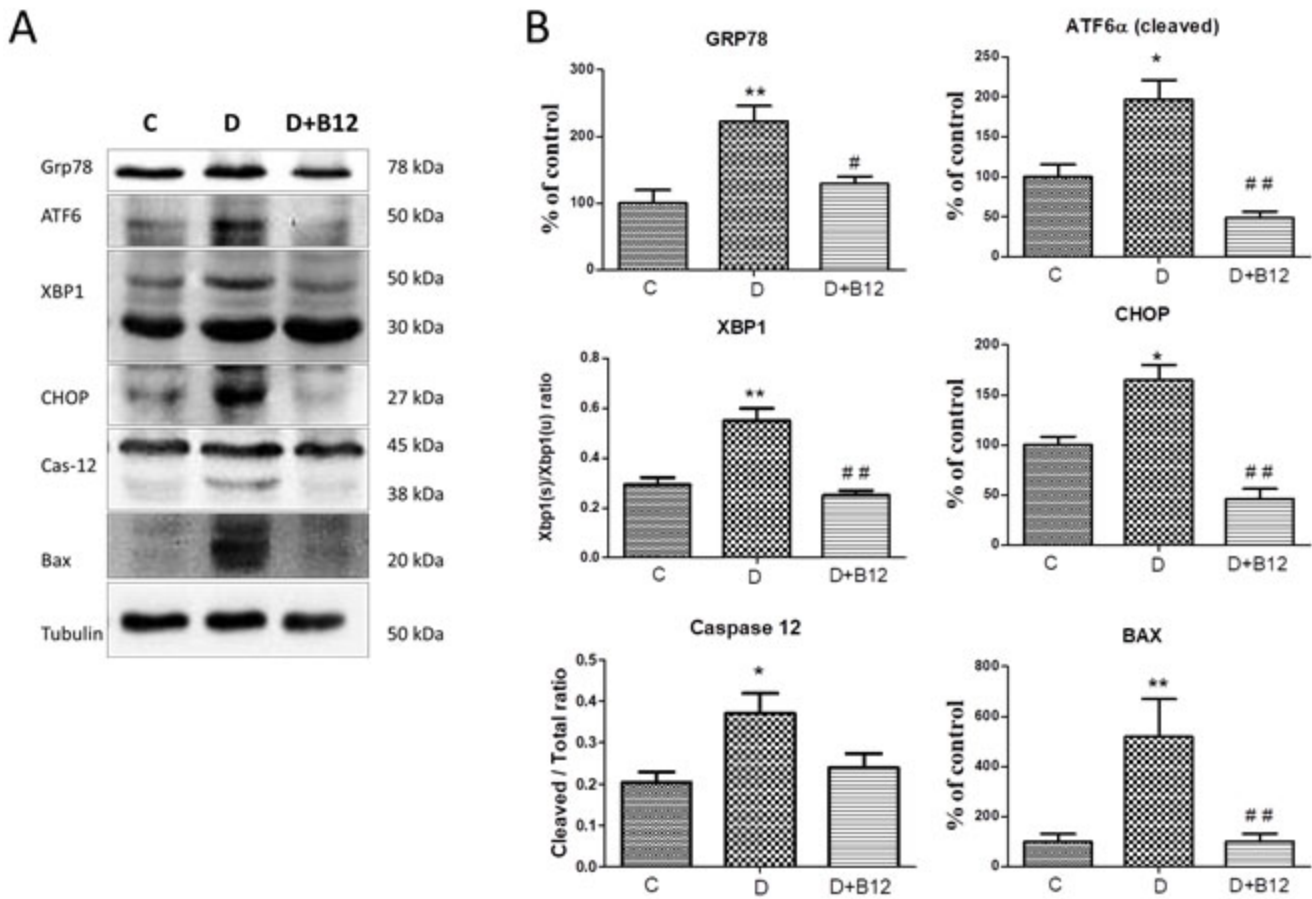
Figure 6. Immunoblotting for ER stress and apoptotic markers in the rat retina. **A**: Representative immunoblots for endoplasmic reticulum (ER) stress markers and apoptotic markers. **B**: Quantification of the corresponding densitometry data. The protein expression was normalized to the tubulin and was represented as %control. Data are mean ± standard error of the mean (SEM, n=3). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B$_{12}$. *Significant difference from the control group; #significant difference from the diabetes group (p<0.05). ** and ## indicate respective statistical significance at p<0.01.

with a post hoc Tukey test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Animal bodyweight, food intake, and blood glucose levels: In line with previous studies [26], the food intake and fasting blood glucose levels of the diabetes group were higher, and the bodyweights were lower, than those of the normal control rats (Figure 1). Further, vitamin B$_{12}$ supplementation of diabetic rats showed no influence on any of the three parameters.

Plasma vitamin B$_{12}$ and homocysteine levels: Although there was no statistically significant change in the plasma vitamin B$_{12}$ levels, the plasma homocysteine levels were found to be lower in the STZ-induced diabetic rats (Figure 1). However,

vitamin B$_{12}$ supplementation of diabetic rats for 4 months led to increased plasma vitamin B$_{12}$ levels over and above those of the control rats. Although the homocysteine levels were slightly higher in the vitamin B$_{12}$ -supplemented diabetic rats than in the diabetic control rats, the level was not statistically significant.

Retinal thickness: Systematic morphological examination of the H&E-stained ocular sections as shown in Figure 2 revealed differences in retinal thickness among the groups. Although the inner plexiform layer was affected predominantly, the remaining layers, including the outer nuclear layer, were also affected significantly in the diabetic rats. The overall thickness of the retina in the diabetic rats was decreased significantly when compared with that of the
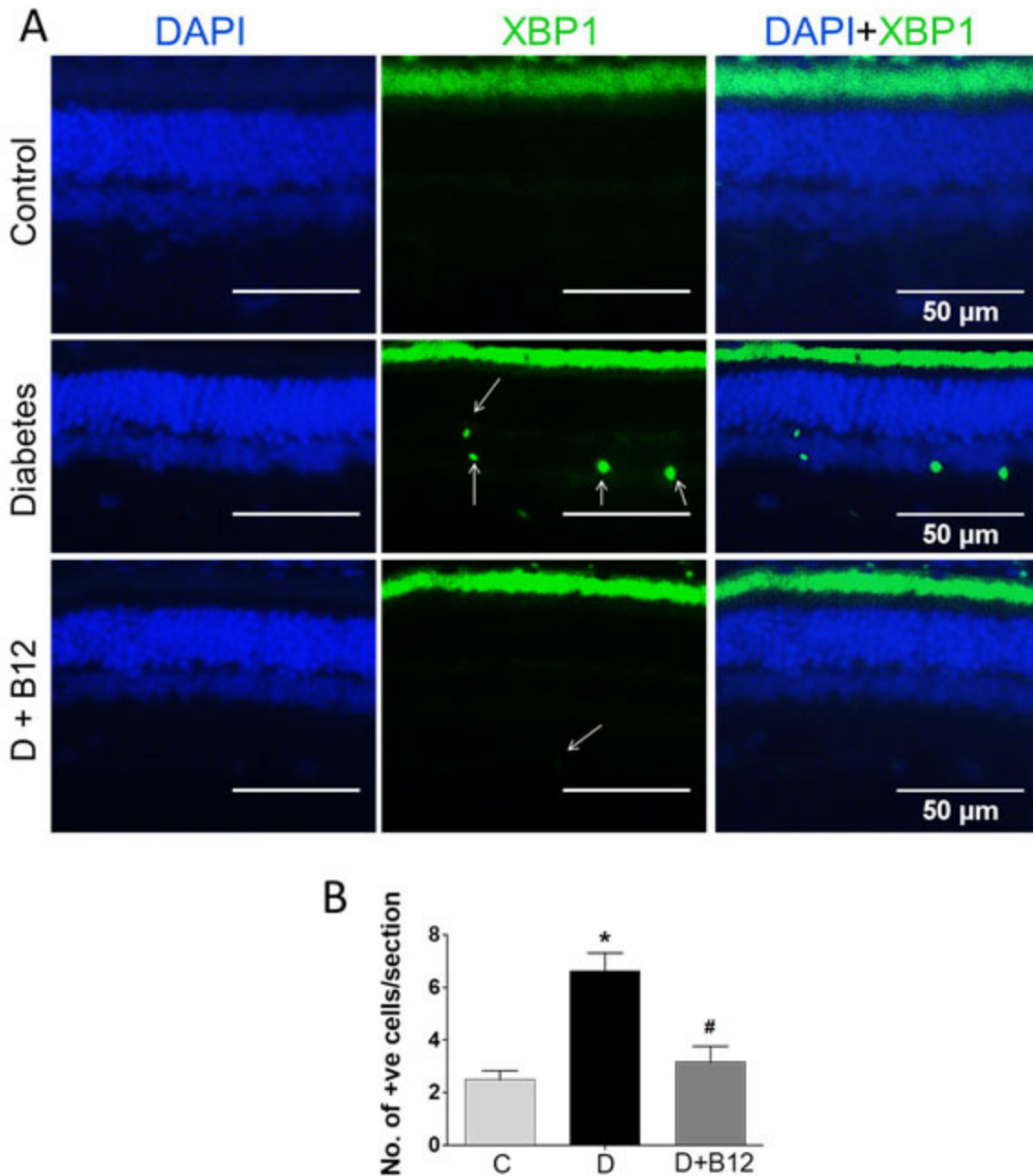
Figure 7. Immunofluorescence staining for XBP1 in the rat retina. **A**: Representative images of immunofluorescence staining for XBP1 (green, indicated by white arrows), counterstained with 4′, 6-diamidino-2-phenylin-dole (DAPI; blue) for cellular nuclei. Magnification = 400X. Scale bar = 50 µm. **B**: Quantification of XBP1 staining. Data are mean ± standard error of the mean (SEM, n=3). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B_{12}. *Significant difference from the control group; #significant difference from the diabetes group (p<0.05).

control rats. Vitamin B_{12} supplementation in the diabetic rats partially prevented retinal thinning.

Rhodopsin and GFAP: The rod cell visual photoreceptor rhodopsin (Rho) mediates the transformation of light into vision. We analyzed Rho levels in diabetic rats with and without vitamin B_{12} supplementation after 4 months and in age-matched non-diabetic control rats with immunofluorescence staining. Figure 3 shows reduced Rho staining (red) in the retinas of the diabetic rats, but vitamin B_{12} treatment considerably prevented loss of Rho in the diabetic rats. GFAP
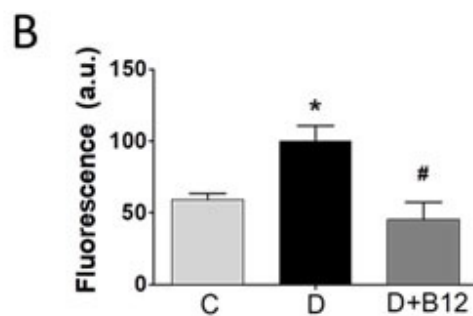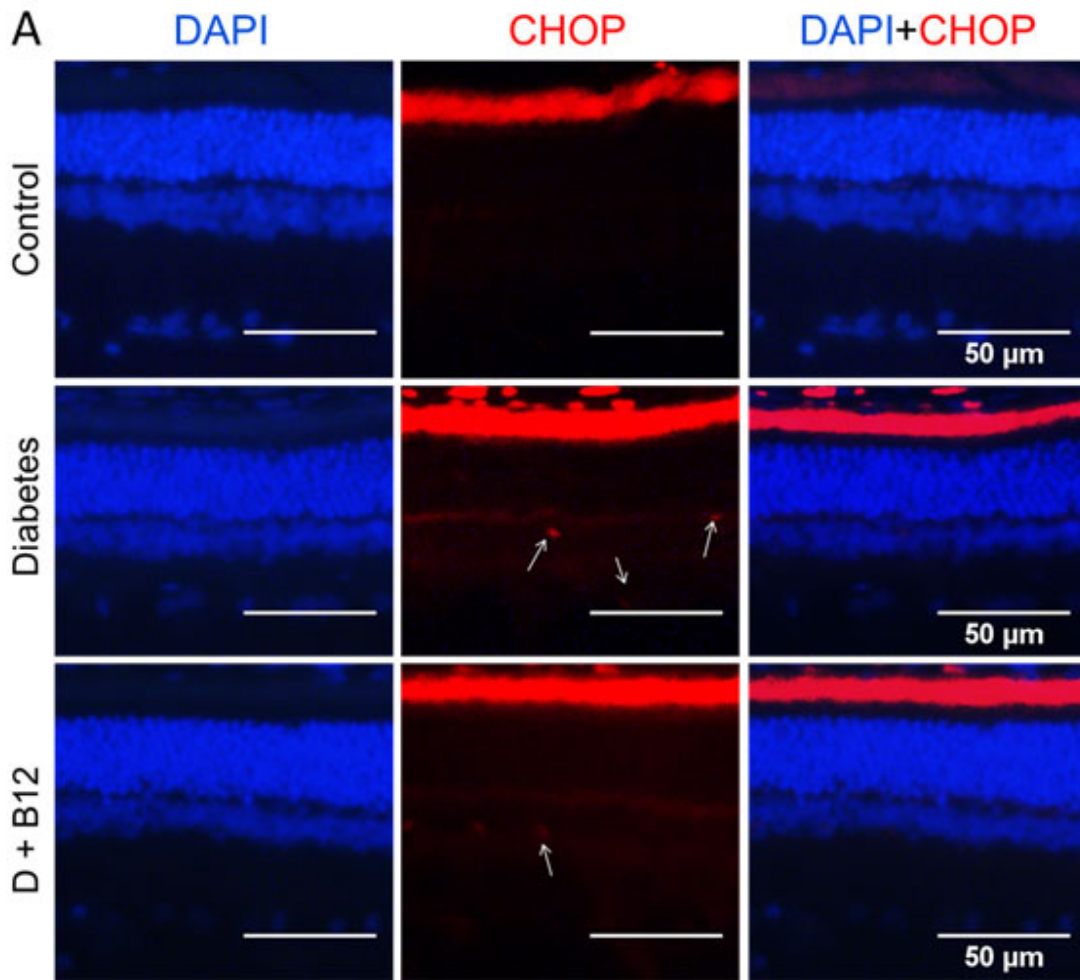
Figure 8. Immunofluorescence staining for CHOP in the rat retina. **A**: Representative images of immunofluorescence staining for CHOP (red, indicated by white arrows), counterstained with 4′, 6-diamidino-2-phenylin-dole (DAPI; blue) for cellular nuclei. Magnification = 400X. Scale bar = 50 μm. **B**: Quantification of CHOP staining. Data are mean ± standard error of the mean (SEM, n=3). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B_{12}. *Significant difference from the control group; #significant difference from the diabetes group (p<0.05).

is an intermediate filament protein present in retinal glial cells. Retinal glial cells respond to retinal injury and have been shown to be activated in diabetes. Immunofluorescence staining for GFAP on the rat retinal sections showed minimal staining (red) in the control rats, especially in the ganglionic cell layer and the nerve fiber layer (Figure 4). In the diabetic rats, the GFAP staining spanned all the retinal layers indicating activation of Müller cells or gliosis. Nevertheless, vitamin B_{12} treatment in the diabetic rats for 4 months completely prevented diabetic gliosis.

HIF1α and VEGF: HIF1α is a sub-unit of HIF1, a master regulator of the cellular response to hypoxia. VEGF is a prominent angiogenic factor that induces vascular permeability. Immunoblotting for HIF1α and VEGF displayed higher protein levels in diabetic rats indicative of hypoxia and elevated vascular permeability in the diabetic rat retina (Figure 5). Vitamin B_{12} intervention in the diabetic rats prevented overexpression of HIF1α and VEGF.

ER stress markers: As endoplasmic reticulum (ER) stress has been shown to cause DR pathology [26], we examined ER stress markers such as GRP78, ATF6, XBP1, CHOP, and caspase 12 with immunoblotting (Figure 6), and XBP1 and CHOP with immunofluorescence (Figure 7 and Figure 8). The diabetic rats showed higher levels of GRP78, ATF6, and XBP1 proteins indicative of ER stress. Immunofluorescence for XBP1 and CHOP showed more staining (green and red, respectively) in the outer nuclear and inner nuclear layers of
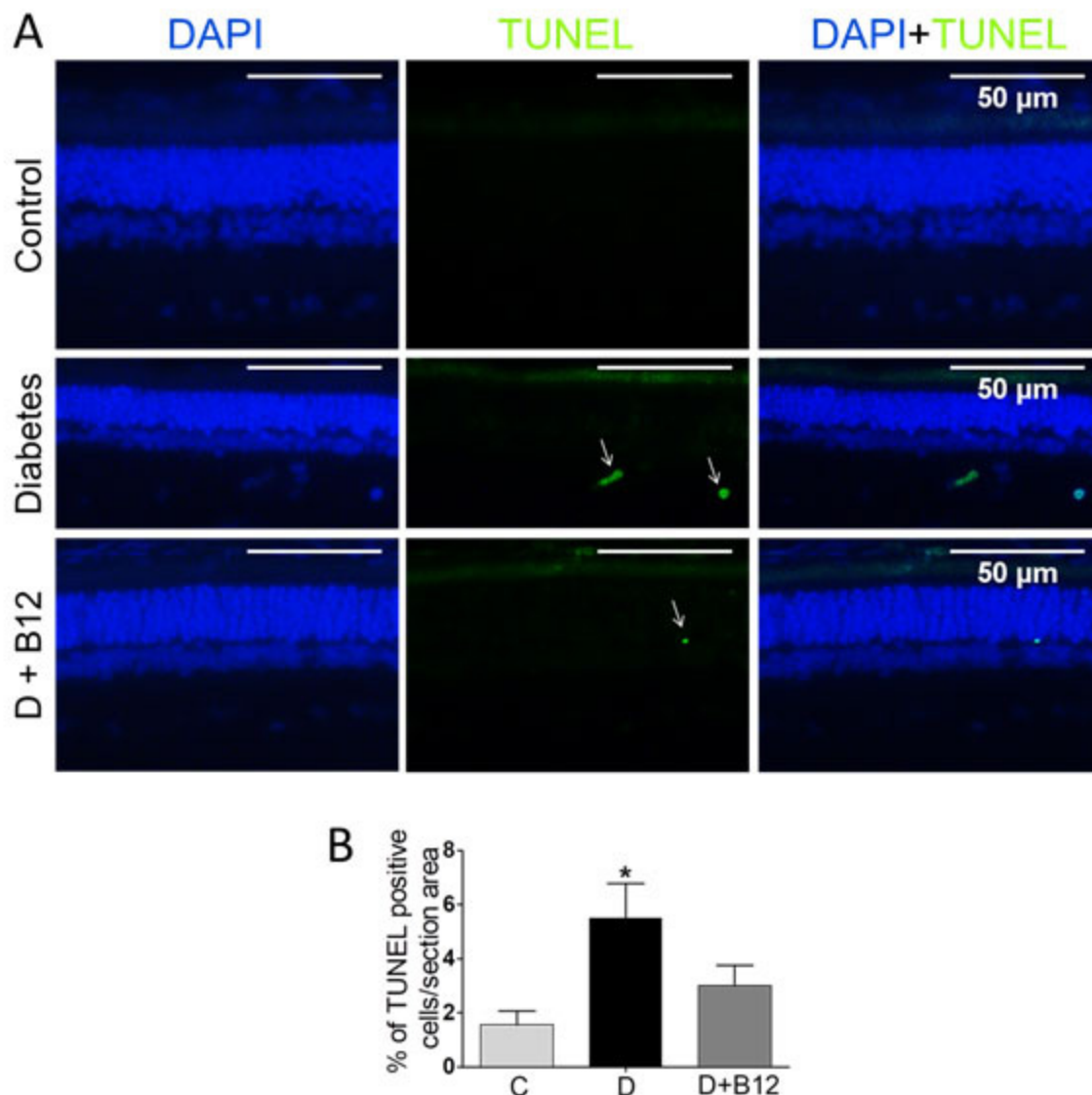
Figure 9. TUNEL labeling of rat retinal sections. **A**: Representative fluorescence microscopic images of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL; green), counterstained with 4′, 6-diamidino-2-phenylin-dole (DAPI; blue) for cellular nuclei. White arrows indicate TUNEL positive cells. Magnification = 400X. Scale bar = 50 μm. **B**: Quantification of TUNEL positive cells. Data are mean ± standard error of the mean (SEM, n=3). C, control; D, diabetes; D+B12, diabetic rats treated with vitamin B_{12}. *Significant difference from the control group ($p<0.05$).

the retina in the diabetic rats. Further, CHOP and caspase 12 overexpression is indicative of maladaptive ER stress, as they trigger apoptosis. Interestingly, vitamin B_{12} supplementation of the diabetic rats prevented overexpression of ER stress markers in the retina (Figure 6, Figure 7, and Figure 8).

Apoptosis: After observing maladaptive ER stress, we next examined the retinas for apoptosis. Immunoblotting for BAX protein showed higher levels in diabetic rats (Figure 6). Further, TUNEL assay was performed to confirm apoptosis, and the results showed increased TUNEL positive cells (green) as shown in Figure 9. Vitamin B_{12} supplementation of diabetic rats considerably prevented apoptosis in the retina.

DISCUSSION

Diabetic retinopathy is the most common microvascular complication of diabetes, and long-term complications of diabetes, including DR, represent the main cause of morbidity and mortality in diabetic patients. Nearly all individuals with type 1 diabetes and more than 60% of people with type 2 diabetes show some degree of DR after 20 years of disease. In particular, India has a high rate of diabetes and thus, DR. Established risk factors for DR include glycemic control, duration of diabetes, hypertension, and dyslipidemia [1,27]. Although strict glycemic control is expected to prevent DR, perfect glycemic control is not always possible. Multiple factors are likely to be involved in predisposing patients with diabetes to complications, as evidenced by the fact that many but not all diabetic patients develop one or more microvascular complications. In addition to genetic factors, nutritional factors (in particular, micronutrients) could play a role in the development of DR. Previously, we reported that vitamin B_{12} deficiency could be an independent risk factor for DR [11]. Therefore, we investigated the effect of vitamin B_{12} supplementation on DR using the STZ-induced diabetic rat model.

In line with previous reports, the plasma vitamin B_{12} levels in the diabetic rats showed a decreased trend with no statistical significance when compared with those of the control rats [28,29]. The diabetic rats consumed double the amount of food, and thus, double the vitamin B_{12} intake, when compared to the control rats. However, vitamin B_{12} supplementation of the diabetic rats was reflected in higher plasma levels compared to those in the control rats. In agreement with previous studies in humans and rodents, there was a decrease in the circulating homocysteine levels in the diabetic rats [21,27,30-35]. This is due to deficit levels of insulin that plays a key role in homocysteine metabolism. A diabetic state, characterized by reduced insulin levels, hyperglycemia, and elevated circulating counter-regulatory hormones, has been shown to be a condition that results in the perturbation of homocysteine and methyl group metabolism. For type 1 and type 2 diabetes without renal complications, hypohomocysteinemia has been found in humans and rats. This altered methyl metabolism drastically affects various cellular processes, including DNA methylation, an epigenetic mechanism to control gene expression that has been implicated in many pathologies. Further, previous reports showed that there exists no correlation between plasma homocysteine and retinopathy [27].

Four months of diabetes resulted in drastic retinal thinning. Interestingly, vitamin B_{12} supplementation prevented retinal thinning. Rhodopsin was decreased in the diabetic rats demonstrating disturbed retinoid metabolism and photoreceptor death. This may cause visual function defects, such as delayed dark adaptation in early DR. Chronic deficiency in visual pigment formation may contribute to photoreceptor degeneration and irreversible retinal pathologies in advanced DR. The present study results suggested that restoration of the normal retinoid level with vitamin B_{12} supplementation may represent a potential therapeutic strategy for early DR.

Retinal Müller cells are principal macroglial cells that spread a considerable width of the retina and play a crucial role in supporting the neurons and their functions. Activation of these cells, as revealed by increased GFAP expression, is an indication of human early DR pathology. In normal conditions, GFAP expression is observed only in astrocytes of the ganglion cell layer and the nerve fiber layer of the retina. GFAP levels are regulated by hormones such as insulin and glucocorticoids, cytokines, and growth factors which are greatly altered in diabetes. However, in DR due to cellular stress and injury, Müller cells start expressing GFAP, which is considered as a marker for reactive gliosis [36,37]. Glial activation may disturb the metabolism and functioning of neurons causing neurodegeneration [38]. In the present study, we observed that vitamin B_{12} supplementation in the diabetic rats prevented overexpression of GFAP in the retina. Vitamin B_{12} is also a good scavenger of reactive oxygen species, can pass through the blood–brain barrier, and is suggested to be a good neuroprotectant [39,40]. Previous studies showed antioxidant, anti-inflammatory, antiapoptotic, and antinecrotic effects of vitamin B_{12} on neurons [41,42]. Further, studies suggested that vitamin B_{12} treatment had a preventive effect on peripheral nerve lesions in experimental diabetic neuropathy [43] and a beneficiary effect in repairing the damaged nerves of rats [44].

The increased expression of HIF1α in the retina of diabetic rats is indicative of retinal hypoxia, which is another contributory element in DR. As the retina is a metabolically active tissue, it is sensitive to hypoxic conditions. HIF1α

further promotes transcription of its target gene *VEGF* (Gene ID: 83785; OMIM 192240), leading to neovascularization and vascular hyperpermeability in the retina [45-48]. Müller cells are the primary source of retinal VEGF, in healthy as well as DR conditions [49,50]. Müller cell-derived VEGF protein overexpression plays a central role in DR pathology by causing blood–retina barrier breakdown, vascular leakage, acellular capillaries, retinal neovascularization and vaso-obliteration, and reduction of preretinal neovascular endo-thelial cells. Thus, VEGF inhibitors are the most successful therapeutic strategies at present. Although we did not show the effects of VEGF in the present study rat model, we observed higher VEGF protein expression in diabetes that is partially ameliorated by vitamin B_{12} supplementation. The status of DR in the present rat model could be in between severe non-proliferative retinopathy and early proliferative retinopathy.

We and others reported ER stress-mediated cell death in diabetic rat retinas and implicated it as one of the major proteostasis factors for DR [26,51]. Studies have shown involvement of ER stress in neuronal and vascular abnor-malities, such as pericyte loss and neovascularization [52,53]. ATF4 that is upregulated during ER stress increases VEGF expression in the retina. ER stress also increases inflam-mation in the retina [51,54]. As shown in the present study, persistent ER stress induces apoptosis mediated by CHOP and caspase 12. CHOP is a key mediator of ER stress-induced cell death, and previous studies showed silencing of CHOP expression prevents ER stress-mediated cell death [55]. Caspase 12 is an ER resident caspase, activated explicitly by ER stress to facilitate apoptosis. Interestingly, vitamin B_{12} supplementation in the present study abolished adaptive (GRP78, ATF6, and XBP1) and maladaptive (CHOP and caspase 12) ER stress responses in the diabetic rat retina.

Supplementation with some antioxidants and micronutri-ents has shown encouraging results in experimental models of DR and human studies [56,57]. Interestingly, a study showed that age-related eye disease study (AREDS)-based micro-nutrients proven to be beneficial in ameliorating the lesions associated with DR in experimental rats [58]. Low levels of vitamin B_{12} have been recognized in Indians for a long time, and recent studies confirmed vitamin B_{12} deficiency and its implications in diabetes and cardiovascular diseases in India [13,15,59]. In conclusion, vitamin B_{12} supplementation of diabetic rats was shown to be beneficial by preventing retinal hypoxia, VEGF overexpression, and ER stress-mediated cell death in the retina. The present study adds another facet of vitamin B_{12} in diabetes. Considering the general prevalence of micronutrient deficiency, and its contribution to many metabolic and age-related disorders (such as diabetes) and cardiovascular diseases in India [59,60], the ameliorative effects of vitamin B_{12} on DR merit attention.

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