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Tocotrienol-rich fraction (TRF) protects against retinal cell apoptosis and preserves visual behavior in rats with streptozotocin-induced diabetic retinopathy

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Abstract

Background Tocotrienol is a vitamin E analogue that is known to exert anti-inflammatory and antioxidant effects. Hence, in the current study, the effects of TRF on the expression of pro- and anti-apoptotic proteins in the streptozotocin-induced diabetic rat retinas were investigated. The effect of TRF on the visual behaviour of rats was also studied.

Methods Diabetes was induced in rats by intraperitoneal injection of streptozotocin and was confirmed by a blood sugar level of at least 20 mmol/L, 48 h, post-injection. Diabetic rats were divided into a group treated with vehicle (DV) and the other treated with TRF (100 mg/kg; DT). A group of non-diabetic rats treated with vehicle (N) served as the control group. All treatments were administered orally for 12 weeks. Rats were then subjected to an assessment of general behaviour in an open field arena and a two-chamber mirror test to assess their visual behaviour. At the end of the experimental period, rats were sacrificed, and their retinas were isolated to measure the expression of pro- (Casp3, Bax) and anti-apoptotic (Bcl2) markers using RT-qPCR and ELISA. TUNEL staining was used to detect the apoptotic retinal cells.

Results Treatment with TRF lowered the retinal expression of Casp3 protein by 2.26-folds ($p < 0.001$) and Bax protein by 2.18-fold ($p < 0.001$) compared to vehicle-treated rats. The retinal anti-apoptotic protein Bcl2 expression was 1.87-fold higher in DT compared to DV rats ($p < 0.001$). Accordingly, the Bax/Bcl2 ratio in the TRF-treated group was significantly greater in DT compared to DV rats. Retinal *Casp3*, *Bax*, and *Bcl2* gene expression, as determined by RT-qPCR, also showed changes corresponding to protein expression. In the open field test, DV rats showed greater anxiety-related behaviour than group N, while the behaviour of DT rats was similar to the N group of rats. DT rats and group N rats preferred the inverse mirror chamber over the mirror-containing chamber in the two-mirror chamber test ($p < 0.01$).

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Conclusion Oral TRF therapy for 12 weeks lowers retinal cell apoptosis by decreasing pro- and increasing anti-apoptotic markers. The preservation of visual behaviour in a two-chamber mirror test supported these retinal molecular alterations in diabetic rats.

Keywords Tocotrienol-rich fraction (TRF), Apoptosis, Diabetic retinopathy, Visual behaviour

Introduction

Diabetic retinopathy, the most common complication of diabetes is known to affect one in three people with diabetes. After 20 years of diabetes, nearly all people with type 1 diabetes and more than half of those with type 2 diabetes are likely to develop retinopathy [1]. Diabetic retinopathy is recognised as one of the leading causes of blindness in the working-age population [2, 3]. It leads to a reduced quality of life, poor mental well-being, and a greater likelihood of additional diabetic complications and mortality. Diabetic retinopathy is strongly associated with deteriorating glycaemic control, hypertension and dyslipidaemia [1–7].

The development of diabetic retinopathy as a microvascular complication has been conceptualized slowly over a long period [8]. It is clinically classified according to structural alterations in the retinal microvasculature since these are visible during fundoscopic examinations [9, 10]. The pathological modifications in the neuroretina were discovered after the detection of degenerating neurons in diabetic patients, post-mortem [11, 12]. Since then, the presence of a variety of apoptotic cells, including vascular endothelial cells, pericytes and neuronal cells, has consistently been observed in the diabetic retinas. One possible explanation for the emergence of acellular capillaries in diabetic retinas is the apoptotic loss of vascular endothelial cells. It contributes to localized capillary nonperfusion and retinal ischemia in diabetic retinas [13, 14]. Apoptosis also accounts for the appearance of ghost pericytes or complete loss of pericytes, a commonly observed finding in diabetic retinas [15]. Importantly, apoptotic loss of neurons, particularly in the inner retina, has been observed to occur at almost a constant rate throughout the progression of diabetes, and in fact, neuronal apoptosis sets in earlier than the apoptosis of vascular elements [16, 17].

In diabetic retinas, apoptosis of various cell types has been observed to occur in various time frames, and a different mechanism may be responsible for the apoptosis of various cell types [18]. In fact, the pathogenetic mechanisms underlying diabetic retinopathy are too many, such as oxidative stress, excitotoxicity, lack of trophic support and inflammation [19]. However, most culminate in cellular apoptosis. Therefore, it would be a reasonable argument that targeting apoptosis, the final end-point where several mechanisms converge may be considered a logical approach for preventing retinal cell apoptosis and preserving vision in diabetic retinopathy. From this standpoint, a substance that acts via several mechanisms that culminate into protection against cellular apoptosis in the retina could be potentially useful in diabetic retinopathy [8, 20–24].

Tocotrienols, a type of vitamin E analogue, are widely known to exert potent anti-inflammatory and antioxidant effects. The oral administration of tocotrienol was shown to prevent oxidative and nitrosative stress in rats with cognitive impairment induced by intracerebroventricular streptozotocin [25]. In diabetic rats treatment with palm-derived TRF was shown to preserve retinal vessel diameter [26]. In rats postnatally exposed to ethanol, treatment with tocotrienol was shown to suppress expression of inflammatory mediators such as TNF-α, IL-1β, and TGF-β1 in the brain tissue [27]. The anti-inflammatory effect of tocotrienol was shown to result from suppression of nuclear factor kappa B activation in various experimental settings [28–30]. Interestingly, Gamma tocotrienol protected radiation exposed intestinal tissue against cellular apoptosis by upregulating the anti and downregulating the pro-apoptotic factors [31]. In fact tocotrienol rich fraction (TRF) protected glutamate exposed astrocytes by reducing oxidative stress [32]. Moreover, it has been demonstrated that orally administered TRF from palm oil, protects against diabetes-induced changes in retinal morphology and retinal cell loss in rats [33]. However, the effects of TRF on the expression of anti- and pro-apoptotic proteins in diabetic retinas remain unknown. Moreover, it is of interest to know if such changes may translate into desirable functional outcomes. In our previous studies, it was observed that the oral administration of TRF reduces retinal oxidative stress and activation caspase-3 (Casp3) protein resulting in reduced retinal cell apoptosis [34]. The results of this study raised further questions because it remained unclear if the anti-apoptotic effect of TRF is also associated with altered expression of pro- and anti-apoptotic markers Bax and Bcl2, respectively. Notably, Bcl2 interacts with the Bax at an upstream checkpoint to cause Casp3 activation to regulate apoptosis and change in their expression indicates mitochondrial involvement in the activation of apoptotic pathways [35]. Furthermore, it remained unclear if the anti-apoptotic effect of TRF is the consequence of protein level interactions or its effects are in fact at the level of gene expression. Hence, the present study investigated the effects of TRF on the expression of pro- and anti-apoptotic proteins in streptozotocin-induced diabetic rat retinas both at the genes and protein levels. We also

investigated the effect of TRF on the general behaviour of diabetic animals in open field and visual behaviour using a two-chamber mirror set up. In view of the differences observed in visual behaviour of animals in the current study compared to those observed in modified open field mirror test [34], the insight into the importance of experimental set up and interpretation of the animal behaviour is of considerable importance.

Materials and methods

Male Sprague Dawley rats weighing 200–250 g and aged 8–12 weeks were purchased from Takrif Bestari Sdn. Bhd, MY. The animal handling and all experiments were carried out in compliance with the ARVO statement for the use of animals in ophthalmic and vision research [36] and animal ethics guidelines of International Medical University and Universiti Teknologi MARA. All procedures in the conduct and reporting of this experiment complied with ARRIVE guidelines. Ethical approval was obtained from International Medical University (BMSI/2020 [5]) and Universiti Teknologi MARA (UiTM care 328/2020) ethical committees. Each rat was individually housed in a polypropylene cage with a stainless-steel top grill in a temperature-controlled room (25±2 °C). The animals were acclimatized to a 12-hour light/dark cycle. Free access to food pellets and reverse osmosis water was provided throughout the experimental period. Animals with no abnormalities on systemic and ophthalmic examination were recruited into the study.

Study design

Intraperitoneal injection (IP) of streptozotocin was used to induce diabetes in rats and was confirmed by a blood sugar level of at least 20 mmol/L, 48 h, post-injection. Subsequently, diabetic rats were divided into a group treated with vehicle (DV) and the other treated with TRF (100 mg/kg; DT). A group of non-diabetic rats that received IP citrate buffer was treated with vehicle (N) and served as the control group. Each group consisted of 18 rats (36 retinas). All treatments were given once daily by oral gavage, starting 48 h post-streptozotocin injection for a duration of 12 weeks. The TRF was formulated in olive oil and 100 mg/kg body weight was administered to rats in group DT. Empty olive oil formulation without TRF was similarly given to groups N and DV. All rats were subjected to measurement of body weight and blood glucose levels once a week during the course of the treatment. The blood samples for measurement of blood glucose were collected by pricking the tail vein using a 26G needle.

Following 12 weeks of treatment, the animals were subjected to behavioural assessment and visual function tests on two consecutive days. Subsequently, the rats were sacrificed using IP pentobarbital sodium (0.14 mg/kg body weight) [37]. Once the process of sacrifice was confirmed to be completed, eyeballs were enucleated. After enucleation, the anterior segment and vitreous were meticulously removed. The retinas were isolated, washed, weighed, and stored at -20 °C. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression of *Casp3*, *Bax* and *Bcl2* genes ($n=6$, each sample was a pool of two retinas of the same animal). The expression of Casp3, Bax and Bcl2 proteins was determined using ELISA ($n=6$ retinas from 6 different animals for each marker) using commercially available ELISA kits. To extent of retinal cell apoptosis was assessed by subjecting the retinas to TUNEL immunostaining ($n=6$ from 6 different animals).

Induction of diabetes

For induction of diabetes, fasting rats were administered with a single IP streptozotocin (55 mg/kg body weight; Santa Cruz Biotechnology Inc., Texas, US) [38] dissolved in ice-cold sodium citrate buffer (10 mmol/L, pH 4.5). The blood sugar levels in tail vein samples were measured 48 h after streptozotocin injections using an Accu Chek Performa glucometer (Roche Diagnostic, Basel, CH). Rats were considered to have diabetes if their blood glucose level was 20 mmol/L or higher. A single IP sodium citrate buffer was similarly administered to rats in the non-diabetic control group.

Formulation of TRF

Palm oil-derived TRF (12.3% α-tocopherol, 13.1% α-tocotrienol, 2.1% β-tocotrienol, 19.4% γ-tocotrienol, 5.8% δ-tocotrienol) was obtained from Excel Vite Sdn Bhd, Perak, MY. For oral administration, it was mixed with olive oil at a ratio of 7:1. Empty olive oil formulation without TRF was used as the vehicle for both the non-diabetic (N) and diabetic untreated groups (DV).

Assessment of the general behaviour of rats in the open field test

An open field arena was used to observe the general locomotor activity, exploratory behaviour, and anxiety-related behaviour of rats as described previously [39–41]. The test arena, which was divided into central and peripheral zones was made up of white acrylic and measured 100 cm (L) x 100 cm (W) with walls of 25 cm height (Fig. 1). The experimental set-up was illuminated with a 100 W tungsten lamp and was carried out in a soundproof room. The rats were allowed ten minutes to explore after initially placing them in the central zone. All experimental sessions were recorded by a digital video camera positioned 2.5 m above the central zone of the arena (Fig. 1A). The following parameters were observed for each animal: total distance travelled, distance travelled by the head, number of line crossings, total rearing episodes (stands

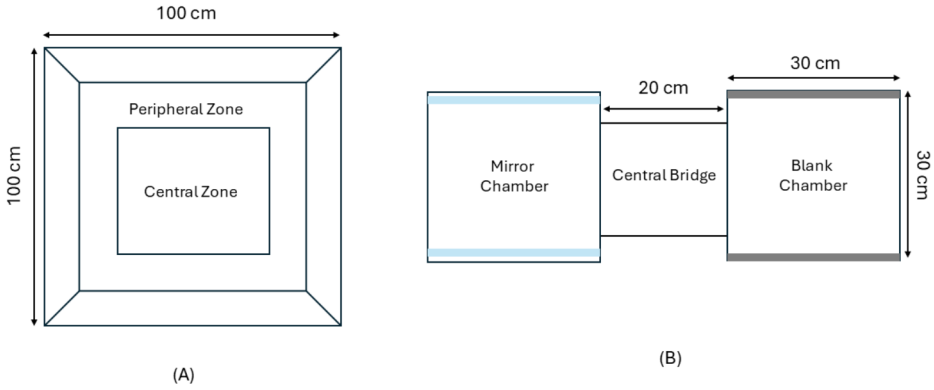

Fig. 1 **A**: Diagrammatic representation of open field arena to assess the behaviour of animals. **B**: Diagrammatic representation of experimental set-up for two-chamber mirror test used to assess the visual behaviour of animals

on hind legs), total time immobile (animal remains in the same place), number of freezing episodes (animal is completely motionless apart from breathing), number of faecal pellets excreted, number of self-groom episodes (animal licks and scratches itself). The test arena was wet-mopped with 70% alcohol between test sessions to prevent any transmission of olfactory cues. Any-maze version 4.0 was used to analyze the recordings.

Two-chamber mirror test

The experimental set-up for the two-chamber mirror test consisted of two identical chambers connected by a central bridge with measurements as described by Yih et al [42]. Within one of the chambers, two mirror panels were inserted along the chamber walls facing each other. The other chamber was inserted with a similar mirror with a non-reflecting surface and was referred to as the inverse mirror chamber (Fig. 1B). The equipment was put in a soundproof room and illuminated by a 100 W tungsten lamp. All experimental sessions were recorded by a digital video camera positioned 2.5 m above the central zone of the arena. The test was carried out in two trials. For the first trial, rats were first placed into the central bridge and then allowed 5 min to explore the apparatus freely. Rats were allowed to rest for 5 min after removing them from the apparatus. The apparatus was then rotated 180 degrees to swap the positions of the two chambers, and the rats were subjected to a 2nd trial like the first one. This helped to eliminate the influence of learning and memory, if any, on the performance of the rats. During the test, we recorded the time spent by animals in the mirror chamber as well as the inverse mirror chamber by each rat. To stop the transmission of any olfactory cues between test sessions, the test arena was wet-mopped with 70% alcohol. The recordings were analysed using ANY-maze software version 4.0.

Retinal expression of Casp3, Bax and Bcl2: RT-qPCR

RNA extraction from retinal samples was done using an RNA purification kit according to the manufacturer's instructions (Macherey Nagel, DE). RNA was quantified using a NanoDropTM Spectrophotometer (ND-1000 Thermo Fisher Scientific, US). Subsequently, one Script Hot cDNA Synthesis Kit (Applied Biological Materials, CA) was used to synthesize cDNA. The conditions for RT-qPCR included denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, and extension at 72 °C for 30 s. Forty cycles were performed to detect genes in cDNA.The amplification was carried out in a Biorad iCycler Machine (Bio-rad Laboratories, US) using 4X CAPITALTM Green Master Mix (Biotechrabbit, DE). The RT-qPCR experiment was carried out in accordance with MIQE recommendations [43]. Six biological and three technical replicates were used for all RT-qPCR reactions. For each reference gene and gene of interest, the RT-qPCR efficiencies were calculated. Using the ΔΔCT method, the relative fold expression of the genes of interest was calculated. The data were normalized to the reference genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and beta-actin *(Actb)*. The primer specificity was authenticated using the Nucleotide Basic Local Alignment Search Tool, which was provided by Sigma-Aldrich (BLAST) [44] (Table 1).

Retinal expression of Casp3, Bax and Bcl2: ELISA

The retinal samples were washed with ice-cold PBS (0.01 M, pH 7.4) and homogenised in PBS at a ratio of 1 g of retinal weight to 9 mL of PBS. Following homogenization, the centrifugation was done at 5000 x *g* for 5 min at 4 °C. The supernatant was subjected to assay using ELISA kits which were commercially available (Elabscience Biotechnology Inc., Texas, US) as directed by the manufacturer. Accordingly, standard or samples, in a volume of 100 µL, were pipetted in the designated wells. Each well was added with 100 µL of biotinylated

Table 1 Primers used to determine the expression of retinal *Bax*, *Bcl2* and *Casp3* genes

Gene	Sequence	NCBI Reference
Gapdh	F: CTAATGACCACAGTCCATTC	NM_008085
	R: GATGGGATGATGTTTTGGTG	
Actb	F: GATGTATGAAGGCTTTGGTC	NM_007393
	R: TGTGCACTTTTATTGGTCTC	
Bax	F: CCTTTTTGCTACAGGGTTTC	NM_007527
	R: ATATTGCTGTCCACTTCATC	
Bcl2	F: ATGATCGAGTACCTGAACC	NM_009741
	R: ATATAGTTCCACAAAGGCATC	
Casp3	F: CATAAGAGCACTGGAATGTC	NM_009810
	R: GCTCCTTTTGCTATGATCTTC	

detection secondary antibody immediately after incubation at 37 °C for 90 min. Plates were again incubated at 37 °C for an hour. After aspirating the solution, the wells were washed three times with 350 µL of wash buffer for 1–2 min and patted dry. After that, 100 µL of the horseradish peroxidase (HRP) conjugate was pipetted into each well and incubation was done for another 30 min at 37 °C. Tetramethylbenzidine (TMB) substrate was then added to the wells after five rounds of washing. The reactions were stopped by adding stop solution after an additional 15 min of incubation at 37 °C, and the optical density (OD) was measured at 450 nm using a microplate reader. (Victor X5™, Perkin Elmer, Waltham, MA, US).

TUNEL staining of retinal tissue

The detection of apoptotic cells in retinal tissue was carried out using the Terminal Transferase-Mediated dUTP Nick End-Labelling (TUNEL) Assay Kit (Merck Millipore, Beijing, CN). After deparaffinization, 3 µM retinal sections were subjected to antigen retrieval by incubation with proteinase K (1:100 in 10 mM Tris, pH 8, 37^{0}C) for 20 min. Subsequently, the sections were washed with tris-buffered saline (TBS) followed by incubation in Terminal Deoxynucleotidyl Transferase (TdT) equilibration buffer (200 mM potassium cacodylate pH 6.6, 25 mM Tris-HCl pH 6.6, 0.2 mM dithiothreitol, 0.25 mg/mL bovine serum albumin (BSA), 2.5 mM cobalt chloride) at room temperature for 20 min. The sections were now washed with TBS and treated with DNase I (1 g/L). This was followed by overnight incubation at room temperature with a labelling reaction mixture containing fluorescein and TdT enzyme and mounting with Fluorescence-FragELTM mounting fluid.

Sections were viewed at a 20X magnification using a fluorescent microscope (BX5 Fluorescence Trinocular Microscope, Olympus, Tokyo, JP). The retinal cell population was viewed using a DAPI filter set at 330 nm, and labelled nuclei were observed using a traditional fluorescence filter set at 465 nm. TUNEL-positive cells were counted using Image J software (version 1.31, National Institutes of Health, Bethesda, MD, US) in six randomly selected fields of view within the GCL in each section. Apoptotic cell count was expressed as cells per mm^2 of GCL.

Statistical analysis

GraphPad Prism 8.0 software was used for the data analysis. A one-way analysis of variance (ANOVA) and the post hoc Tukey test were used for statistical comparison among groups. For comparisons within groups in the visual chamber test, paired t-test was used. Statistical significance was assumed at a confidence level of at least 95% ($p<0.05$).

Results

Effect of TRF on body weight and blood glucose level

The DV group rats showed significantly less weight gain than the N group of rats over 12 weeks of the treatment period. TRF-treated diabetic rats also showed significantly lower weight gain from week 8–12 post-treatment compared to the N group of rats. Throughout the course of the experiment, the blood glucose levels in both diabetic groups remained significantly higher than those in the N group of rats ($p<0.0001$) (Fig. 2).

Effect of TRF on the retinal expression of pro-and anti-apoptotic markers: RT-qPCR

The diabetic vehicle-treated rats showed a 4.69-fold higher retinal *Casp3* expression than the normal control rats ($p<0.05$). Retinal *Casp3* expression was, however, 4.77-fold lower in diabetic rats treated with TRF ($p<0.05$). Similarly, retinal *Bax* expression was 4.18-fold higher in vehicle-treated diabetic rats compared to the N group of rats ($p<0.05$), but the same was 3.73-folds lower in TRF-treated diabetic rats compared to vehicle-treated diabetic rats ($p<0.05$). Compared to normal control rats, the anti-apoptotic *Bcl2* gene expression in the vehicle-treated diabetic rat retinas was 3.15-fold higher ($p<0.05$). Following treatment with TRF, diabetic rats showed 1.62-fold greater retinal expression of *Bcl2* compared to the N group of rats (Fig. 3).

Effect of TRF on the retinal expression of pro-and anti-apoptotic markers: ELISA

The retinal Casp3 protein expression in DV was significantly higher than in group N (2.25-fold, $p<0.001$) but it was significantly lower in DT compared to group DV (2.26-fold, $p<0.001$) (Fig. 4A). When compared to the N group, the retinal Bax expression in the group DV was 2.43-fold higher ($p<0.001$). The same in the group DT was, however, 2.18-fold lower than group DV ($p<0.001$) (Fig. 4B). Rats in group DV had 2.72 times lower retinal Bcl2 expression compared to group N ($p<0.001$), whereas in group DT it was 1.87 times higher than that in

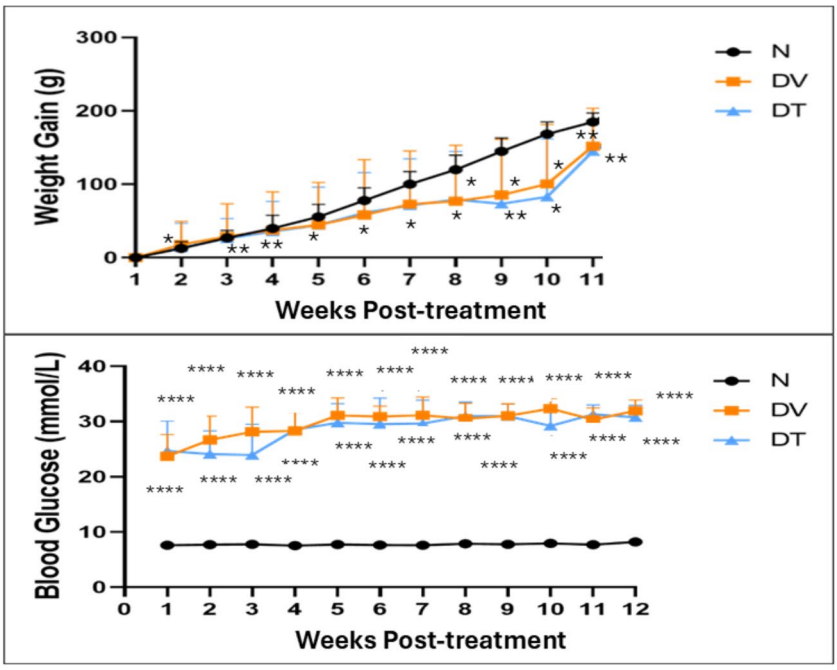

Fig. 2 **A**. Effect of TRF on body weight of rats with streptozotocin-induced diabetes following 12 weeks of treatment **B**. Effect of TRF on blood glucose in streptozotocin-induced diabetic rats over a period of 12 weeks. Data points represent mean ± SD, $n = 6$. *$p < 0.05$ vs. N, **$p < 0.01$ vs. N; ****$p < 0.001$ vs. N. ANOVA and the post hoc Tukey test were used for statistical comparison among groups. N: Rats that received the vehicle, DV: Diabetic rats that received the vehicle, DT: TRF treated diabetic rats

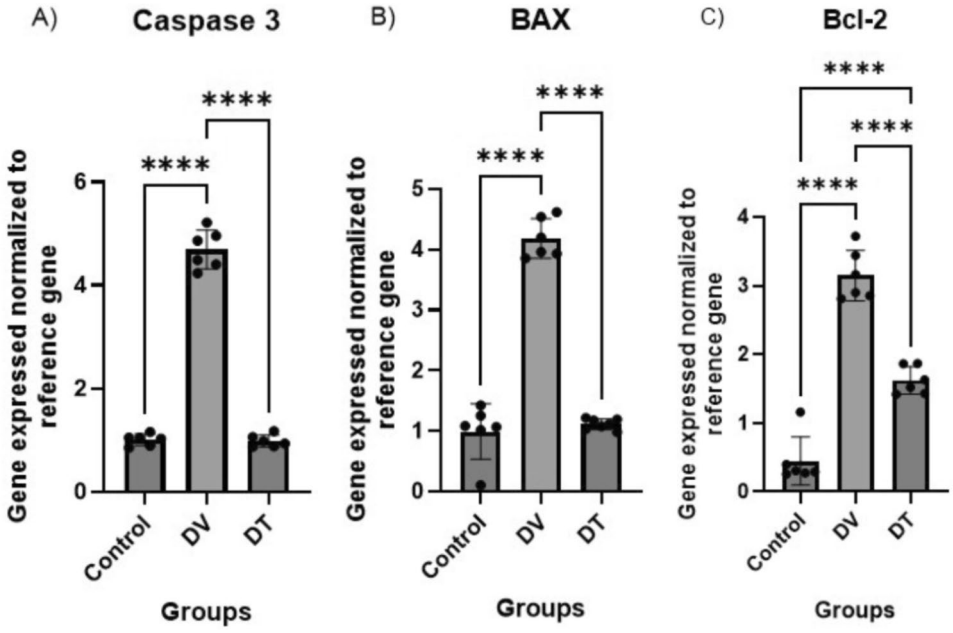

Fig. 3 Effect of TRF on the retinal expression of pro-and anti-apoptotic genes in rats with diabetic retinopathy. **A**. *Casp3*; **B**. *Bax*; **C**. *Bcl2*. Bars represent mean ± SD. $n = 6$. *$p < 0.05$ versus N; # $p < 0.05$ versus DV. ANOVA and the post hoc Tukey test were used for statistical comparison among groups. N: Control rats treated with vehicle, DV: Diabetic rats treated with vehicle, DT: Diabetic rats treated with TRF

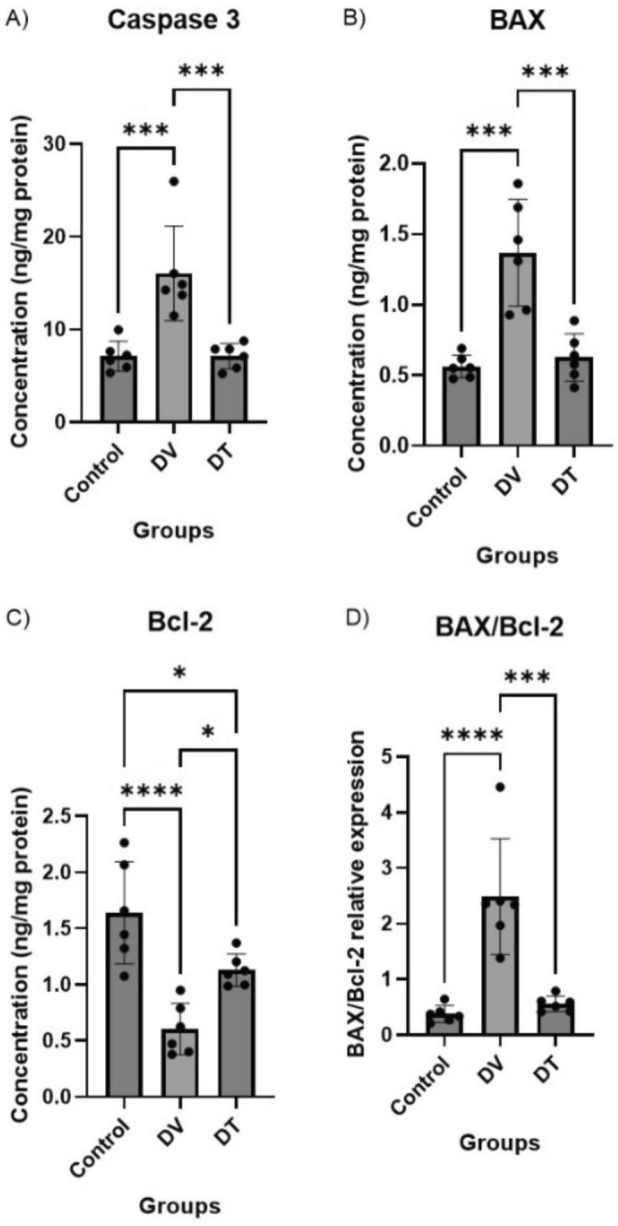

Fig. 4 Effect of TRF on the retinal expression of pro- and anti-apoptotic proteins in diabetic rat retinas. **A**. Casp3; **B**. Bax; **C**. Bcl2; **D**. Bax: Bcl2 ratio. Bars represent mean ± SD. $n = 6$., $^{**}p < 0.01$ versus N; $\#\#p < 0.01$ versus DV. ANOVA and the post hoc Tukey test were used for statistical comparison among groups. N: Rats that received the vehicle, DV: Diabetic rats that received the vehicle, DT: TRF treated diabetic rats

group DV ($p<0.001$) (Fig. 4C). The ratio of Bax/Bcl2 protein expression was therefore significantly higher in the DV group than in group N ($p<0.001$). As compared to the group DV, the same in the DT group was significantly lower ($p<0.001$) (Fig. 4D).

Effect of TRF on diabetes-induced retinal cell apoptosis: TUNEL

The degree of retinal cell apoptosis in the GCL was assessed using TUNEL labelling of the retinal sections (Fig. 5A). Group DV had a significantly higher number of TUNEL-positive retinal cell counts than group N (1.67-fold, $p<0.001$). In group DT, there were significantly fewer apoptotic cells in the ganglion cell layer than the group DV (1.55-fold, $p<0.001$). The number of apoptotic cells in group DT was comparable to that in group N (Fig. 5B).

Effect of TRF on the general behaviour of rats in the open field test

The open-field arena was used to assess general movement, exploratory behaviour, and anxiety-related behaviour of animals (Fig. 6I). The total distance travelled by rats from group DV was significantly less than that of the rats in group N ($p<0.05$). Although DT rats appeared to travel a longer distance than the group DV, the difference was insignificant. Additionally, the total distance travelled by rats from groups DT and N was comparable (Fig. 6A).

The distance travelled by head and number of line crossings were found to be the lowest in the group DV. In comparison to the DV rats, rats from group N showed greater distance travelled by ($p<0.001$). The number of line crossings also showed a similar trend, with a significantly greater number of crossings by group N than DV ($p<0.0001$). The distance travelled by the head for group DT was significantly greater than that in group DV ($p<0.05$) but these groups did not differ for the number of line crossings ($p>0.05$). There were no notable differences ($p>0.05$) among groups N, DV and DT for the number of rearing episodes (Fig. 6B, C, D).

The rats in group DV displayed the highest levels of anxiety-related behaviour, as shown by the highest total time immobile, the number of faecal pellets excreted and the number of freezing episodes. The total time immobile and the number of faecal pellets excreted by DV rats were significantly higher when compared to group N ($p<0.001$ and <0.01, respectively), and the same was observed for the number of freezing episodes ($p<0.001$). Notably, there were no statistically significant differences for the same parameters between groups N and DT. We did not observe significant differences in grooming episodes among the three groups of rats (Fig. 6E, F, G, H).

Effect of TRF on the visual behaviour of rats in the two-chamber mirror test

In the two-chamber mirror test, the time spent by the rats in group DV in the mirror and inverse mirror chambers was comparable ($p>0.05$). However, both the N and DT groups of rats displayed a preference for the inverse

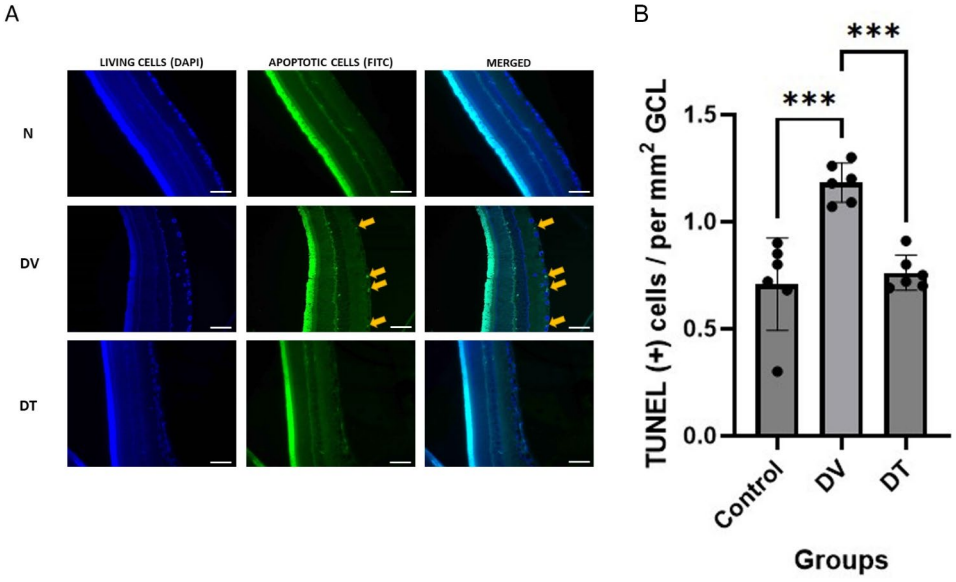

Fig. 5 **A**. Microphotographs of retinal sections showing the effect of TRF on diabetes-induced retinal cell apoptosis as detected using TUNEL staining (20× magnification). Scale bar represents 100 µm. Yellow arrow: TUNEL positive cells; Left panel: DAPI; Middle panel: FITC; Right panel: Merged (DAPI and FITC). **B**. Quantitative estimation of the retinal cell apoptosis in the ganglion cell layer showing the effect of TRF in diabetic rats. Bars represent mean ± SD. $n = 6$, ***$p < 0.001$. ANOVA and the post hoc Tukey test were used for statistical comparison among groups. N: Rats that received the vehicle, DV: Diabetic rats that received the vehicle, DT: TRF treated diabetic rats

mirror chamber and spent significantly greater time in the inverse mirror chamber than in the mirror chamber. (Fig. 7).

Discussion

Our study observed the effect of TRF on the expression of pro- and anti-apoptotic markers in the retina and the behaviour of rats with diabetic retinopathy for the first time. The neuroprotective effect of tocotrienols has been reported earlier [45]. The cytotoxic effect of H_2O_2 on rat striatal neurons was attenuated by tocotrienols [46]. In has also been observed that at nanomolar concentration, tocotrienol protects neurons via an antioxidant-independent mechanism [47]. It also protects against chronic cerebral hypoperfusion-induced neurodegeneration in rats [48]. Since, hypoperfusion is one of the prominent pathologies associated with diabetes related neuronal loss, it could be hypothesized that TRF is likely to prevent retinal neuron apoptosis in diabetic retinopathy as [49].

In the current study, TRF was administered orally and its bioavailability in retina could not be speculated due to lack of relevant studies. However, the retina is an extension of the central nervous system (CNS) and is protected by retinal blood barrier akin to blood brain barrier. Therefore, it will be logical to draw a parallel from the data on the bioavailability of oral TRF in the brain. In a previous study following chronic oral administration of TRF containing 28.5% γ-tocotrienol, 22.9% δ-tocotrienol, 22.1% α-tocotrienol, and 20.0% α-tocopherol, α-tocotrienol was found to be the primary isomer that increased in the brain despite constituting only a third of the total TRF composition [47]. This preferential permeation of α-tocotrienol through blood brain barrier may be due to its unsaturated side chain, which allows flexibility and better penetration [48]. In line with these observations, various in vivo studies have shown the neuroprotective effects of TRF in brain pathologies including age-related deficits in spatial learning and memory, Alzheimer disease and vascular dementia [49–51].

Current study showed that in diabetic rats, TRF treatment significantly decreased the expression of the pro-apoptotic markers, Casp3 and Bax, while increasing that of the anti-apoptotic marker Bcl2. We observed that this effect of TRF was evident both at the gene and protein levels. Importantly, these effects of TRF correlated with the preservation of visual functions in diabetic rats. Notably, these protective effects of TRF against diabetes-induced retinal cell apoptosis were evident despite continued hyperglycaemia.

It is well known that diabetic retinopathy causes selective loss of vascular endothelial cells, pericytes, glial and Muller cells, as well as ganglion cells. In fact, this loss occurs much earlier than the typical morphological changes in the retina or clinically noticeable vision loss [13, 52, 53]. Therefore, apoptosis, a damaging irreversible cellular event, set in during the early clinically silent phase of the disease. The detectable clinical features as a consequence of structural changes are evident only at a late phase in the progression of the disease [54]. Hence,

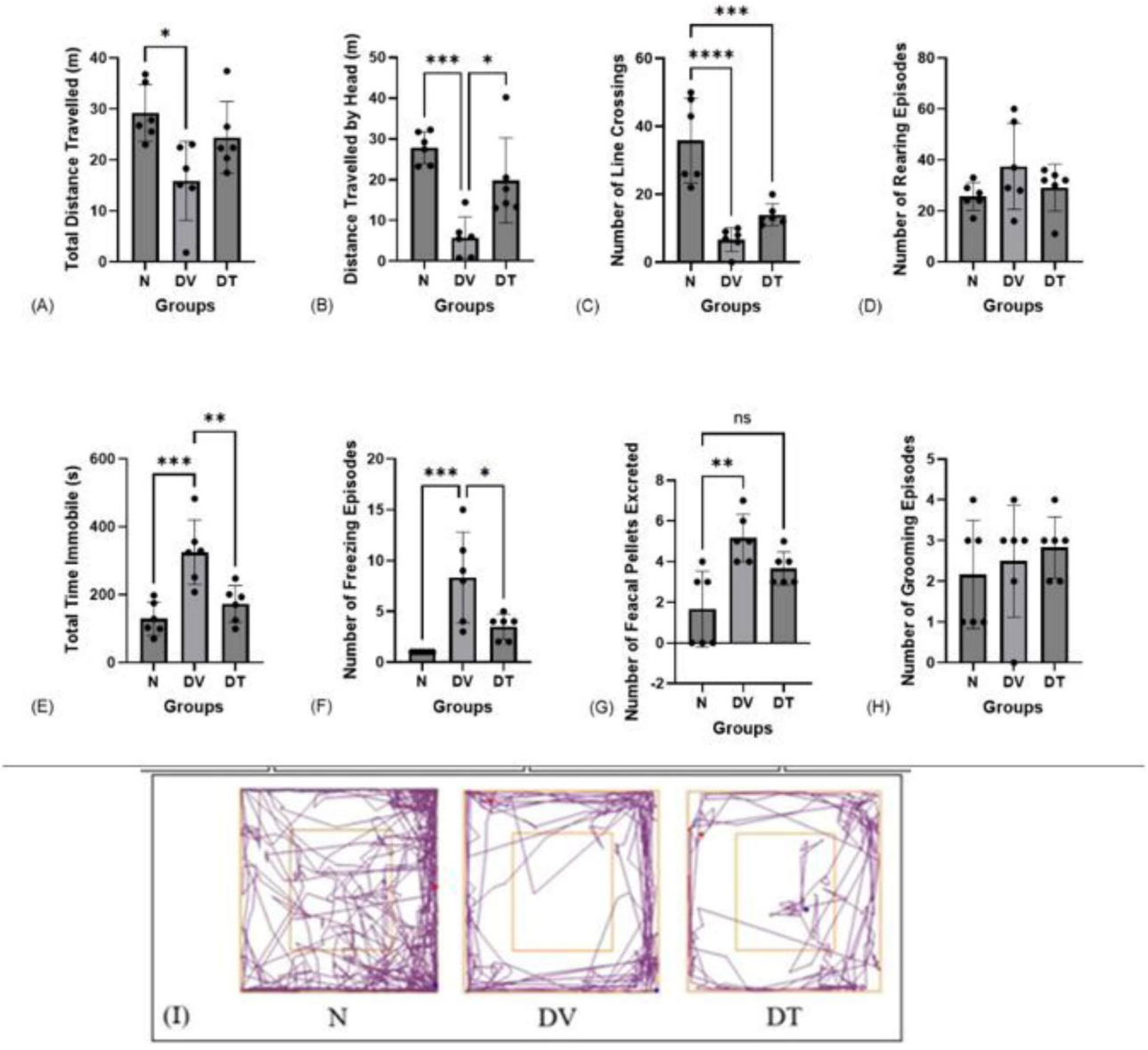

Fig. 6 The effect of TRF on general locomotor activity, exploratory behaviour and anxiety-related behaviour of diabetic rats in the open field arena. Bars represent mean ± SEM (**A**) Total distance travelled; (**B**) Distance travelled by the head; (**C**) Number of line crossings; (**D**) Number of rearing episodes; (**E**) Total time immobile; (**F**) Number of freezing episodes; (**G**) Number of faecal pellets excreted; (**H**) Number of grooming episodes; (**I**) Track plots., $n = 6$, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. ANOVA and the post hoc Tukey test were used for statistical comparison among groups.N: Rats that received the vehicle, DV: Diabetic rats that received the vehicle, DT: TRF treated diabetic rats

investigations into therapies that can halt the apoptotic process early in the disease are clearly of great significance.

Apoptosis refers to programmed cell death that results from a series of biochemical events and is characterized by specific morphological changes in the affected cells. It is mediated either directly via extrinsic or non-mitochondrial pathways as well as via intrinsic pathways involving mitochondria. In one of the studies, microarray analysis showed upregulation of several pro-apoptotic genes, particularly those related to tumour necrosis alpha (TNF-a) ligand and receptor, *Casp3* and *Bcl2* family [53], indicating that the cell apoptosis in diabetic retinas takes place via both non-mitochondrial and mitochondrial pathways. In fact, TNF-a induces apoptosis via both mitochondrial and non-mitochondrial pathways. It binds with its cell membrane receptors that activate Fas/FasL pathway. Binding of Fas with its ligand FasL activates zymogen forms of caspases (Casp), particularly Casp10, a cysteine protease. This in turn activates downstream caspases and then effector Casp3, which is involved in carrying out the last steps of apoptosis. TNF-a may also directly

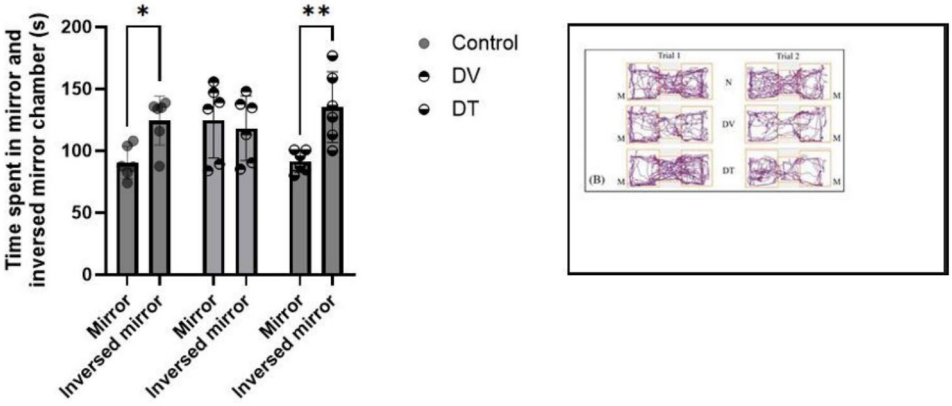

Fig. 7 Effect of TRF on visual behaviour of diabetic rats using two-chamber mirror test. (**A**) Total time spent in the mirror and inverse mirror chambers; (**B**) Track plots. Bars represent mean ± SEM. $n = 6$, $^{**}p < 0.001$. Paired t test was used to calculate the significant differences. N: Rats that received the vehicle, DV: Diabetic rats that received the vehicle, DT: TRF treated diabetic rats

activate caspase recruitment domains and, subsequently caspase pathway [54, 55]. When TNF-α activates the mitochondrial pathway, mitochondrial cytochrome c is released, activating Casp9, and producing apoptosomes. Activation of the executioner caspase, Casp3, is finally made possible by Casp9 [56]. Therefore, it is clear that the activation of Casp3 by various mechanisms following the activation of intrinsic and extrinsic pathways as the result. Moreover, Casp3 reinforces caspase-8 activation and amplifies the feedback loop [57].

In addition to caspases, the mitochondrial pathway also mediates cell death through a caspase-independent pathway, with the Bcl2 family of proteins playing a major role. Bax, a pro-apoptotic protein, and Bcl2, an anti-apoptotic protein, have been shown to be connected to hyperglycaemia-related retinal cell apoptosis. The Bcl2 family of proteins contains both pro- and anti-apoptotic proteins [58]. Following activation of the caspase-independent pathway, a cell's fate is significantly determined by the balance of pro- and anti-apoptotic signals from the Bcl2 family [56]. Hence, in this study, measurement of retinal Casp3, Bax and Bcl2 expression, both at gene and protein levels, allows us an evaluation of the possible involvement of both caspase-dependent and -independent pathways in the anti-apoptotic effects of TRF. We found that diabetic control rats had a significantly higher retinal expression of the pro-apoptotic genes *Casp3* and *Bax* than the normal rats. We also observed that the expression of the retinal *Bcl2* gene, which is an anti-apoptotic marker, was also significantly greater in diabetic rats treated with a vehicle than in the normal rats. This upregulation of *Bcl2* seems to be a protective response to counteract the pro-apoptotic signalling. Interestingly, protein expression as measured by ELISA for Casp3 and Bax corresponded to their gene expression; however, we did not observe a corresponding increase in *Bcl2* protein expression in the diabetic control group despite increased *Bcl2* gene expression. It is likely that due to post-translation modifications, the expression of *Bcl2* gene expression did not translate into protein expression. Therefore, it can be hypothesised that both caspase-dependent and independent signalling activated via extrinsic and/or intrinsic pathways are involved in retinal cell apoptosis in diabetic rats as indicated by increased expression of Casp3 and Bax, and reduced expression of Bcl2 in the retinas of vehicle-treated diabetic rats. Moreover, vehicle-treated diabetic rats had a significantly higher ratio of Bax/Bcl2 proteins than untreated diabetic rats, indicating a shift in the balance towards pro-apoptotic signalling. Additionally, diabetic animals given vehicle treatment exhibited a significantly higher number of TUNEL-positive cells correlating the molecular changes with the morphological outcome.

It is a widely reported observation that oxidative stress and inflammation, the two key pathogenetic mechanisms in diabetic retinopathy, may act as an initial stimulant of apoptotic pathways such as by stimulating TNF-a activation. The current study showed a significantly lower gene expression for both the *Casp3* and *Bax* in TRF-treated rats compared to DV rats. This effect of TRF on gene expression translated at the protein level, and accordingly, we observed an attenuation of the diabetes-induced increase in the expression of *Casp3* and *Bax* in the TRF-treated group. The *Bcl2* gene expression in TRF-treated rat retinas did not differ significantly from that in the normal or vehicle-treated diabetic rats; however, the difference from group N amounted to 1.62-fold. Interestingly, the Bcl2 protein expression after treatment with TRF was significantly greater than in diabetic rats treated with vehicle and this was reflected in the significantly lower Bax/Bcl2 protein ratio in TRF-treated rats than that in diabetic controls indicating a shift towards suppression of anti-apoptotic pathways by TRF. This effect of TRF on pro-and anti-apoptotic proteins and retinal

cell apoptosis may be attributed to its antioxidant and anti-inflammatory properties. Tocotrienols prevent the non-enzymatic oxidation of various cell components and preserve membrane integrity [59]. Prior studies have shown that topically administered tocotrienol prevents lenticular oxidative stress induced by galactosemia and hyperglycaemia in rats [60, 61]. TRF has also been shown to exert potent anti-inflammatory activity and suppress TNF-a activation and pro-inflammatory activity of the transcription factor nuclear factor kappa B (NF-κB) [62]. Although previous studies indicate the possibility that TRF could improve insulin sensitivity and metabolic derangements in diabetes, as observed in diabetic db/db mice [63], the current study did not show a reduction in the blood glucose level of diabetic rats. Additionally, we found no difference in body weight gain between diabetic rats treated with TRF and diabetic rats treated with the vehicle. Therefore, we could rule out the possibility that the changes in the expression of pro- and anti-apoptotic markers and retinal cell apoptosis are a result of improved metabolic status.

By observing the behaviour of rats in an open field arena and a two-mirror chamber test, we also investigated the functional effects of the TRF treatment in this study. Various parameters noted in the open field indicated an increased anxiety-related behaviour among diabetic rats treated with a vehicle, and the same behaviour was suppressed among TRF-treated rats. These behavioural findings were interpreted under the presumption that visually impaired rats find it challenging and take longer to acclimatize to unfamiliar environments. In the current study, this was reflected in the total time immobile, freezing episodes, and faecal pellets excreted. Previous studies have also made similar observations [42, 64–66]. However, these earlier studies have shown that anxious rats exhibit greater locomotor and exploratory behaviour. Contrary to this, our observation was that the locomotor and exploratory activity was suppressed among vehicle-treated diabetic rats, although they showed other anxiety-related behaviour. This could be attributed to relatively poor muscle strength due to high catabolic status, as reflected by poor body weight gain among these animals. This finding is in concurrence with previous studies [67]. The TRF-treated diabetic rats showed greater locomotor and exploratory activity despite the poor body weight gain, perhaps indicating that the animals were at relative ease with the environment due to better visual abilities and hence were motivated to explore the arena despite persisting metabolic abnormalities [34].

In the two-chamber mirror test, we further explored the visual abilities of animals. The experimental set-up used was different from our prior study, which used open field arena installed with mirror and inverse mirror [34]. The set up used in the current study has been described earlier [42]. Briefly, it consisted of mirror chamber, and inverse-mirror chamber with dimensions of 30 cm (L) x 30 cm (W) x 40 cm (H) connected by bridging chamber. Mirror chamber had mirrors placed on the opposite walls, whereas reversed mirrors with non-reflective surface were similarly placed in the inverse-mirror chamber. In contrast to earlier observations [34], we noticed that both normal and TRF-treated rats preferred to remain in the inverse mirror chamber rather than the mirror chamber. The assumption used to interpret this finding is that visually capable rats tend to avoid interaction with another animal that they mistake for being real without realising that it is a reflection of themselves. For the same reason, it can be interpreted that vehicle-treated diabetic rats showed no chamber preference as they could not see their image as well as TRF-treated rats. In agreement with our observations, a previous study has reported that two visually healthy rats show a reduction in social interaction when placed in an unfamiliar test environment [68].

Interestingly, these findings in the current study are in contrast to the observation made earlier [33], in which visually competent animals preferred to stay in the area close to the mirror and interact with their image, assuming it to be another rat. The reason for these contrasting findings may lie in the differences in the experimental setup. Earlier studies used an open-field arena with mirrors or inverse mirrors installed on its walls [35]. Whereas the experimental set-up used in the current study had two separate chambers with mirror or inverse mirror, separated from each other by a passage. It is likely that in the open field arena, visually competent animals were aware of the area available around them to run away from in a threat situation. On the other hand, enclosed chambers give animals a sense of greater isolation and threat in the presence of other rats leading to preference for inverse-mirror chamber. Clearly, animal behaviour is highly sensitive to experimental setups and requires careful interpretation.

Conclusions

Our results demonstrate that oral administration of TRF for 12 weeks reduces retinal cell apoptosis by enhancing the expression of anti-apoptotic markers while decreasing the expression of pro-apoptotic markers. These molecular changes in the retinas of diabetic rats are associated with the preservation of their visual behaviour. The findings of this study may also find applications for targeting apoptosis in conditions not associated with hyperglycaemia.

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Author contributions
GY and ZS performed the experiments, HJ conceived and designed the study, wrote the manuscript, supervised the study, NA conceived and designed the study, analysed the data, supervised the study, RA and II conceived and designed the study, revised the manuscript, and supervised the study, NI supervised the study.

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Data availability
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The animal handling and all experiments were carried out in compliance with the ARVO statement for the use of animals in ophthalmic and vision research (Association for Research in Vision and Ophthalmology, 2022), animal ethics guidelines of International Medical University and Universiti Teknologi MARA. All procedures in the conduct and reporting of this experiment complied with ARRIVE guidelines. Ethical approval was obtained from International Medical University (BMSI/2020 [5]) and Universiti Teknologi MARA (UiTM care 328/2020) ethical committees.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Wong TY, Sabanayagam C. Strategies to tackle the global burden of diabetic retinopathy: from epidemiology to artificial intelligence. Ophthalmologica. 2020;243(1):9–20.
2. Yau JWY, Rogers SL, Kawasaki R, Lamoureux EL, Kowalski JW, Bek T, et al. Global prevalence and major risk factors of diabetic retinopathy. Diabetes Care. 2012;35(3):556–64.
3. Cavan D, Makaroff L, da Rocha Fernandes J, Sylvanowicz M, Ackland P, Conlon J, et al. The diabetic retinopathy barometer study: global perspectives on access to and experiences of diabetic retinopathy screening and treatment. Diabetes Res Clin Pract. 2017;129:16–24.
4. Fenwick EK, Xie J, Ratcliffe J, Pesudovs K, Finger RP, Wong TY, et al. The impact of diabetic retinopathy and diabetic macular edema on health-related quality of life in type 1 and type 2 diabetes. Invest Ophthalmol Vis Sci. 2012;53(2):677–84.
5. Rees G, Xie J, Fenwick EK, Sturrock BA, Finger R, Rogers SL, et al. Association between diabetes-related eye complications and symptoms of anxiety and depression. JAMA Ophthalmol. 2016;134(9):1007–14.
6. Kramer CK, Rodrigues TC, Canani LH, Gross JL, Azevedo MJ. Diabetic retinopathy predicts all-cause mortality and cardiovascular events in both type 1 and 2 diabetes: meta-analysis of observational studies. Diabetes Care. 2011;34(5):1238–44.
7. Federation ID. Idf diabetes atlas. Int Diabetes Fed. 2013. https://diabetesatlas.org/101289/imageehpv119i03.
8. Simó R, Stitt AW, Gardner TW. Neurodegeneration in diabetic retinopathy: does it really matter? Diabetologia. 2018;61(9):1902–12.
9. Aiello LM. Perspectives on diabetic retinopathy. Am J Ophthalmol. 2003;136(1):122–35.
10. Wilkinson CP, Ferris FL III, Klein RE, Lee PP, Agardh CD, Davis M, et al. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology. 2003;110(9):1677–82.
11. Wolter JR. Diabetic capillary microaneurysms of the retina. Arch Ophthalmol. 1961;65(6):847–54.
12. Engerman R, Bloodworth JMB Jr, Nelson S. Relationship of microvascular disease in diabetes to metabolic control. Diabetes. 1977;26(8):760–9.
13. Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. J Clin Invest. 1996;97(12):2883–90.
14. Joussen AM, Poulaki V, Mitsiades N, Cai W, Suzuma I, Pak J, et al. Suppression of Fas-FasL-induced endothelial cell apoptosis prevents diabetic blood-retinal barrier breakdown in a model of streptozotocin-induced diabetes. FASEB J. 2003;17(1):76–8.
15. Kern TS, Engerman RL. Comparison of retinal lesions in alloxan-diabetic rats and galactose-fed rats. Curr Eye Res. 1994;13(12):863–7.
16. El-Remessy AB, Al-Shabrawey M, Khalifa Y, Tsai N-T, Caldwell RB, Liou GI. Neuroprotective and blood-retinal barrier-preserving effects of cannabidiol in experimental diabetes. Am J Pathol. 2006;168(1):235–44.
17. Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest. 1998;102(4):783–91.
18. Barber AJ, Gardner TW, Abcouwer SF. The significance of vascular and neural apoptosis to the pathology of diabetic retinopathy. Invest Ophthalmol Vis Sci. 2011;52(2):1156–63.
19. Sadikan MZ, Abdul Nasir NA. Diabetic retinopathy: emerging concepts of current and potential therapy. Naunyn-Schmiedeberg's Archives of Pharmacology. 2023;396(12):3395-406.
20. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107(9):1058–70.
21. Ola MS, Nawaz M, Ahsan H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem. 2011;351(1):41–58.
22. Blinder KJ, Dugel PU, Chen S, Jumper JM, Walt JG, Hollander DA, et al. Anti-VEGF treatment of diabetic macular edema in clinical practice: effectiveness and patterns of use (ECHO study report 1). Clin Ophthalmol (Auckland NZ). 2017;11:393.
23. Duh EJ, Sun JK, Stitt AW. Diabetic retinopathy: current understanding, mechanisms, and treatment strategies. JCI Insight. 2017;2(14).
24. Du X-L, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, et al. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. Proc Natl Acad Sci. 2000;97(22):12222–6.
25. Tiwari V, Kuhad A, Bishnoi M, Chopra K. Chronic treatment with tocotrienol, an isoform of vitamin E, prevents intracerebroventricular streptozotocin-induced cognitive impairment and oxidative–nitrosative stress in rats. Pharmacol Biochem Behav. 2009;93(2):183–9.
26. Abdul Ghani NA, Abdul Nasir NA, Lambuk L, Sadikan MZ, Agarwal R, Ramli N. The effect of palm oil-derived tocotrienol-rich fraction in preserving normal retinal vascular diameter in streptozotocin-induced diabetic rats. Graefe's Arch Clin Exp Ophthalmol. 2023;261(6):1587–96.
27. Tiwari V, Arora V, Chopra K. Attenuation of NF-κβ mediated apoptotic signaling by tocotrienol ameliorates cognitive deficits in rats postnatally exposed to ethanol. Neurochem Int. 2012;61(3):310–20.
28. Theriault A, Chao J-T, Gapor A. Tocotrienol is the most effective vitamin E for reducing endothelial expression of adhesion molecules and adhesion to monocytes. Atherosclerosis. 2002;160(1):21–30.
29. Noguchi N, Hanyu R, Nonaka A, Okimoto Y, Kodama T. Inhibition of THP-1 cell adhesion to endothelial cells by α-tocopherol and α-tocotrienol is

dependent on intracellular concentration of the antioxidants. Free Radic Biol Med. 2003;34(12):1614–20.
30. Qureshi AA, Reis JC, Papasian CJ, Morrison DC, Qureshi N. Tocotrienols inhibit lipopolysaccharide-induced pro-inflammatory cytokines in macrophages of female mice. Lipids Health Dis. 2010;9:1–15.
31. Suman S, Datta K, Chakraborty K, Kulkarni SS, Doiron K, Fornace AJ Jr, et al. Gamma tocotrienol, a potent radioprotector, preferentially upregulates expression of anti-apoptotic genes to promote intestinal cell survival. Food Chem Toxicol. 2013;60:488–96.
32. Selvaraju TR, Khaza'ai H, Vidyadaran S, Abd Mutalib MS, Vasudevan R. The neuroprotective effects of tocotrienol rich fraction and alpha tocopherol against glutamate injury in astrocytes. Bosn J Basic Med Sci. 2014;14(4):195.
33. Sadikan MZ, Nasir NAA, Iezhitsa I, Agarwal R. Open field mirror test as a tool for the assessment of visual functions in rats with streptozotocin-induced diabetes. Neurosci Res Notes. 2021;4(3):11–20.
34. Sadikan MZ, Nasir NAA, Iezhitsa I, Agarwal R. Antioxidant and anti-apoptotic effects of tocotrienol-rich fraction against streptozotocin-induced diabetic retinopathy in rats. Biomed Pharmacother. 2022;153:113533.
35. Oltval ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. Cell. 1993;74(4):609–19.
36. Ophthalmology A. for R in V and. Statement for the use of animals in ophthalmic and vision research. Invest Ophthalmol Vis Sci. 1994;35:v–vi.
37. Zatroch KK, Knight CG, Reimer JN, Pang DSJ. Refinement of intraperitoneal injection of sodium pentobarbital for euthanasia in laboratory rats (Rattus norvegicus). BMC Vet Res. 2016;13(1):1–7.
38. Sadikan MZ, Nasir NA, Ghani NA, Lambuk L, Iezhitsa IN, Agarwal R. The use of Fiji image J as an image analysis tool for measuring retinal vessel diameter in rodent model of diabetic retinopathy. Asian Journal of Medicine and Biomedicine. 2021;5(1):61-6.
39. Spasov AA, Iezhitsa IN, Kharitonova MV, Kravchenko MS. Depression-like and anxiety-related behaviour of rats fed with magnesium-deficient diet. Zhurnal Vyss Nervn Deiatelnosti Im IP Pavlov. 2008;58(4):476–85.
40. Iezhitsa IN, Spasov AA, Kharitonova MV, Kravchenko MS. Effect of magnesium chloride on psychomotor activity, emotional status, and acute behavioural responses to clonidine, d-amphetamine, arecoline, nicotine, apomorphine, and L-5-hydroxytryptophan. Nutr Neurosci. 2011;14(1):10–24.
41. Mohd Lazaldin MA, Iezhitsa I, Agarwal R, Bakar NS, Agarwal P, Mohd Ismail N. Neuroprotective effects of brain-derived neurotrophic factor against amyloid beta 1-40-induced retinal and optic nerve damage. Eur J Neurosci. 2020;51(12):2394–411.
42. Yih TH, Abd Ghapor AA, Agarwal R, Razali N, Iezhitsa I, Ismail NM. Effect of trans-resveratrol on glutamate clearance and visual behaviour in rats with glutamate induced retinal injury. Exp Eye Res. 2022;109104.
43. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: M inimum I nformation for publication of Q uantitative real-time PCR E xperiments. Oxford University Press; 2009.
44. Sadikan MZ, Nasir NA, Ibahim MJ, Iezhitsa I, Agarwal R. Identifying the stability of housekeeping genes to be used for the quantitative real-time PCR normalization in retinal tissue of streptozotocin-induced diabetic rats. International Journal of Ophthalmology. 2024;17(5):794.
45. Sadikan MZ, Abdul Nasir NA, Bakar NS, Iezhitsa I, Agarwal R. Tocotrienol-rich fraction reduces retinal inflammation and angiogenesis in rats with streptozotocin-induced diabetes. BMC Complementary Medicine and Therapies. 2023;23(1):179.
46. Musalmah M, Leow KS, Nursiati MT, Hanis Raja IRN, Syah AF, Renuka S et al. Selective uptake of alpha-tocotrienol and improvement in oxidative status in rat brains following short-and long-term intake of tocotrienol rich fraction. 2013.
47. Suzuki YJ, Tsuchiya M, Wassall SR, Choo YM, Govil G, Kagan VE, et al. Structural and dynamic membrane properties of. alpha.-tocopherol and. alpha.-tocotrienol: implication to the molecular mechanism of their antioxidant potency. Biochemistry. 1993;32(40):10692–9.
48. Mazlan M, Hamezah HS, Taridi NM, Jing Y, Liu P, Zhang H, et al. Effects of aging and tocotrienol-rich fraction supplementation on brain arginine metabolism in rats. Oxid Med Cell Longev. 2017;2017(1):6019796.
49. Durani LW, Hamezah HS, Ibrahim NF, Yanagisawa D, Nasaruddin ML, Mori M, et al. Tocotrienol-rich fraction of palm oil improves behavioral impairments and regulates metabolic pathways in AβPP/PS1 mice. J Alzheimer's Dis. 2018;64(1):249–67.
50. Shaikh SA, Varatharajan R, Muthuraman A. Palm oil derived tocotrienol-rich fraction attenuates vascular dementia in type 2 diabetic rats. Int J Mol Sci. 2022;23(21):13531.
51. Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. Programmed cell death and the control of cell survival: lessons from the nervous system. Sci (80-). 1993;262(5134):695–700.
52. Kowluru RA, Chan P-S. Metabolic memory in diabetes–from in vitro oddity to in vivo problem: role of apoptosis. Brain Res Bull. 2010;81(2–3):297–302.
53. Chen QM, Tu VC. Apoptosis and heart failure. Am J Cardiovasc Drugs. 2002;2(1):43–57.
54. Park HH, Lo Y-C, Lin S-C, Wang L, Yang JK, Wu H. The death domain superfamily in intracellular signaling of apoptosis and inflammation. Annu Rev Immunol. 2007;25.
55. Khosravi-Far R. Death receptor signals to the mitochondria. Cancer Biol Ther. 2004;3(11):1051–7.
56. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, et al. Ordering the cytochrome c–initiated caspase cascade: hierarchical activation of caspases-2,-3,-6,-7,-8, and-10 in a caspase-9–dependent manner. J Cell Biol. 1999;144(2):281–92.
57. Certo M, Moore VDG, Nishino M, Wei G, Korsmeyer S, Armstrong SA, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell. 2006;9(5):351–65.
58. Sadikan MZ, Abdul Nasir NA, Lambuk L, Mohamud R, Reshidan NH, Low E, Singar SA, Mohmad Sabere AS, Iezhitsa I, Agarwal R. Diabetic retinopathy: a comprehensive update on in vivo, in vitro and ex vivo experimental models. BMC ophthalmology. 2023;23(1):421.
59. Atkinson J, Epand RF, Epand RM. Tocopherols and tocotrienols in membranes: a critical review. Free Radic Biol Med. 2008;44(5):739–64.
60. Nasir NAA, Agarwal R, Vasudevan S, Tripathy M, Alyautdin R, Ismail NM. Effects of topically applied tocotrienol on cataractogenesis and lens redox status in galactosemic rats. Mol Vis. 2014;20:822.
61. Abdul Nasir NA, Agarwal R, Sheikh Abdul Kadir SH, Vasudevan S, Tripathy M, Iezhitsa I, et al. Reduction of oxidative-nitrosative stress underlies anticataract effect of topically applied tocotrienol in streptozotocin-induced diabetic rats. PLoS ONE. 2017;12(3):e0174542.
62. Nasir NAA, Sadikan MZ, Agarwal R. Modulation of NFκB signalling pathway by tocotrienol: a systematic review. Asia Pac J Clin Nutr. 2021;30(3):537–55.
63. Fang F, Kang Z, Wong C. Vitamin E tocotrienols improve insulin sensitivity through activating peroxisome proliferator-activated receptors. Mol Nutr Food Res. 2010;54(3):345–52.
64. Fazel MF, Abu IF, Mohamad MHN, Agarwal R, Iezhitsa I, Bakar NS, et al. Philanthotoxin-343 attenuates retinal and optic nerve injury, and protects visual function in rats with N-methyl-D-aspartate-induced excitotoxicity. PLoS ONE. 2020;15(7):e0236450.
65. Lambuk L, Iezhitsa I, Agarwal R, Agarwal P, Peresypkina A, Pobeda A, et al. Magnesium acetyltaurate prevents retinal damage and visual impairment in rats through suppression of NMDA-induced upregulation of NF-κB, p53 and AP-1 (c-Jun/c-Fos). Neural Regen Res. 2021;16(11):2330.
66. Arfuzir NNN, Agarwal R, Iezhitsa I, Agarwal P, Ismail NM. Magnesium acetyltaurate protects against endothelin-1 induced RGC loss by reducing neuroinflammation in Sprague Dawley rats. Exp Eye Res. 2020;194:107996.
67. Sadikan MZ, Abdul Nasir NA, Agarwal R, Mohd Ismail N. Protective effect of palm oil-derived tocotrienol-rich fraction against retinal neurodegenerative changes in rats with streptozotocin-induced diabetic retinopathy. Biomolecules. 2020;10(4):556.
68. File SE, Hyde JR. Can social interaction be used to measure anxiety? Br J Pharmacol. 1978;62(1):19.

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