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Effects of topically applied tocotrienol on cataractogenesis and lens redox status in galactosemic rats

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Purpose: Oxidative and nitrosative stress underlies cataractogenesis, and therefore, various antioxidants have been investigated for anticataract properties. Several vitamin E analogs have also been studied for anticataract effects due to their antioxidant properties; however, the anticataract properties of tocotrienols have not been investigated. In this study, we investigated the effects of topically applied tocotrienol on the onset and progression of cataract and lenticular oxidative and nitrosative stress in galactosemic rats.

Methods: In the first part of this study, we investigated the effects of topically applied microemulsion formulation of tocotrienol (TTE) using six concentrations ranging from 0.01% to 0.2%. Eight groups of Sprague-Dawley rats (n = 9) received distilled water, vehicle, or one of the six TTE concentrations as pretreatment topically twice daily for 3 weeks while on a normal diet. After pretreatment, animals in groups 2–8 received a 25% galactose diet whereas group 1 continued on the normal diet for 4 weeks. During this 4-week period, topical treatment continued as for pretreatment. Weekly slit-lamp examination was conducted to assess cataract progression. At the end of the experimental period, the animals were euthanized, and the proteins and oxidative stress parameters were estimated in the lenses. In the second part of the study, we compared the anticataract efficacy of the TTE with the liposomal formulation of tocotrienol (TTL) using five groups of Sprague-Dawley rats (n = 15) that received distilled water, TTE, TTL, or corresponding vehicle. The mode of administration and dosing schedule were the same as in study 1. Weekly ophthalmic examination and lens protein and oxidative stress estimates were performed as in study 1. Lens nitrosative stress was also estimated.

Results: During the 4-week treatment period, the groups treated with 0.03% and 0.02% tocotrienol showed slower progression of cataract compared to the vehicle-treated group ($p<0.05$), whereas the group treated with 0.2% tocotrienol showed faster progression of cataract compared to the vehicle-treated group ($p<0.05$). The lenticular protein content, malondialdehyde, superoxide dismutase, and catalase levels were normalized in the groups that received 0.03% and 0.02% tocotrienol. The lenticular reduced glutathione also showed a trend toward normalization in these groups. In contrast, the group treated with 0.2% tocotrienol showed increased lenticular oxidative stress. When the microemulsion and liposomal formulations were compared, the effects on cataract progression, lens oxidative and nitrosative stress, and lens protein content did not show significant differences.

Conclusions: Topically applied tocotrienol within the concentration range of less than 0.05% and more than 0.01% tends to delay the onset and progression of cataract in galactose-fed rats by reducing lenticular oxidative and nitrosative stress. However, topical tocotrienol at a concentration of 0.2% and higher aggravates cataractogenesis in galactose-fed rats by increasing lens oxidative stress. The anticataract efficacy of 0.03% microemulsion of tocotrienol did not differ from its liposomal formulations at the same concentration.

Cataract, characterized by the development of lenticular opacities, is a leading cause of blindness worldwide [1]. Cataract-related blindness is a particularly important public health issue especially in developing countries due to illiteracy, lack of access to services, and the high cost of surgical management [1,2]. The prevalence of cataract further increases in patients with underlying metabolic abnormalities such as diabetes mellitus, Lowe's syndrome, hypoparathyroidism, abnormalities of lactose absorption, and galactosemia [3].

Among several pathophysiological mechanisms known to underlie cataractogenesis, increased lenticular oxidative stress has a central role [4-6]. Emerging evidence has also demonstrated the role of nitrosative stress in cataractogenesis [7-9]. Excessive production of nitric oxide (NO) secondary to induction of inducible nitric oxide synthase (iNOS) has been shown to be cytotoxic to lenticular cells [7-11].

Therefore, substances that possess potent antioxidant properties are of particular importance as potential anticataract agents [12,13]. Vitamin E is known for its antioxidant

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effects, and some of its analogs have been investigated for anticataract effects. Tocopherols were the first vitamin E analog to be discovered in 1922 [14] and were shown to possess anticataract properties in animal models [15-17]. However, clinical trials showed variable results [18-20]. Tocotrienols, the other vitamin E analogs, were discovered 40 years later by Pennock and Whittle [21]. Tocotrienols differ from tocopherols in structure as well as properties. Tocotrienols exist as four isomers; α-, β-, γ-, and δ. Although tocotrienols and tocopherols have a chroman head and an isoprenoid tail, only tocotrienols possess three double trans bonds in the isoprenoid side chain and thus have an unsaturated tail. This unique molecular structure makes tocotrienols more flexible and allows easy permeation through the cell membranes [22-24]. Tocotrienols are found in the seeds of endosperm of monocotyledon and dicotyledon plants, cereals, and edible oils such as rice bran and palm oil [25]. Annatto seeds predominantly contain δ-tocotrienol, minimal γ-tocotrienol, and no tocopherol [26,27]. This may be advantageous since α-tocopherol was reported to decrease the cellular uptake of tocotrienol [28,29]. Tocotrienols have distinct molecular targets and more potent antioxidant effects than tocopherols [30,31]. Tocotrienols also reduce nitrosative stress by reducing iNOS activity, which leads to decreased nitric oxide production [32,33].

In this study, for the first time we investigated the effects of topically applied tocotrienol on the onset and progression of cataract and lenticular oxidative and nitrosative stress. We used the galactose-induced model of experimental cataract in rats. Galactosemic animal models are widely used to study sugar-induced complications. Although this model is not an exact representation of human diabetic cataract and there are differences between the two, some of the common features include activation of aldose reductase, polyol accumulation, and oxidative stress [34-38]. Since galactose feeding can rapidly produce cataract and animal survival is better due to less severe systemic metabolic changes, the animal model is often favored over the diabetic model, particularly for initial screening of new investigational agents. In this study, first we studied the anticataract effect of Annatto tocotrienol using a wide concentration range in a microemulsion formulation. Subsequently, using the concentration that showed the best anticataract effect, we formulated a liposomal tocotrienol preparation and compared its anticataract efficacy with microemulsion.

METHODS

Animals: Three-week-old Sprague-Dawley rats were obtained from the Laboratory Animal Care Unit of Universiti Teknologi MARA. Animals were housed under standard laboratory conditions (12 h:12 h light-dark cycle) and were given food and water ad libitum. All animals were subjected to systemic and ophthalmic examination, and those found normal were included in the study. All experiments and animal handling were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research as well as the local institutional ethical guidelines.

Microemulsion formulation of tocotrienol: Microemulsion of tocotrienol was formulated as described by Valdivia et al. [39]. Kolliphor P188 (Sigma Aldrich, St. Louis, MO) was added to double-distilled water to create an aqueous phase. Annatto tocotrienol, which contains 90% δ-tocotrienol and 10% γ-tocotrienol with no tocopherol, was a gift from American River Nutrition (Hadley, MA). Tocotrienol was added to Miglyol 812 (AXO Industry, Wavre, Belgium) to create an oily phase. The oily phase was then added to the aqueous phase under moderate agitation. Subsequently, the particle size was reduced using an ultrasound sonicator (Fisher Scientific, FB120, Hampton, NH) for 40 min at the setting of 80% amplitude with cycles of 50 s on and 20 s off. After sonication, sorbitol and disodium edetate (EDTA; Fisher Chemical, Hampton, NH) were added as an isotonizing agent and stabilizer, respectively. The microemulsion was prepared once every 8 weeks.

Characterization of microemulsion formulation: Microemulsion formulation was characterized for particle size, zeta potential, and viscosity using an acoustic and electroacoustic spectrometer (Dispersion Technology – Acousto Phor Zeta Size DT-1201, Bedford Hill, NY). The size, zeta potential, and viscosity were measured again after 60 days of preparation to assess stability.

Liposomal formulation of tocotrienol: Cholesterol (5 µmol), phosphatidylcholine (20 µmol; Sigma Aldrich), and tocotrienol were dissolved in a 5:1 solution of chloroform and methanol. The lipid solution was subjected to evaporation for 2 h to obtain a thin lipid film. The lipid film was rehydrated with 2 ml of PBS (1X; 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, 0.0027 M KCl, 0.137 M NaCl, pH 7.4), and after 30 min of shaking, another 2 ml of PBS was added. The multilamellar vesicle solution was obtained, which was then sonicated for 10 min. The solution then underwent further reduction in particle size using a hand-held extruder ten times through each of the 400-nm, 200-nm, and 100-nm polycarbonate membranes. Fresh liposomes were prepared every other day.

Characterization of liposome formulation: Liposome formulation was characterized for particle size and zeta potential using a dynamic light scattering-based zetasizer (Malvern Zetasizer, Nano ZS, Worcestershire, UK). Entrapment efficiency was determined with a modified minicolumn centrifugation method using Sephadex G-25 minicolumns [40]. Sephadex G-25 gel in column was allowed to swell in PBS for 15 min, and then the column was centrifuged for 5 min at 1,000 ×*g* using a microcentrifuge to remove excess PBS. The dry column was loaded with empty liposomes to saturate the column and minimize adsorption of the actual sample. The loaded column was centrifuged for 15 min at 1,520 ×*g* to expel the liposomes. Subsequently, the tocotrienol-loaded liposomes were introduced into the column and centrifuged at 1,520 ×*g* for 15 min to separate untrapped tocotrienol from the liposome-entrapped drug. One hundred microliters of 20% Triton X (Sigma Aldrich) was added to the eluted sample to destroy the liposomes. The tocotrienol content was measured using a microplate reader at absorbance of 297 nm. The entrapment efficiency was calculated using the following formula: Entrapment Efficiency (%) = (Entrapped drug/Total drug) × 100. Liposome stability was determined by measuring and comparing the entrapment efficiency at different time points: 0 min, 30 min, 60 min, 3 h, 6 h, 24 h, and 48 h.

Study design:

Study 1: Dose–response study using microemulsion formulation—The animals were divided into eight groups of nine animals each. All animals were pretreated for 3 weeks, topically, bilaterally, and twice daily in a volume of 10 µl using a micropipette. Group 1 received distilled water (normal group) while group 2 received vehicle (vehicle group). Groups 3 to 8 received tocotrienol microemulsion (TTE) 0.2%, 0.1%, 0.05%, 0.03%, 0.02%, and 0.01%, respectively. During this 3-week pretreatment period, all animals were fed normal rat chow.

After the 3-week pretreatment period was completed, the treatment was continued over the next 4 weeks as outlined for pretreatment. However, during the treatment period, the normal group received a normal diet while the other groups received a 25% galactose diet.

Anterior segment imaging was performed at the beginning of the experiment, at the end of the pretreatment, and, subsequently, weekly during the treatment period. After 4 weeks of treatment, the animals were euthanized with overdose of ketamine (250 mg/kg) and xylazine (50 mg/kg) intraperitoneally. The lenses were carefully dissected out with the posterior approach to estimate the lens protein content and oxidative stress parameters.

Study 2: Anticataract efficacy of microemulsion versus liposome—The animals were divided into five groups of 15 animals each. As in the dose–response study, the animals were given a pretreatment for 3 weeks, topically, bilaterally, and twice daily in a volume of 10 µl using a micropipette. Group 1 received distilled water (normal group) while groups 2 and 3 received vehicle used for microemulsion (VE) and liposome (VL) formulation, respectively. Group 4 received TTE at the most effective concentration based on the dose–response study, and group 5 received tocotrienol liposomal formulation (TTL) in a similar concentration as group 4. After the 3-week pretreatment was completed, the treatment was continued over the next 4 weeks as in the dose–response study. During the treatment period, the normal group received a normal diet while all other groups received a 25% galactose diet.

Anterior segment imaging was performed as in study 1. At the end of experiment, the lens protein content and oxidative and nitrosative stress parameters were estimated.

Anterior segment imaging: Anterior segment imaging was performed using a Hawkeye Portable Slit Lamp (Optotek Medical, Ljubljana, Slovenia) equipped with a digital camera (Pentax Optio, S60, Denver, CO). Topically applied 1% tropicamide (Alcon Laboratories, Fort Worth, TX) was used as the mydriatic. The lenticular changes observed were categorized into eight stages as described previously [41]: Stage 0, normal lenses; Stage 1a, appearance of vacuoles as an equatorial ring; Stage 1b, vacuolization covering one-third of the anterior cortex; Stage 1c, vacuolization covering more than two-thirds of the anterior cortex; Stage 2a, early coalescence and liquefaction of vacuoles; Stage 2b, late coalescence and liquefaction of vacuoles and appearance of haziness; Stage 3, uniform opalescence; Stage 4, nuclear opacity (Figure 1). This semiquantitative method of assessing the severity of cataractous changes had insignificant intraobserver and interobserver variability [41]. Additionally, each cataract stage was given a score of 0 to 8, to calculate the opacity index as described by Vats et al. [42].

Estimate of lens protein levels and the parameters indicating oxidative stress: Each lens was homogenized in 0.5 ml of 50 mM cold phosphate buffer, pH 7.4, containing 1 mM EDTA. The homogenate was centrifuged at 890 ×g for 15 min. Supernatant was separated to quantify the proteins and antioxidant parameters. All estimates were done in duplicate.

Lens proteins: The lens protein level was determined using the Bradford method, which detects change in the color of

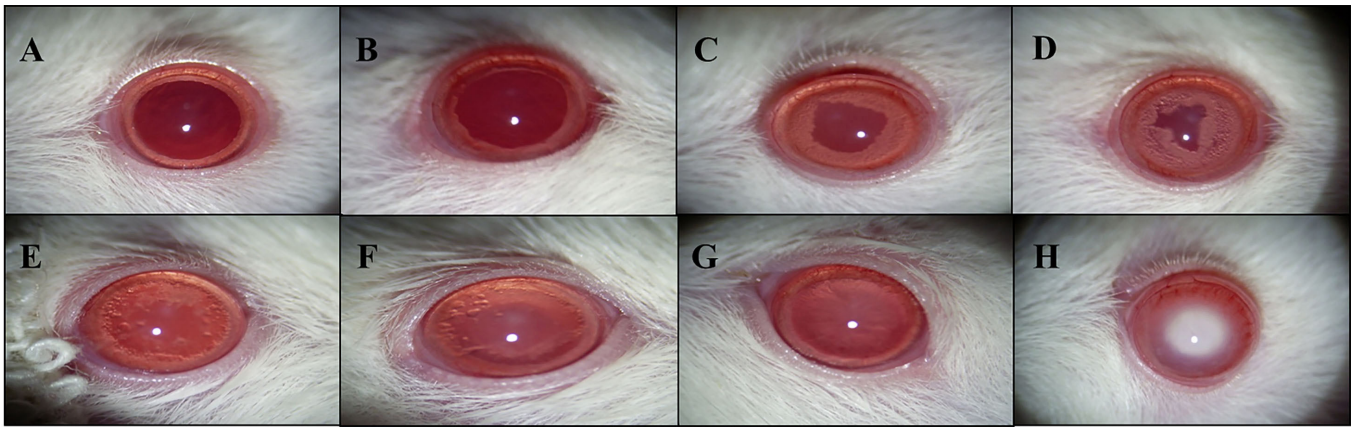

Figure 1. Retroillumination anterior segment photographs showing progression of cataract from stage 0 to stage 4. **A**: Stage 0. **B**: Stage 1A. **C**: Stage 1B. **D**: Stage 1C. **E**: Stage 2A. **F**: Stage 2B. **G**: Stage 3. **H**: Stage 4.

Coomassie dye from brown to blue as a result of binding to proteins in acidic medium. The total protein level was determined using 100 µl of the homogenized sample (before centrifugation) while soluble protein was quantified using 100 µl of the supernatant.

Lens reduced glutathione estimation: Glutathione (GSH) estimation was based on the enzymatic recycling method [43] and was done using a commercially available assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, 100 µl of supernatant from lens homogenate was deproteinated by adding 100 µl metaphosphoric acid (MPA) reagent (5 g of MPA in 50 ml of high-performance liquid chromatography–grade water). Triethanolamine (TEAM) reagent (50 µl/ml, 4M) was then added to the solution and vortexed. Standards or samples (50 µl) were pipetted into the designated wells. This was followed by adding 150 µl of freshly prepared assay cocktail consisting of 11.25 ml of MES Buffer (0.4 M 2-(N-morpholino) ethanesulphonic acid, 0.1 M phosphate, and 2 mM EDTA), 0.45 ml of reconstituted cofactor mixture (lyophilized powder of NADP+ and glucose-6-phosphate reconstituted with 0.5 ml water), 2.1 ml of reconstituted enzyme mixture (glutathione reductase and glucose-6-phosphate dehydrogenase reconstituted in 2 ml of MES buffer), 2.3 ml of water, and 0.45 ml of Ellman's Reagent (5,50-dithio-bis-(2-nitrobenzoic acid)). The absorbance was read at 405 nm after 25 min of incubation.

Lens superoxide dismutase activity: Superoxide dismutase (SOD) activity was quantified using a commercially available assay kit (Cayman Chemical), which utilizes tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. Two hundred microliters of diluted tetrazolium salt solution (50 µl tetrazolium salt solution added to 19.95 ml diluted assay buffer containing 50 mM Tris-HCl, pH 8.0), 1 mM diethylenetriaminepentaacetic acid, and 0.1 mM hypoxanthine were added to each well followed by 10 µl of the standard or sample to the designated wells. Reaction was initiated by adding 20 µl of reconstituted xanthine oxidase (50 µl xanthine oxidase added to 19.95 ml of 50 mM Tris-HCl, pH 8.0). The plate was incubated for 20 min, and then the absorbance was read at 440–460 nm using a microplate reader.

Lens catalase activity: Lens catalase (CAT) activity was quantified using a commercially available assay kit (Cayman Chemical) based on the CAT reaction with methanol in the presence of the optimal H_2O_2 concentration. The production of formaldehyde was measured by adding 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole, a chromagen (Purpald). Purpald forms a cyclic derivative with aldehyde, which upon oxidation turns from colorless to purple. One hundred microliters of diluted assay buffer (100 mM potassium phosphate, pH 7.0) was added followed by 30 µl methanol and 20 µl standard or sample to the designated wells. The reaction was initiated by adding 20 µl diluted H_2O_2 (40 µl of 8.82 M H_2O_2 with 9.96 ml water). The plate was incubated on a shaker for 20 min. Reaction was terminated by adding 30 µl diluted potassium hydroxide (10 M), followed by 30 µl catalase Purpald. After 10 min of incubation, 10 µl catalase potassium periodate in 0.5 M potassium hydroxide was added. After 5 min of incubation, absorbance was read at 540 nm using a microplate reader.

Lens malondialdehyde level: The extent of lens lipid peroxidation was determined using a commercially available assay kit (Cayman Chemicals), which indirectly measures malondialdehyde (MDA), a byproduct of lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) at high temperature in an acidic medium to produce a colored complex. For the assay, the lenses were homogenized with radioimmunoprecipitation

assay (RIPA) lysis buffer containing a protease inhibitor in a ratio of 1 mg lens weight:10 µl RIPA buffer. The samples were then centrifuged at 1,600 ×*g* at 4 °C for 10 min, and supernatant was used for analysis. One hundred microliters of samples or standards were added to 100 µl sodium dodecyl sulfate (SDS) solution followed by 4 ml color reagents (530 mg TBA with 50 ml diluted TBA acetic acid and 50 ml diluted TBA sodium hydroxide). Solutions were boiled for 1 h and then incubated in an ice bath for 10 min to stop the reaction. This was followed by centrifugation of solutions at 1,600 ×*g* and 4 °C for 10 min. Absorbance was then read at 540 nm using a microplate reader.

Lens inducible nitric oxide synthase activity: Inducible nitric oxide synthase (iNOS) activity was determined using a commercially available iNOS enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science, Wuhan, China), which has a microtiter plate coated with monoclonal antibody specific to iNOS. One hundred microliters of standards, blank, or samples were added to the designated wells and incubated for 2 h at 37 °C. After incubation, the solution in the well plate was removed. One hundred microliters of detection reagent A (containing detection antibody, Tris buffer saline, 1% BSA, and 0.01% sodium azide) was added to each well and incubated for 1 h at 37 °C. The solution in the well plate was aspirated, and each well was washed with 350 µl Tris buffer saline three times. One hundred microliters of detection reagent B (containing horseradish peroxidase-linked avidin, Tris buffer saline, 1% BSA, and 0.01% sodium azide) was then added and incubated for 30 min at 37 °C. Then, the solution in the well plate was aspirated and washed five times. Ninety microliters of 3,3′,5,5′-tetramethylbenzidine (0.05%) was added and incubated for 15 min at 37 °C. Fifty microliters of 1 M sulphuric acid were then added, and absorbance was read at 450 nm using a microplate reader.

Lens 3-nitrotyrosine content: Measuring 3-nitrotyrosine (3-NT) indirectly provides an estimate of peroxynitrite in the samples. 3-NT activity was determined using a commercially available 3-nitrotyrosine ELISA kit (Abcam, Cambridge, England), which has the microplates coated with a nitrotyrosine containing antigen. Fifty microliters of standards and samples was added to the designated wells followed by 50 µl horseradish peroxidase-conjugated anti 3-nitrotyrosine antibody. Plate was incubated on a shaker for 2 h at room temperature. The solution in each well was aspirated and washed with 300 µl Tris buffer saline four times. One hundred microliters of H_2O_2 was added, and the plate was read in the kinetic mode for 15 min at 1 min intervals at absorbance of 600 nm using a microplate reader.

Statistical analysis: All values are expressed as mean ± standard deviation (SD). Statistical comparison was done using two-way ANOVA with the Bonferroni correction. $p<0.05$ was considered significant.

RESULTS

Characterization of microemulsion formulation: The mean particle size for microemulsion formulation was 147.93±22.90 nm. The zeta potential recorded was 255.88±23.05 mV, and the viscosity was 3.96±0.10 cP. There was no significance difference in the parameters observed at day 0 and day 60 (Table 1).

Characterization of liposomal formulation: The mean particle size for liposomal formulation was 315.57±68.58 nm while the zeta potential recorded was 2.43±0.5 mV. The entrapment efficiency of tocotrienol in the liposomal formulation was 58.56±7.5% at 0 h and 44.39±9.6% at 48 h. There was no significant difference in the entrapment efficiency at two time points (Table 2).

Table 1. Particle size, zeta potential and viscosity of microemulsion formulation at day 0 and day 60.

Parameters	Day 0	Day 60
Particle size	147.93±22.90 nm	152.47±20.62 nm
Zeta potential	255.88±23.05 mV	244.81±41.43 mV
Viscosity	3.96±0.10 cP	4.08±0.04 cP

All values are mean ± SD (n=3).

Table 2. Entrapment efficiency of tocotrienol in liposomal formulation.

Hours	0 h	0.5 h	1 h	3 h	6 h	24 h	48 h
Entrapment efficiency (%)	58.57±7.5	52.78±3.3	46.46±7.1	45.98±13.4	42.70±3.0	43.27±3.4	44.39±9.6

All values are mean ± SD (n=3).

Study 1:

Effect of tocotrienol on the onset and progression of cataract—Anterior segment imaging did not show any changes in the lens after 3 weeks of pretreatment with TTE in any of the groups. During the treatment period, after starting the galactose diet, all galactose-fed groups showed cataractous changes in the lens that progressed over 4 weeks. However, we observed slower progression of cataract in groups that received 0.03% and 0.02% TTE compared to vehicle group. The 0.03% TTE concentration had a significantly lower opacity index compared to 0.02% TTE at weeks 2, 3, and 4. Conversely, the group that received the 0.2% TTE concentration showed significantly greater cataractous changes than the vehicle-treated group. The groups that received 0.1%, 0.05%, and 0.01% did not show any differences compared to vehicle-treated group. This trend in the progression of cataract was observed throughout the 4-week experimental period (Figure 2).

Effect of tocotrienol on lens protein level: The ratio of lens soluble to insoluble protein was 3.97-fold lower in the vehicle-treated groups compared to the normal group ($p<0.001$). The groups treated with 0.03% and 0.02% TTE showed a trend toward normalization of the lens soluble to insoluble protein ratio. The soluble to insoluble protein ratio in the 0.03% TTE group had a significantly higher value compared to the 0.02% TTE group ($p<0.01$). However, all other treatment groups showed significantly lower soluble to insoluble protein ratios with a maximum decrease of 1.87-fold in the group treated with 0.2% TTE compared to the vehicle-treated group (Table 3).

Effect of tocotrienol on lens redox status: The lens GSH content remained significantly low in all galactose-fed groups including those that received 0.03% and 0.02% TTE compared to group 1. However, these two TTE-treated groups showed 2.3-fold higher GSH content compared to the galactose-fed vehicle-treated group ($p<0.05$).

Quantification of the antioxidant enzyme activity in the lenses showed that CAT activity was restored to normal in groups treated with 0.03% and 0.02% TTE, whereas the vehicle-treated group and the groups treated with 0.2% and 0.1% TTE showed a decrease in CAT activity by 2.30-, 1.91-, and 1.5-fold, respectively, compared to the normal group. SOD activity was restored to normal in the groups treated with 0.03% and 0.02% TTE. SOD activity was reduced by 1.61-, 2.76-, 1.67-, 1.32-, and 1.25-fold in the vehicle-treated group and the groups treated with 0.2%, 0.1%, 0.05%, and 0.01%, respectively, compared to the normal group ($p<0.05$). The lens MDA levels were normalized in the groups treated with 0.03% and 0.02% TTE whereas the other galactose-fed

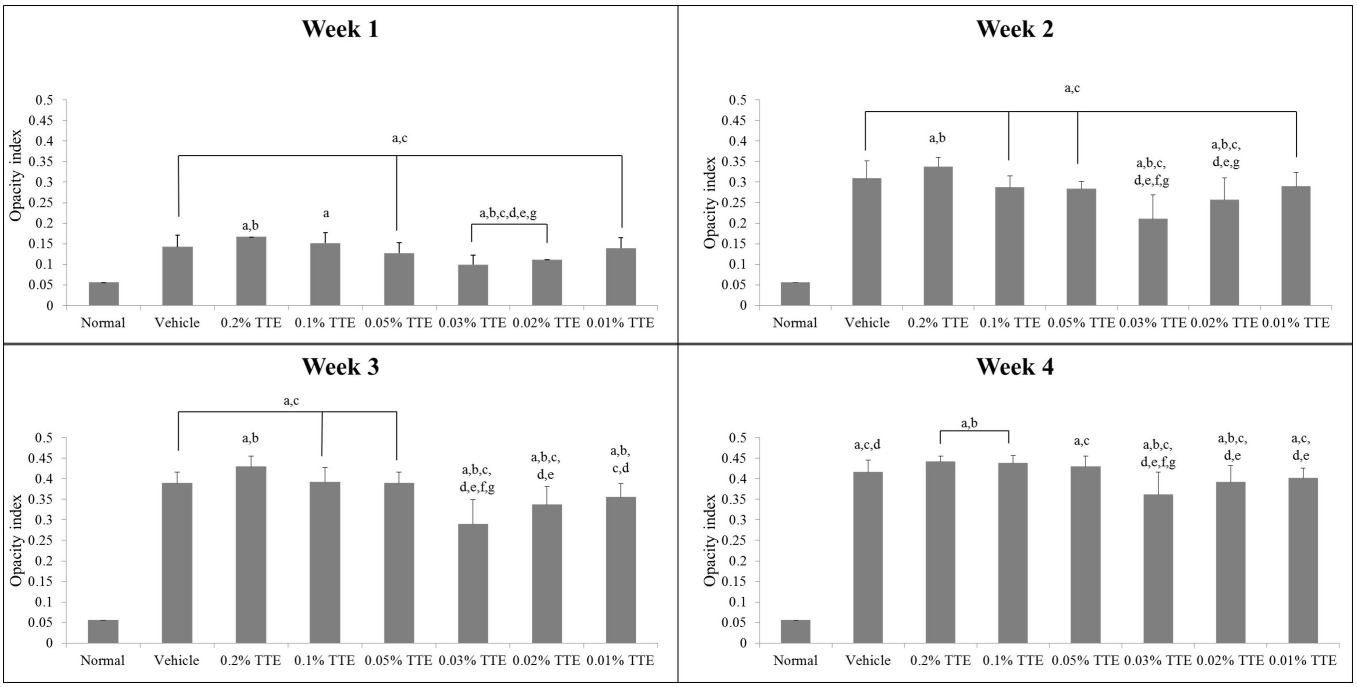

Figure 2. Effect of microemulsion formulation of tocotrienol (TTE) in various concentrations on the opacity index of galactose-fed rats during 4 weeks of treatment. All values are mean ± standard deviation (SD; n = 18). [a]$p<0.001$ versus normal; [b]$p<0.01$ versus vehicle; [c]$p<0.05$ versus 0.2% tocotrienol (TTE); [d]$p<0.05$ versus 0.1% TTE; [e]$p<0.05$ versus 0.05% TTE, [f]$p<0.05$ versus 0.02% TTE, [g]$p<0.05$ versus 0.01% TTE.

Table 3. Effect of topical tocotrienol microemulsion (TTE) in different concentrations on lens proteins (Study 1: dose–response study using microemulsion formulation).

Groups	Total protein (mg/g lens weight)	Soluble protein (mg/g lens weight)	Insoluble protein (mg/g lens weight)	Soluble: Insoluble protein (Ratio)
Normal	523.43±37.6	491.06±25.8	32.37±17.3	17.23±7.7
Vehicle	428.61±45.7 [a]	340.40±49.1 [a]	88.21±30.7 [a,c]	4.33±1.7 [a, c]
0.2% TTE	426.61±28.3 [a]	295.95±36.5 [a]	130.66±16.3 [a]	2.32±0.5 [a, b]
0.1% TTE	446.63±57.3 [a]	342.64±65.1 [a]	103.99±46.8 [a]	3.92±2.0 [a]
0.05% TTE	432.61±73.5 [a]	365.16±61.2 [a, c]	67.45±17.5 [a, c]	5.60±1.2 [a, c]
0.03% TTE	556.88±116.4 [b, c, e]	524.61±130.8 [b, c, d, e, g]	32.28±15.3 [b, c, d, e, g]	18.77±6.6 [b, c, d, e, f, g]
0.02% TTE	480.50±21.9 [a, b, c, g]	425.20±42.2 [a, b, c, g]	55.31±27.5 [b, c, d,]	9.22±3.8 [a, b, c, d, g]
0.01% TTE	433.93±34.5 [a]	361.97±41.5 [a, c]	71.95±14.5 [a, c]	5.30±1.7 [a, c]

All values are mean ± SD (n=6). [a]p<0.05 versus Normal; [b]p<0.05 versus Vehicle; [c]p<0.05 versus TTE 0.2%; [d]p<0.05 versus TTE 0.1%; [e]p<0.05 versus TTE 0.05%, [f]p<0.05 versus TTE 0.02%, [g]p<0.05 versus TTE 0.01% .

groups showed significantly higher lens MDA content compared to the normal group (Table 4).

Study 2:

Anticataract efficacy of microemulsion versus liposome—In study 1, we observed higher anticataract efficacy of 0.03% TTE compared to 0.02% TTE in weeks 2, 3, and 4. Additionally, the soluble to insoluble protein ratio in 0.03% TTE showed a significantly higher value compared to the 0.02% TTE concentration. Thus, we selected 0.03% TTE for study 2.

As in the dose–response study, anterior segment imaging did not show any changes in the lens after 3 weeks of pretreatment with tocotrienol in any of the groups. All galactose-fed groups showed significant cataractous changes in the lens that progressed over 4 weeks during the treatment period. As observed before, slower progression of cataract was seen in groups that received 0.03% TTE and 0.03% TTL compared to the corresponding vehicle-treated groups. However, there was no significance difference in the opacity index between the TTE- and TTL-treated groups at all time points, during the 4-week treatment period (Figure 3, Table 5).

Effect of different formulation of tocotrienol on lens protein level: In this study, we also observed normalization of the soluble to insoluble protein ratio in the tocotrienol-treated groups (p<0.05). However, there was no significant difference

Table 4. Effect of topical tocotrienol microemulsion (TTE) in different concentrations on lens MDA, GSH, CAT and SOD. (Study 1: dose–response study using microemulsion formulation).

Groups	Lens MDA (µmol/g lens weight)	Lens GSH (µmol/g lens weight)	Lens CAT (µmol/g lens protein)	Lens SOD (Units/mg lens protein)
Normal	60.26±7.1	5.00±1.4	86.63±26.9	9.41±1.8
Vehicle	81.24±12.8 [a]	0.48±0.1[a]	36.73±14.4[a]	5.86±0.5[a, c]
0.2% TTE	93.48±8.4 [a]	0.53±0.1[a]	45.44±22.1[a]	3.40±0.8[a]
0.1% TTE	82.65±8.3 [a, c]	0.65±0.3[a]	55.29±5.2[a]	5.63±1.6[a, c]
0.05% TTE	73.40±10.9 [a, c]	0.71±0.3[a, b]	71.89±26.0[b,c,d]	7.14±3.2[c]
0.03% TTE	53.04±15.2 [b, c, d, e, f]	1.02±0.5[a, b, c]	85.12±13.9[b, c, d, f]	10.13±3.0[b, c, d]
0.02% TTE	59.77±17.4[b, c, d, f]	1.00±0.5[a, b, c]	85.18±22.7[b, c, d, f]	10.0±1.5[b, c, d]
0.01% TTE	79.68±15.2[a]	0.85±0.3[a, b, c]	58.62±6.7 [a, b, c]	7.5±2.9[b, c, d]

All values are mean ± SD (n=6). [a]p<0.05 versus Normal; [b]p<0.05 versus Vehicle; [c]p<0.05 versus TTE 0.2%; [d]p<0.05 versus TTE 0.1%; [e]p<0.05 versus TTE 0.05%, [f]p<0.05 versus TTE 0.01%. MDA- malondialdehyde, GSH- reduced glutathione, CAT- catalase, SOD – superoxide dismutase.

Table 5. Effect of microemulsion and liposomal formulation of tocotrienol (0.03%) on the progression of cataract during 4 weeks period of treatment (Study 2: anticataract efficacy of microemulsion versus liposome).

Groups	Week 1		Week 2		Week 3		Week 4	
	Stage of cataract	% of lenses	Stage of cataract	% of lenses	Stage of cataract	% of lenses	Stage of cataract	% of lenses
Normal	0	100	0	100	0	100	0	100
VE	1A	40	2A	66.67	2B	10 90	3	43.33
	1B	60	2B	26.67	3		4	56.67
			3	6.67				
VL	1A	46.67	1B	3.33	2A	6.67	3	40
	1B	53.33	2A	60	2B	40	4	60
			2B	33.33	3	43.33		
0.03% TTE	0	6.67	3	3.33	4	10	2B	16.67
	1A	83.33	1B	20	2A	33.33	3 4	63.33
	1B	10	1C	3.33	2B	40		20
			2A	76.67	3	26.67	1A	6.67
0.03% TTL	0	33.33	1A	13.33	1A	6.67	2A	13.33
	1A	30	1B	33.33	1B	6.67	2B	36.67
	1B	36.67	1C	10	2A	26.67	3 4	36.67
			2A	30	2B	50		6.67
			2B	13.33	3	10		

VE – vehicle for microemulsion; VL – vehicle for liposomes; TTE – tocotrienol in microemulsion; TTL – tocotrienol in liposomal formulation

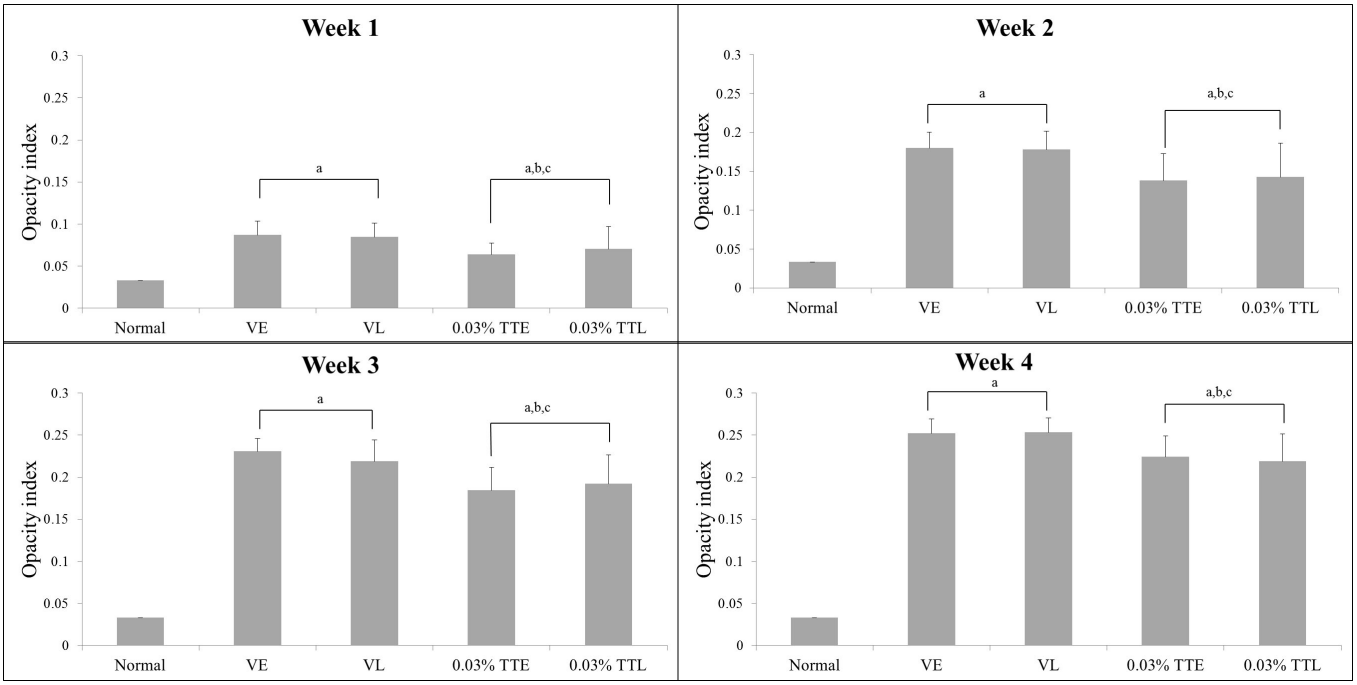

Figure 3. Effect of tocotrienol (0.03%) in microemulsion (TTE) and liposomal (TTL) formulation of on the opacity index of galactose fed rats during 4 weeks period of treatment. VE - vehicle for microemulsion, VL - vehicle for liposomes. All values are mean ± standard deviation (SD; n = 6). [a]p<0.05 versus normal; [b]p<0.05 versus VE; [c]p<0.05 versus VL.

Table 6. Effect of microemulsion and liposomal formulation of tocotrienol (0.03%) on the lenticular protein during 4 weeks period of treatment (Study 2: anticataract efficacy of microemulsion versus liposome).

Groups	Total protein (mg/g lens weight)	Soluble protein (mg/g lens weight)	Insoluble protein (mg/g lens weight)	Soluble: Insoluble protein (Ratio)
Normal	523.43±37.6	491.06±25.8	32.37±17.3	17.23±7.7
VE	428.61±45.7 [a]	340.40±49.1 [a]	88.21±30.7 [a]	4.33±1.7 [a]
VL	422.08±76.4 [a]	344.25±78.8 [a]	77.83±19.0 [a]	4.45±0.3 [a]
0.03% TTE	556.88±116.4 [b]	524.61±130.8 [b]	32.28±15.3 [b]	18.77±6.6 [b]
0.03% TTL	529.96±30.8 [c]	499.60±42.3 [c]	30.36±13.5 [c]	18.58±6.0 [c]

All values are mean ± SD (n=6). [a]p<0.05 versus normal; [b]p<0.05 versus VE; [c]p<0.05 versus VL. VE – vehicle for microemulsion; VL – vehicle for liposomes; TTE – tocotrienol in microemulsion; TTL – tocotrienol in liposomal formulation

Table 7. Effect of microemulsion and liposomal formulation of tocotrienol (0.03%) on the lenticular MDA, GSH, CAT and SOD during 4 weeks period of treatment (Study 2: anticataract efficacy of microemulsion versus liposome).

Groups	Lens MDA (µmol/g lens weight)	Lens GSH (µmol/g lens weight)	Lens CAT (µmol/g lens protein)	Lens SOD (Units/mg lens protein)
Normal	60.26±7.1	5.00±1.4	86.63±29.9	9.41±1.8
VE	81.24±12.8 [a]	0.48±0.1 [a]	36.73±14.4 [a]	5.16±1.3 [a]
VL	95.28±19.3 [a]	0.48±0.1 [a]	35.29±6.9 [a]	5.23±1.2 [a]
0.03% TTE	53.04±15.2 [b]	1.02±0.5 [a, b]	85.12±13.9 [b]	10.13±3.0 [b]
0.03% TTL	61.94±7.0 [c]	1.16±0.2 [a, c]	82.33±26.5 [c]	11.98±5.8 [c]

All values are mean ± SD (n=6). [a]p<0.05 versus normal; [b]p<0.05 versus VE; [c]p<0.05 versus VL. MDA- malondialdehyde; GSH- reduced glutathione; CAT- catalase; SOD – superoxide dismutase; VE – vehicle for microemulsion; VL – vehicle for liposomes; TTE – tocotrienol in microemulsion; TTL – tocotrienol in liposomal formulation.

between the groups treated with microemulsion and liposome (Table 6).

Effect of different formulation of tocotrienol on lens redox status: Both formulations at the 0.03% concentration showed a tendency to restore the lenticular GSH content toward normal. The GSH content was 2.1- (p<0.05) and 2.4- (p<0.01) fold higher in the TTE- and TTL-treated groups, respectively, compared to the corresponding vehicle-treated groups. A similar observation was made for the lens CAT and SOD activity. CAT activity increased by 2.32- and 2.33-fold in the TTE- and TTL-treated groups, respectively, compared to the corresponding vehicle-treated groups (p<0.01). SOD activity increased by 2.0- and 2.3-fold in TTE- and TTL-treated groups, respectively, compared to the corresponding vehicle-treated groups (p<0.01). The lenticular MDA content

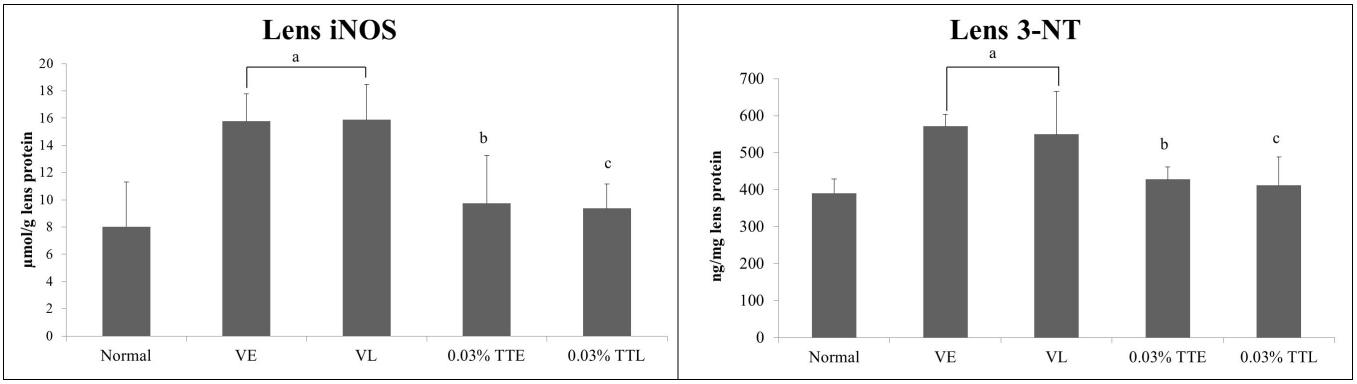

Figure 4. Effect of microemulsion and liposomal formulation of tocotrienol (0.03%) on lens iNOS and 3-NT during 4 weeks period of treatment. iNOS- inducible nitric oxide synthase; NT- nitrotyrosine; VE – vehicle for microemulsion; VL – vehicle for liposomes. All values are mean ± SD (n=6). [a]p < 0.05 versus normal; [b]p < 0.05 versus VE; [c]p < 0.05 versus VL.

was 1.53- and 1.54-fold lower in the TTE- and TTL-treated groups, respectively, compared to the corresponding vehicle-treated groups ($p<0.05$; Table 7).

Effect of different formulation of tocotrienol on lens nitrosative stress: The TTE- and TTL-treated groups showed a tendency toward normalization of lens iNOS activity. Compared to the respective vehicle-treated groups, we observed 1.6- and 1.7-fold lower ($p<0.01$) iNOS activity in the TTE- and TTL-treated groups, respectively. Similar observations were made for 3-NT levels with 1.34- and 1.33-fold lower values ($p<0.05$) in the TTE- and TTL-treated groups, respectively, compared to the corresponding vehicle-treated groups (Figure 4).

DISCUSSION

In the present study, for the first time we demonstrated the anticataract effects of topically applied Annatto tocotrienol. Tocotrienol is a lipophilic substance, which makes it insoluble in tear film. This prevents its close contact with the cornea, thus causing poor ocular bioavailability. Therefore, in the first part of this study we used microemulsion and observed that TTE delayed the onset and progression of cataract in galactose-fed rats at 0.02% and 0.03% concentrations. The anticataract effect of the 0.03% concentration was higher than that of the 0.02% TTE concentration in weeks 2, 3 and 4. The 0.2% TTE concentration aggravated cataractogenesis, whereas the effects of the 0.1%, 0.05%, and 0.01% TTE concentrations were comparable to that of vehicle. Since the unique structure of liposomes allows them to entrap a significant amount of the lipophilic drug in particular, we also prepared the liposomal formulation of tocotrienol for topical application. In the second part of the study, we compared the efficacy of the microemulsion and liposomal formulations of tocotrienol in a 0.03% concentration. Between these two groups, we did not observe any significant difference in the rate of progression of cataract.

In the 0.03% and 0.02% TTE-treated groups, delayed cataractogenesis was associated with decreased lenticular oxidative stress. Previous studies have shown that tocotrienols act as potent antioxidants by donating their phenolic hydrogen to free radicals and neutralize them, thus sparing the endogenous antioxidants [44,45]. Experimental galactosemia as well as diabetes in rats have been shown to increase oxidative stress [46-48]. Similar observations were made in the *Drosophila melanogaster* model of classic galactosemia [49]. Increased oxidative stress resulting from excessive production of free radicals or reduced lenticular antioxidant defense has been shown to underlie the pathogenesis of cataract [50-52]. The lens antioxidant enzymes include SOD, CAT, and glutathione peroxidase. SOD eliminates superoxide ions by converting them to H_2O_2, while catalase and glutathione peroxidase detoxify H_2O_2 [53]. The lens contains an unusually high concentration of GSH, which protects against denaturation of thio-group-containing proteins in the presence of oxidative stress [54,55]. Increased oxidative stress causes lipid peroxidation that results in increased MDA levels. Patients with cataract have been shown to have increased plasma levels of lipid peroxidation products and decreased levels of glutathione [56,57].

Significant quantities of peroxynitrite, a metabolite of nitric oxide and a prooxidant, have been detected in cataractous lens. Örnek et al. showed that the nitric oxide level in the cataractous lens is higher than in the normal lens [10]. Ito et al. showed a high level of iNOS in selenite-induced cataracts [7]. Furthermore, aminoguanidine, a NOS inhibitor, was shown to have an inhibitory effect on the development of cataract [8]. These studies suggest that nitrosative stress, resulting from iNOS activation and overproduction of nitric oxide, has a role to play in cataractogenesis. In the current study, we demonstrated that tocotrienol at 0.03% concentration, in microemulsion and liposomal formulations, decreases lens iNOS activity and NT content compared to the corresponding vehicle-treated groups. The effect of tocotrienol on iNOS and NT observed in our study is in line with other studies that showed the ability of tocotrienol to reduce the activity of iNOS in human monocytic cells [58] and the murine macrophage cell line [59].

Increased oxidative stress alters membrane permeability, thus affecting the cellular ionic balance, particularly intracellular calcium [60]. Increased intracellular calcium results in activation of calpain, which causes degradation of soluble lens proteins, especially crystalline, into insoluble proteins. An increased ratio of insoluble to soluble proteins results in the loss of lens transparency and the development of cataract. Our study has shown that treatment with 0.03% and 0.02% TTE tends to restore the lens soluble to insoluble protein ratio; however, 0.03% TTE had a significantly greater effect compared to the 0.02%. Restoration of lens proteins could be attributed to preservation of lens redox status and thus reduced cataractogenesis. Significant differences in the ratio of the lens proteins between the 0.03% and 0.02% TTE-treated groups despite comparable effects on lens oxidative stress is perhaps due to additional mechanisms underlying the anticataract effects of TTE. Similar changes in the lens protein ratio were also observed when tocotrienol was administered in the liposomal formulation without any significant difference from the microemulsion-treated group.

Importantly, the current study showed that increasing the concentration of TTE beyond 0.03% caused, at first, the loss of anticataract effects and further increases enhanced cataractogenesis. TTE at the 0.1% and 0.2% concentrations had prooxidant effects. Previous studies have also shown that tocotrienols exert a prooxidant effect at high doses in vitro [61,62]. In vivo studies and clinical trials have also reported reversal of its cholesterol-lowering effects at high doses [63,64]. The prooxidant effect of TTE at higher doses might be attributed to its conversion to α-tocopherol in vivo, which has been shown to be a highly reactive prooxidant at a high concentration [65,66]. Furthermore, cell culture studies using osteoblasts [65], fibroblasts [67], myoblasts [68], and neuronal cells [69] have shown that γ-tocotrienol has cytotoxic activity at higher doses, which might be attributed to its prooxidant activity. In our study, the Annatto tocotrienol contained 10% γ-tocotrienol, and this might have contributed to the prooxidant effect. However, the exact mechanism of the prooxidant effect of γ-tocotrienol remains unknown. One limitation of this study was that we could not determine the major constituent contributing to prooxidant effects. Further studies using only the δ or γ isomer of tocotrienol would be beneficial in determining the same.

Another important observation made in this study was the lack of significant differences between the microemulsion- and liposome-treated groups for any of the parameters measured. Thus, the effectiveness of microemulsion in delivering tocotrienol to ocular tissue such as the lens seems to be comparable to that of liposomes. Previous studies have shown variable results in this regard. Hironaka et al. demonstrated that liposomes have better penetration in ocular tissues compared to microemulsion in vivo using a lipophilic dye as a marker [70]. However, Cortessi et al. showed that the formulations exerted similar effects [71]. In one study, microemulsion, as a drug carrier, showed better tissue permeation for a lipophilic drug in mice skin compared to liposomes [72]. In the current study, however, we did not determine the relationship between the extent of ocular tissue penetration by tocotrienol and its anticataract effects. More elaborate ocular pharmacokinetic studies are needed to study the ocular bioavailability of tocotrienol.

In summary, this study demonstrated the anticataract effects of topically applied tocotrienol in the concentration range of less than 0.05% and more than 0.01% in galactose-fed rats. At the 0.03% concentration, the microemulsion and liposomal formulations of tocotrienol showed comparable anticataract effects. The anticataract effect of tocotrienol could be attributed to reduced lenticular oxidative stress and attenuation of nitrosative stress. At a concentration of 0.2% and higher, TTE aggravates cataractogenesis in galactose-fed rats by increasing lens oxidative stress. Precise molecular targets that lead to the prooxidant effects and mechanisms involved in decreasing the nitrosative or oxidative stress of tocotrienol remain to be determined.

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