Molecular Vision 2016; 22:734-747 <http://www.molvis.org/molvis/v22/734>
Received 16 December 2015 | Accepted 8 July 2016 | Published 10 July 2016

Mechanism of the anticataract effect of liposomal magnesium taurate in galactose-fed rats

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Purpose: Increased lenticular oxidative stress and altered calcium/magnesium (Ca/Mg) homeostasis underlie cataractogenesis. We developed a liposomal formulation of magnesium taurate (MgT) and studied its effects on Ca/Mg homeostasis and lenticular oxidative and nitrosative stress in galactose-fed rats.

Methods: The galactose-fed rats were topically treated with liposomal MgT (LMgT), liposomal taurine (LTau), or corresponding vehicles twice daily for 28 days with weekly anterior segment imaging. At the end of the experimental period, the lenses were removed and subjected to analysis for oxidative and nitrosative stress, Ca and Mg levels, ATP content, Ca^{2+}-ATPase, Na^{+},K^{+}-ATPase, and calpain II activities.

Results: The LTau and LMgT groups showed significantly lower opacity index values at all time points compared to the corresponding vehicle groups ($p<0.001$). However, the opacity index in the LMgT group was lower than that in the LTau group ($p<0.05$). Significantly reduced oxidative and nitrosative stress was observed in the LTau and LMgT groups. The lens Ca/Mg ratio in LMgT group was decreased by 1.15 times compared to that in the LVh group. Calpain II activity in the LMgT group was decreased by 13% compared to the LVh group. The ATP level and Na^{+},K^{+}-ATPase and Ca^{2+}-ATPase activities were significantly increased in the LMgT group compared to the LVh group ($p<0.05$).

Conclusions: Topical liposomal MgT delays cataractogenesis in galactose-fed rats by maintaining the lens mineral homeostasis and reducing lenticular oxidative and nitrosative stress.

Cataract, the leading cause of blindness worldwide, remains an important public health issue, particularly for developing countries, due to the economic burden posed by surgical management. Several pharmacological strategies, particularly those that can restore the lens redox status, have been investigated. Our earlier study demonstrated that topical application of magnesium taurate (MgT) delays the onset and progression of cataract in galactose-fed rats [1]. We also observed that this anticataract effect of MgT is associated with reduced oxidative stress and a significant improvement in the lenticular calcium /magnesium (Ca^{2+}/Mg^{2+}) ratio. However, it remains to be determined whether this effect of MgT on the lens ionic balance helps to restore the lens ATP and ATPase functions. Improved Na^{+}-K^{+}-ATPase functions prevent cellular swelling while improved Ca^{2+}-ATPase functions maintain a low cytoplasmic Ca^{2+} and thus prevent activation of the enzyme calpain that causes aggregation of soluble to insoluble lens proteins resulting in the development of cataract [2-4]. Furthermore, studies have shown that magnesium prevents nitrosative stress [2], and therefore, it is likely that MgT–induced inhibition of cataractogenesis involves reduction of nitrosative stress.

In our previous study, as we observed that treatment of isolated lenses with MgT provides higher correction in the Ca^{2+}/Mg^{2+} ratio and the lens redox status [1], it is likely that the increase in ocular bioavailability could further enhance the MgT–induced delay in the onset and progression of cataract. Ocular bioavailability is often a concern due to anatomic and physiologic barriers on the ocular surface. The unique structure of the cornea consisting of epithelial cells and hydrophilic stroma restricts the transcorneal permeation of hydrophilic drugs. Furthermore, the tear film with its proteins and enzymes also acts as a barrier for transcorneal drug permeation. Although the cornea consists of several transporters to facilitate the movement of different molecules, such as amino acids, special drug delivery systems are useful for

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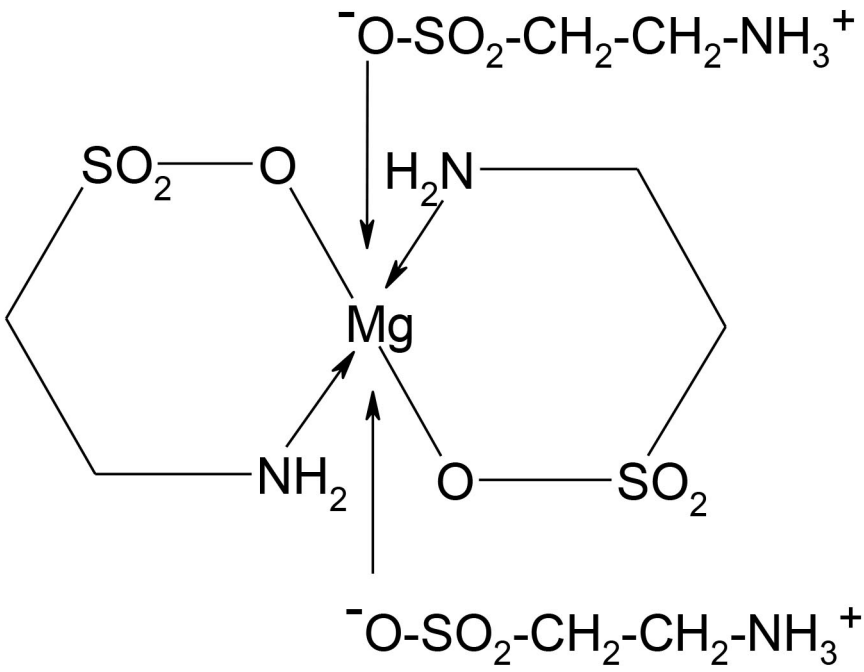

Figure 1. Structural formula of MgT.

enhancing the intraocular bioavailability of various classes of drugs. Liposomes as the corpuscular carriers are one such delivery system that enhance the permeation of topically delivered drugs across the cornea [5,6].

In the current study, we developed a liposomal formulation of MgT and studied the anticataract efficacy of the topical application of MgT in comparison with the aqueous solution of MgT. Further, we investigated the effects of MgT on lens Ca/Mg homeostasis, lenticular oxidative and nitrosative stress, lens ATP content, ATPase functions, and calpain activity in galactose-fed rats.

METHODS

Animals: All experiments and animal handling in this study were performed in accordance with the ARVO Statement for the Use of Animals in Research and approved by Institutional Animal Care and Use Committees (IACUC) of University Teknologi MARA, Malaysia (ACUC approval number – ACUC-6/12 on 30 October 2012). Sprague-Dawley rats of either sex weighing 80–100 g were procured from the Laboratory Animal Care Unit of Universiti Teknologi MARA. The animals were housed under standard laboratory conditions (23 ± 2 °C and a 12 h:12 h light-dark cycle) with food and water given ad libitum. All rats were subjected to ophthalmic examination, and those with any ophthalmic abnormalities were excluded from the study.

Study design:

Study 1: Anticataract effect of liposomal MgT versus aqueous MgT—In study 1, the effect of the liposomal formulation of MgT was compared with that of aqueous MgT. The animals were divided into five groups of nine rats each. The grouping, diet, and treatment given were as follows: Group 1, normal diet (ND); Group 2, 25% galactose diet + empty liposomal vehicle topically (LVh); Group 3, 25% galactose diet + aqueous vehicle topically (AVh); Group 4, 25% galactose diet + liposomal MgT (LMgT); and Group 5, 25% galactose diet + aqueous 1% MgT (AMgT). All rats were treated topically, bilaterally, and twice daily with a 10 µl solution in a single drop. Diet and treatment were continued for a total of 28 days with weekly anterior segment imaging.

Study 2: MgT versus taurine—In study 2, we used the formulation (aqueous or liposomal) that showed higher anticataract efficacy based on the anterior segment imaging in study 1. The effect of the chosen formulation of MgT was compared with the same formulation of taurine. The animals' grouping, diet, and treatment given were as follows: Group 1, normal diet (ND); Group 2, 25% galactose diet + vehicle (L/AVh); Group 3, 25% galactose diet + MgT (L/AMgT); and Group 4, 25% galactose diet + taurine (L/ATau).

As in study 1, all rats were treated topically, bilaterally, and twice daily with a 10 µl solution in a single drop. The diet and treatment were continued for a total of 28 days

with weekly anterior segment imaging. At the end of the experimental period, all rats were euthanized using overdose of IP ketamine (250 mg/kg) and xylazine (50 mg/kg). The lenses were removed and subjected to analysis for reduced glutathione (GSH) and catalase (n = 6), superoxide dismutase (SOD; n = 6), and malondialdehyde (MDA; n = 6) using enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemicals, Ann Arbor, MI). Lens Ca and Mg levels (n = 5) were determined using Cobas Integra as described previously [1]. Lens ATP content (n = 6) was determined with the chemiluminescence method. The lenticular inducible nitric oxide synthase (iNOS) activity and the nitrotyrosine level (n = 6) were determined using ELISA kits. Lens Ca^{2+}-ATPase (n = 6), Na^{+},K^{+}-ATPase (n = 6), and calpain II activities (n = 6) were analyzed using colorimetric methods.

Preparation for topical formulations:

Aqueous MgT—The taurine salt of magnesium (Figure 1) was synthesized based on McCarty's method [7] and as described in our previous study [1]. The aqueous preparation of MgT was prepared by dissolving its appropriate quantity in 1% hydroxypropyl methylcellulose (HPMC) to obtain a 1% solution of MgT.

MgT and taurine loaded liposomes: MgT, taurine, or deionized water was incorporated into the liposomes by the dehydration–rehydration method [8,9]. A lipid mixture of phosphatidylcholine (PC) and cholesterol (8:1) was dissolved in chloroform:methanol (5:1) and dried to a thin film of lipids in a round bottom flask using a rotatory evaporator at 40 °C in a water bath for 2 h. The lipids were rehydrated by adding the MgT or taurine (1% in deionized water) or deionized water. This dispersion was frozen at −80 °C for 5 min followed by thawing in a water bath at 40 °C for 1 min. This freeze–thaw cycle was repeated five times. Then the solution was sonicated for 10 min (Sonic Dismembrator, Fisher Scientific, Loughborough, UK). Freeze–thaw and sonication were performed to obtain the small unilamellar vesicles from the multilamellar vesicles of liposomes. Then, the liposomes were extruded through two stacks each of 400, 200, and 100 nm pore size polycarbonate membranes (Avanti Polar Lipids, Inc., Alabaster, AL) using a handheld extruder. The extrusion through the handheld extruder was repeated ten times for each pore size.

Characterization of size and surface charge of liposomal dispersions: The size and the zeta (ζ) potential of the liposomes were determined using the Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), operating with a helium-neon laser at a wavelength of 633 nm at a scattering detection angle of 175° at 25 °C. Results were the means of triplicate runs performed on different days using freshly prepared samples. In each run, three measurements were made. Standard deviations were determined from the replicate runs instead of the replicate measurements.

Anterior segment imaging and assessment of the progression of cataract: The anterior segment imaging was performed by two independent investigators using a Hawkeye Portable Slit-Lamp equipped with a digital camera, and tropicamide 1% was used as the mydriatic. Both investigators were blinded to the grouping and the treatment given. Lenticular changes

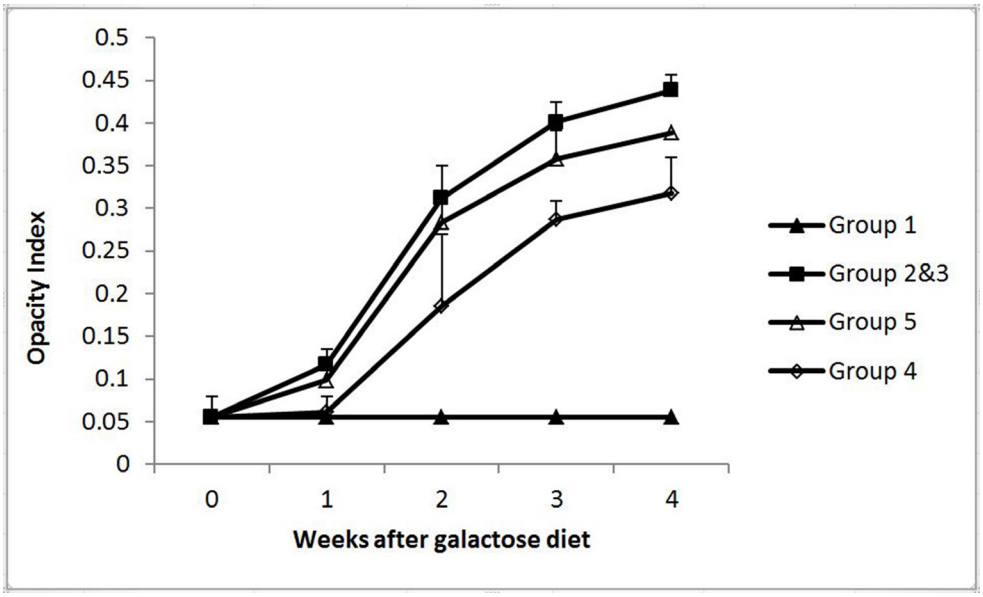

Figure 2. Effect of aqueous and liposomal MgT on the onset and progression of cataract in galactose-fed rats. Group 1, normal rats; groups 2 and 3, aqueous and liposomal vehicle-treated groups; Group 4, liposomal MgT–treated group; and Group 5, aqueous magnesium-treated group. *p<0.0001 versus Group 5; **p<0.0001 versus Groups 2 and 3; ***p<0.01 versus Groups 2 and 3; #p<0.05 versus groups 2 and 3.

Figure 3. Effect of liposomal MgT and taurine on the onset and progression of cataract in galactose-fed rats. Group 1, normal rats; Group 2, empty liposome-treated group; Group 3, liposomal MgT–treated group; and Group 4, liposomal taurine-treated group. *p<0.0001 versus Group 4; **p<0.0001 versus Group 2.

were graded according to a modified version of Sippel's method [10] for a more objective assessment. This method of grading for semiquantitative assessment of the progression of cataract has been standardized in our laboratory and has previously been described [1,11]: Stage 0 (Grade 1), lenses similar to healthy lenses; Stage 1a (Grade 2), lenses show a narrow equatorial band of vacuoles; Stage 1b (Grade 3), vacuoles cover one third or less of the periphery of the anterior cortex; Stage 1c (Grade 4), vacuoles cover more than one third of the periphery of the anterior cortex; Stage 2a (Grade 5), cortical opacity with some clear areas and vacuoles still present; Stage 2b (Grade 6), the cortex is largely opaque with some clear areas, but vacuoles are not present; Stage 3 (Grade 7), uniform opalescence; and Stage 4 (Grade 8), mature nuclear cataract.

The opacity index was calculated as described by Vats et al. [12]. Accordingly, all eyes were given a grade of 0–8 depending on the stage of cataract (0–4). The mean of the scores observed by the two independent investigators was considered the final score recorded by a blinded investigator:

$$\text{Opacity index (OI)} = \frac{\text{Score} \times \text{number of eyes in that stage (within group)}}{\text{Total number of eyes (within group)}}$$

Calcium and magnesium assay: Five lenses from each group were individually homogenized in 0.6 ml of deionized water and centrifuged at 890 ×g for 15 min. The supernatant was separated and was processed according to the Cobas Integra manufacturer's protocol for Ca^{2+} and Mg^{2+} estimation in lens (COBAS Integra 400, Roche Diagnostics, Basel, CH) [13,14].

The protocol for Ca^{2+} estimation is based on the staining reaction of *o*-cresolphthalein complexone described by Schwarzenbach [13]. The Ca^{2+} reacts with *o*-cresolphthalein complexone (Roche Diagnostics) under alkaline conditions and forms a violet complex. The addition of 8-hydroxyquinoline (Roche Diagnostics) prevents interference with magnesium and iron. The color intensity of the complex formed is directly proportional to the calcium concentration and is determined by measuring the increase in absorbance at 552 nm.

Magnesium was measured with colorimetric assay using the method based on the staining reaction of Mg^{2+} and chlorophosphonazo III (CPZ III). CPZ III (Roche Diagnostics) binds to Mg^{2+} and causes an absorbance increase at 659 nm. Ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA; Roche Diagnostics) is used to inhibit Ca^{2+} binding to CPZ III. Nonspecific absorbance interferences were reduced by the addition of EDTA (Roche Diagnostics) that removes Mg^{2+} from the Mg^{2+}-CPZ III complex and allows for an accurate sample measurement. The difference in absorbance between the Mg^{2+}-CPZ III complex and the EDTA-treated complex is the absorbance of Mg^{2+} alone [14].

Estimation of lens proteins: Six lenses from each group were individually homogenized in 0.5 ml of PBS (50 mM, pH 7.4, containing 1 mM EDTA). One hundred microliters of homogenate were used for the total protein estimation, and the remainder was centrifuged at 890 ×g for 15 min at 4 °C. Then 100 µl of the supernatant was used for soluble protein estimation. Quantification of the protein in the samples was performed using a colorimetric protein assay kit (Cayman

Table 1. Effect of the liposomal Mg taurate and taurine on lenticular calcium-to-magnesium ratio, ATP level and ATPases activity in the lenses of galactosemic rats.

Group	Control diet (Group 1)	Galactose diet + vehicle (Group 2)	Galactose diet + Mg taurate (Group 3)	Galactose diet + Taurine (Group 4)
Lenticular Ca^{2+}/Mg^{2+}	0.57±0.17	3.66±0.38*	2.37±0.81*,^	3.12±0.35*,$
ATP level (µmol Pi/mg protein)	34.4±2.2	11.23±3.7*	20.0±5.1*,^	13.5±4.7*
Na^{+}/K^{+}-ATPase (µmol/g wet weight)	7.86±3.87	2.16±1.23*	6.74±3.73	2.47±1.06*,$
Ca^{2+}-ATPase activity (µmol Pi/mg protein)	0.22±0.01	0.14±0.03*	0.22±0.03^	0.16±0.01*,$

Footnote: All values are mean ± SD; *p<0.001 versus Group 1, ^p<0.05 versus Group 2, $p<0.05 versus Group 3; n=5

Chemical , Ann Arbor, MI) based on the Bradford method. The reaction in this method utilizes a staining reaction upon binding of the dye Coomassie Brilliant Blue to the proteins in the sample. The absorbance was measured colorimetrically at 595 nm using a SpectraMax 190 microplate reader (Molecular Devices , Sunnyvale, CA).

Estimation of lenticular oxidative stress: The total GSH and MDA content and SOD and catalase activity in the lenses (n = 6) were estimated using commercially available ELISA kits (Cayman Chemical) and a Spectra Max 190 microplate reader (Molecular Devices). The lenses were rinsed with PBS (pH 7.4), and after the wet weight was recorded, the lenses were homogenized in cold 50 mM PBS with 1 mM EDTA, pH 7.0, for determination of catalase activity and GSH and in cold 20 mM HEPES buffer, pH 7.2, for the SOD assay. The mixtures were then centrifuged at 2970 ×g for 5 min at 4 °C, and the supernatants were removed and stored at −80 °C until used. For estimation of the lens MDA levels, the lenses were homogenized with radioimmunoprecipitation assay lysis (RIPA) buffer containing protease inhibitor in a ratio of 1 mg lens weight to 10 µl RIPA buffer. The samples were then centrifuged at 1,600 ×*g* at 4 °C for 10 min, and the supernatant was used for analysis.

GSH: GSH estimation in the supernatant was based on the enzymatic recycling method. Supernatant from lens homogenate (n = 6) was deproteinated by adding an equal volume of metaphosphoric acid reagent consisting of 5 g of metaphosphoric acid in 50 ml of water, and the mixture was centrifuged at 890 ×g for 2 min. GSH was estimated spectrophotometrically in the deproteinized homogenate.

SOD: The SOD activity assay used xanthine oxidase and hypoxanthine to generate superoxide radicals that were detected by tetrazolium salt, and absorbance was read at 440 nm. One unit of enzyme activity was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Catalase: Estimation of catalase activity was based on the reaction of methanol with the enzyme in the presence of an optimal concentration of hydrogen peroxide, and absorbance was read at 540 nm. One unit of CAT activity was defined as the amount of enzyme causing formation of 1 nmol of formaldehyde per minute.

MDA: For estimation of MDA, 100 µl of samples or standards were added to 100 µl of sodium dodecyl sulfate solution followed by 4 ml of color reagent consisting of thiobarbituric acid (TBA). Solutions were boiled for 1 h and incubated in an ice bath for 10 min to stop the reaction, and the solutions were centrifuged at 4 °C for 10 min. The MDA-TBA complex formed under high temperature (90–100 °C) and acidic conditions was measured colorimetrically at 540 nm. All estimations were performed in duplicate.

Estimation of lenticular nitrosative stress:

Inducible nitric oxide synthase—For sample preparation, six lenses from each group were individually homogenized in 1 ml of 50 mM cold PBS, pH 7.4, containing 1 mM EDTA, and centrifuged at 890 ×g for 15 min. The iNOS level was determined using a commercially available ELISA kit (USCN Life Sciences Inc., Houston, TX) according to

Table 2. Effect of liposomal Mg taurate and taurine on oxidative and nitrosative stress in the lens of galactosemic rats

Group	Control diet (Group 1)	Galactose diet + vehicle (Group 2)	Galactose diet + Mg taurate (Group 3)	Galactose diet + Taurine (Group 4)
GSH (umol/g lens weight)	4.38±0.56	0.44±0.09*	0.73±0.18*,#	0.64±0.20*
Catalase (umol/g lens protein)	54.85±8.84	37.96±8.72**	53.75±9.56#	49.61±8.04^,$
SOD (Units/mg lens protein)	6.65±1.45	10.55±0.68*	7.37±2.73^	8.22±1.81^
MDA (umol/g lens weight)	62.72±4.75	92.57±9.31*	59.05±6.62@	57.42±14.36@
iNOS (ng/mg lens protein)	109.76±5.58	196.58±13.19+	96.13±4.66^	115.38±5.45^
Nitrotyrosine (ng/mg lens weight)	29.80±4.01	58.35±3.13+	26.84±4.34^	26.36±5.94^

All values are mean ± SD; *p<0.001 versus Group 1, **p<0.01 versus Group 1, +p<0.05 versus Group 1, @p<0.001 versus Group 2, #p<0.01 versus Group 2, ^p<0.05 versus Group 2, $p<0.01 versus Group 3

the manufacturer's instructions. The microtiter plate had been precoated with an antibody specific to iNOS. Samples and standards were added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific to iNOS. Avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well and incubated. After the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added, only wells that contained iNOS, biotin-conjugated antibody, and enzyme-conjugated avidin exhibited a color change. The enzyme–substrates reaction was terminated by the addition of sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm using a Tecan Safire microplate reader (Tecan, Männedorf, CH). All estimations were performed in duplicate.

Nitrotyrosine: The lens 3-nitrotyrosine (3-NT) content was determined using an ELISA kit that utilizes polyclonal anti-3-NT antibody (Abcam, Cambridge, UK). Briefly, the lens homogenate (n = 6) was suspended to 25 mg/ml in PBS and was solubilized by adding four volumes of sample extraction buffer to a sample protein concentration of 5 mg/ml. The samples were incubated on ice for 20 min and centrifuged at 1,200 ×*g* at 4 °C for 20 min. The supernatants were used to quantify 3-NT, a product of tyrosine nitration resulting from oxidative damage to proteins by peroxynitrite. The provided microplates were coated with nitrotyrosine containing antigen. The ELISA was performed by adding the test sample mixed with the provided HRP-conjugated anti-3-NT antibody. All the anti-3NT detector antibody is available to bind to the immobilized 3-NT containing protein coating the wells if no 3-NT modified protein is present in the samples. In contrast, if soluble 3-NT modified protein is present in the sample, the protein will compete for binding with the anti-3NT detector antibody. The degree of competition is proportional to the concentration of soluble 3-NT modified proteins in the sample. Therefore, the signal in each well has an inverse relationship to the amount of 3-NT in each sample. The color change was detected at 450 nm using a Tecan Safire microplate reader. All estimations were performed in duplicate.

Estimation of ATP level: Five lenses from each group were individually homogenized in 1 ml of 70% ethanol (v/v) containing 2 mM EDTA (pH 10.9). Then, the homogenate was centrifuged at 890 ×g for 15 min at 4 °C to get the supernatant. Subsequently, the supernatant were diluted ten times with 0.1 M Tris-HCL/2 mM EDTA buffer (pH 7.8). The ATP content was measured using the ATPlite™ Bioluminescence Assay Kit from Perkin Elmer (Waltham, MA) and the 96-well Victor X5 microplate reader (Perkin Elmer) against an ATP calibration curve.

Determination of Na^+/K^+-ATPase and Ca^{2+}-ATPase activities: Tissue preparation was based on a modified version of Zarros et al.'s method [15]. Five lenses from each group were individually homogenized in 600 µl ice-cold (0–4 °C) medium containing 50 mM Tris-HCl, pH 7.4, and 300 mM sucrose using sonicator, followed by centrifugation at 4 °C for 5 min. The protein content was determined with 100 µl of supernatant as previously described. The remainder of the supernatant was used to estimate Na^+/K^+-ATPase and Ca^{2+}-ATPase activity.

Na^+/K^+-ATPase: The enzymatic activity was measured based on Chun-Fa Huang et al.'s [16] and Zarros et al.'s [15] methods. Briefly, 30 µl of assay buffer (118 mM NaCl, 1.67 mM KCl, 1.2 mM $MgCl_2$, 12.3 mM $NaHCO_3$, 11 mM glucose, 0.5 mM EGTA, pH 7.4) was added to the lens homogenate equivalent to 2 µg lens protein in each well. The assay was started with

Table 3. Effect of liposomal Mg taurate and taurine on calpain activity and protein level in galactosemic rats.

Parameter	Control diet (Group 1)	Galactose diet + vehicle (Group 2)	Galactose diet + Mg taurate (Group 3)	Galactose diet + Taurine (Group 4)
Calpain activity (µmol/mg wet weight)	0.42±0.03	0.61±0.06^{+}	0.53±0.02$^{+,\wedge}$	0.56±0.02^{+}
Total protein (mg/g lens weight)	475.93±51.94	462.20±77.88	474.24±37.59	473.68±76.43
Soluble protein (mg/g lens weight)	383.44±45.71	303.82±18.84**	389.72±12.00$^{@}$	339.39±61.68
% Soluble protein	0.8057	0.6573	0.7814	0.7165
% Insoluble protein	0.1943	0.3427	0.2186	0.2835
Ratio of soluble to insoluble protein	4.15	1.92	3.57	2.53

All values are mean ± SD; **p<0.01 versus Group 1, $^{+}$p<0.05 versus Group 1, $^{@}$p<0.001 versus Group 2, ^p<0.05 versus Group 2

the addition of 10 µl of ATP (5 mM) making the final reaction volume of 100 µl and was performed in the presence or absence of 10 mM ouabain (Na^+ K^+ ATPase-specific inhibitor). The reaction was terminated after incubation at 37 °C for 20 min by the addition of 200 µl of 0.75% ammonium molybdate in 0.9 M sulfuric acid solution. The inorganic phosphate (Pi) released from the substrate ATP was colorimetrically assayed with a Tecan Safire microplate reader at 390 nm at 37 °C. The absorbance values were converted to activity values with linear regression using a standard curve of sodium monobasic phosphate from 20 µM to 0 µM that was included in the assay procedure. The specific ATPase activities were expressed as the micromoles of inorganic phosphate (Pi µmol) released per milligram of protein per hour. The Na^+/K^+-ATPase activity was determined by subtracting the ouabain (1.25 mM) insensitive Mg^{2+}-ATPase activity from the overall $Na^+/K^+/Mg^{2+}$-ATPase activity.

Ca^{2+}-ATPase: The Ca^{2+}-ATPase assay were performed based on a modified version of Nagai et al.'s method [17]. Briefly, 125 µl of standard solution (200 mM KCl, 100 mM HEPES, 10 mM $MgCl_2$, 2 mM EGTA, 2 mM ATP) with or without 250 µl of 2.2 mM $CaCl_2$ was added to each sample (125 µl), and the samples were incubated for 1 h in a 37 °C water bath. The enzymatic reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid (TCA), and the tubes were centrifuged at 1190 ×g for 10 min at 4 °C. One hundred microliters of supernatant and 200 µl of 0.75% ammonium molybdate dissolved

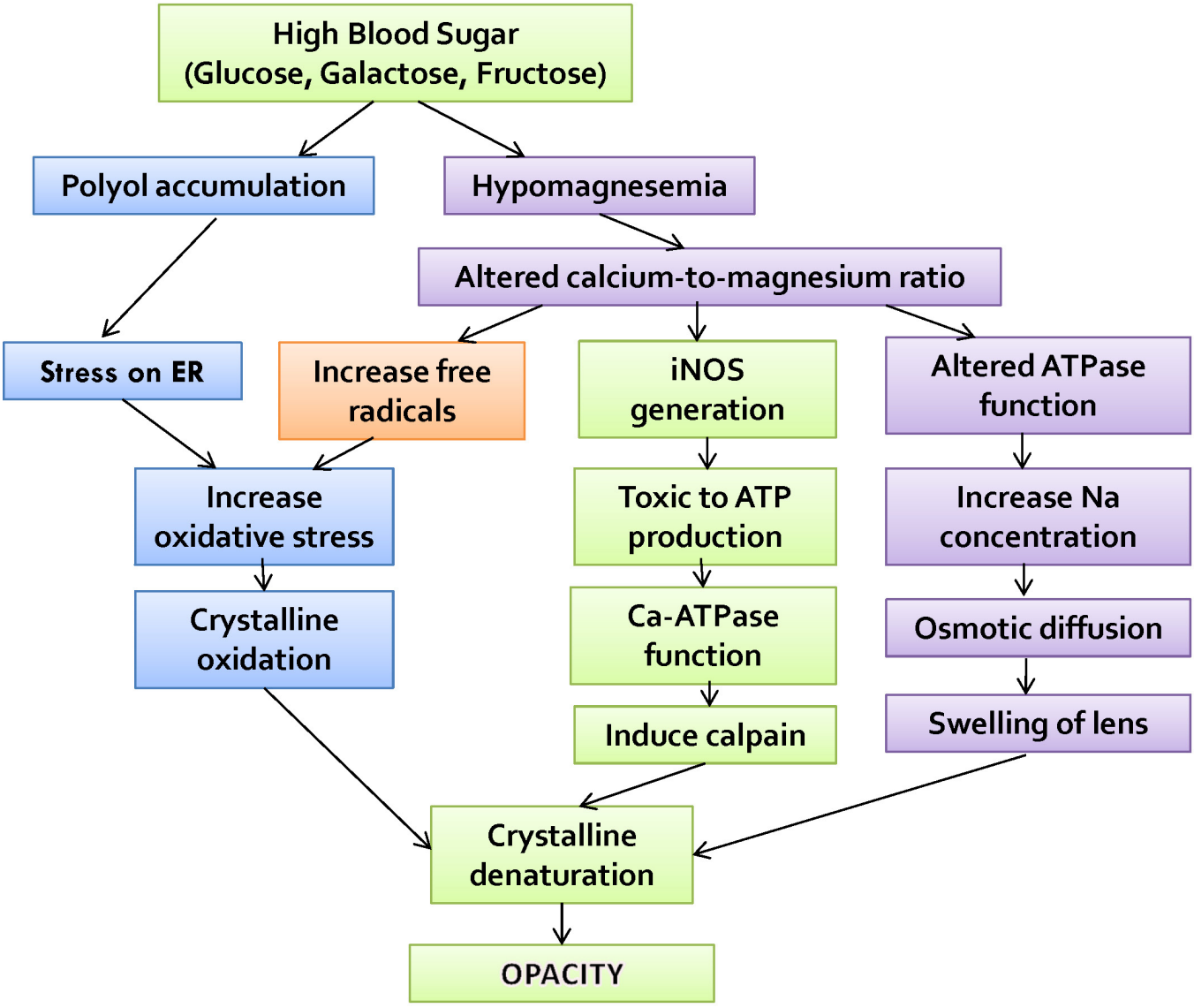

Figure 4. Pathogenesis of sugar-induced cataract.

in 0.9 M sulfuric acid were added to each well. The plate was incubated at 37 °C for 10 min and then was subjected to gentle orbital movement for 1 min before the absorbance of the supernatant was measured at 390 nm using a Tecan Safire microplate reader. A standard curve was plotted using sodium monobasic phosphate from 50 mM to blank. Ca^{2+}-ATPase activity were calculated as the difference in inorganic phosphate liberation measured in the presence and absence of Ca^{2+}.

Calpain activity: Calpain activity was determined using the SensoLyte® 7-amino-4-methylcoumarin (AMC) Calpain Assay Kit (Perkin Elmer). This kit contains a fluorogenic peptide substrate, Suc-LLVY-AMC, which generated the AMC fluorophore that emits bright blue fluorescence upon cleavage by calpain protease. The fluorescence was monitored at excitation and emission of 354 and 442 nm using a Tecan Safire microplate reader. The standard curve was plotted using the AMC fluorescence reference standard.

Statistical analysis: All values were expressed as mean ± standard deviation (SD). Statistical comparison was conducted using two-way ANOVA with Bonferroni's test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Size and surface charge of liposomal dispersions: An adequate characterization of liposome preparations and formulations is necessary, to ensure that the liposomes encompass the required and expected properties for their specific application. The liposomes in our preparation had a homogenous size distribution with a mean size of 114.67±0.15 nm (n = 3). The liposomes had a positive surface charge with a mean value of 1.54±0.34 mV (n = 3).

Study 1: Effect of liposomal MgT versus aqueous MgT on the onset and progression of cataract: Although all lenses in Group 1 remained clear throughout the experimental period, there was a rapid increase in the opacity index in the Group 2 rats that received the galactose diet and vehicle treatment. The group treated with aqueous MgT showed a significantly lower opacity index at week 1 ($p<0.05$), week 2 ($p<0.01$), week 3 ($p<0.0001$), and week 4 ($p<0.0001$). The rats that received MgT in the liposomal formulation showed a significantly lower opacity index compared to those that received aqueous MgT at all four time points ($p<0.0001$) after starting the galactose diet, and at the end of week 1, the opacity index in the liposomal MgT group was comparable to that in the normal rats ($p>0.05$; Figure 2).

Study 2: Effect of liposomal MgT versus liposomal taurine on the onset and progression of cataract: As MgT in the liposomal formulation showed higher anticataract efficacy compared to the aqueous form, we selected the MgT-loaded liposomes for further study. We also included a group treated with liposomes loaded with only taurine to assess the effect of the taurine content of MgT.

Treatment with liposomal taurine prevented an increase in the opacity index as was observed in the vehicle-treated group ($p<0.0001$) at the end of week 3 and week 4 post-galactose diet. At the end of weeks 1 and 2, the opacity index in the taurine-treated group was comparable to that in the vehicle-treated group ($p<0.05$). When compared to the MgT-treated group, the taurine-treated group had a significantly higher opacity index at all time points ($p<0.0001$; Figure 3).

Effect of liposomal MgT versus liposomal taurine on the altered Ca^{2+}-Mg^{2+} homeostasis: The lenticular Ca^{2+} to Mg^{2+} ratio was significantly higher in Group 2 compared to the normal group. However, Group 4 showed no significant difference from Group 2 and had a significantly higher Ca^{2+} to Mg^{2+} ratio compared to Group 1. Group 3 that received MgT showed a significantly lower Ca^{2+} to Mg^{2+} ratio compared to Group 2 and Group 4 (Table 1).

Effect of liposomal MgT on the lenticular ATP level: The lenticular ATP level was significantly reduced in all galactose-fed groups (groups 2, 3, and 4) compared to the normal group (Group 1). Interestingly, the lenticular ATP level was significantly increased in the MgT-treated group (Group 3) when compared to that of the vehicle-treated group (Group 2). The taurine-treated group (Group 4) showed a higher mean value compared to Group 2; however, the difference did not reach the significant level. Similarly, the MgT-treated group showed a relatively higher mean ATP level compared to the taurine-treated group, but the difference did not reach the significant level (Table 1).

Effect of liposomal MgT on lenticular ATPase activities: The lenticular Na^+/K^+-ATPase and Ca^{2+}-ATPase activities in the galactose-fed vehicle-treated group (Group 2) were more than threefold lower compared to those of the normal group (Group 1), and the differences were significant. The increase in the Na^+/K^+-ATPase and Ca^{2+}-ATPase activities in the MgT-treated group (Group 3) was found to be significantly increased compared to the galactose diet untreated group (Group 2). There was no significant difference in the Na^+/K^+-ATPase and Ca^{2+}-ATPase activities between the MgT-treated group and the normal diet group. The Na^+/K^+-ATPase and Ca^{2+}-ATPase activities in the taurine-treated group did not show significant differences from the vehicle-treated group (Group 2); moreover, the same activities in the taurine-treated group were significantly lower than in the MgT-treated group (Table 1).

Effect of liposomal MgT and taurine on lenticular oxidative and nitrosative stress:

Effect of liposomal MgT and taurine on lenticular oxidative stress—The GSH content in Group 2 showed a 10.05-fold reduction compared to the normal group. Although groups 3 and 4 also showed significantly lower lens GSH content compared to the normal group, when compared to Group 2, the content was 1.67- and 1.46-fold higher, respectively. Additionally, in the MgT–treated group, the mean GSH value was significantly higher than that of the vehicle-treated group. Lenticular catalase activity was 1.44-fold lower in Group 2 compared to that in the normal group. However, the same level was restored to normal in Group 3 that received MgT. The taurine treatment group showed a significantly higher mean value compared to Group 2 but was significantly lower compared to the group that received MgT. Lens SOD showed upregulation of the enzyme activity possibly to counteract oxidative stress with a 1.59-fold higher value in Group 2. The mean SOD values in groups 3 and 4 showed no significant difference compared to that of Group 1 but were significantly lower compared to that of Group 2. The lens MDA levels were significantly higher in Group 2 compared to those of Group 1. Groups that received treatment with either MgT or taurine showed significantly lower mean MDA values compared to those of Group 2 (Table 2).

Effect of liposomal MgT and taurine on nitrosative stress—Group 2 also showed a significantly high level of iNOS activity and nitrotyrosine levels compared to those of the normal group. Both treatment groups showed significantly lower iNOS activity and nitrotyrosine levels compared to those of Group 2, and there was no difference for both parameters between the normal group and the two treatment groups (Table 2).

Effect of liposomal MgT and taurine on lenticular calpain activity and protein level: The calpain activity in all galactose-treated groups was significantly higher than that of the normal group; however, the MgT–treated group showed significantly lower calpain activity compared to that of the vehicle-treated group (Group 2). In accordance with the increased calpain activity, Group 2 also showed a significant decrease in the soluble protein contents of lens compared to that of the normal group. Treatment with either MgT or taurine improved the soluble protein level, but only Group 3 showed significantly higher values compared to those of Group 2. Both treatment groups showed no significant differences compared to the normal group. The insoluble protein fraction was highest in Group 2 compared to all other groups. In the MgT–treated group, the ratio of soluble to insoluble proteins was comparable to that of the normal group (Table 3).

DISCUSSION

An adequate characterization of liposome preparations and formulations is necessary, to ensure that liposomes encompass the required and expected properties for their specific application [5,6,18]. In the present study, the mean particle size for liposomal formulation was 116.3 nm and 115.2 nm for the MgT and taurine liposomes, respectively. The zeta potential was +2.64 mV and +2.60 mV for the MgT and taurine liposomes, respectively, which resulted in a favorable binding effect of the positively charged liposomes to the corneal surface [18-20]. Schaeffer and Krohn [21] have shown that positively charged liposomes increase corneal drug delivery fourfold. Additionally, liposomes ranging in size from 100 to 400 nm have been shown to provide greater drug delivery compared to smaller or larger liposomes [22].

In the present study, we evaluated the anticataract effect of MgT in a liposomal formulation using a galactose-induced rat model. This model is not an exact representation of diabetic cataract; however, the key pathophysiological changes in the lens, such as activation of aldose reductase, polyol accumulation, and oxidative and nitrosative stress, mimic the same in diabetic cataract [23-26]. Moreover, galactose feeding results in rapid and predictable cataractous changes with lesser mortality due to less severe systemic metabolic changes.

The current study demonstrated that the delay in the progression of cataract was more pronounced in the group treated with a liposomal formulation compared to the aqueous form indicating higher ocular bioavailability of MgT in the liposomal formulation. As taurine has been shown to reduce lenticular oxidative stress and protein glycation in the presence of high glucose levels, it is important to investigate whether the addition of magnesium to taurine provides greater benefits than taurine alone. This study demonstrated that the liposomal formulation of MgT delays cataractogenesis more than taurine alone in the liposomal formulation thus supporting the role of magnesium in delaying cataractogenesis. Our findings are in accordance with previous studies that showed the role of MgT in delaying the development of cataract [1].

Cataractogenesis is a multifactorial disease involving various pathogenetic mechanisms that have not been completely clarified. A large body of evidence has demonstrated that oxidative stress is an important contributor to the development of cataract [27-31]. Glutathione in its reduced form (GSH) is an important part of lens antioxidant defenses

as GSH prevents the protein-thiol mixed disulfide formation that has been implicated as a possible mechanism for the protein–protein aggregation in cataractogenesis [32,33]. In addition, glutathione functions as an electron donor for glutathione peroxidase that reduces hydrogen peroxide (H_2O_2) to water and contributes to reactive oxygen species (ROS) degradation [33,34]. In the present study, treatment with MgT significantly increased the lenticular GSH levels compared to the vehicle-treated group. Previous studies have also shown that Mg^{2+} pretreatment restores renal and testicular GSH levels in the tissue of mice that were exposed to cadmium intoxication [35]. SOD and catalase are two other important enzymes that remove oxygen free radicals and reduce lenticular oxidative stress. We observed that in rats treated with vehicle, lenticular SOD activity was significantly higher, whereas catalase activity was lower indicating the upregulation of antioxidant defenses. However, in the MgT and taurine treatment groups, the SOD and catalase activities were comparable to those of the control group. These results indicate the efficacy of MgT and taurine in restoring a normal lens redox status. In contrast to our findings, Matkovics et al. [36] demonstrated that treatment with magnesium chloride results in a significant increase (10–50%) in SOD, catalase, and GSH activities in *Drosophila melanogaster*, which may perhaps be attributed to a compensatory tissue reaction to counteract oxidative stress. Magnesium deficiency is also associated with increased lipid peroxidation due to the increased susceptibility of body organs to free radical injury [37-39]. Israa et al. [40] revealed the presence of a high MDA level in response to hyperglycemia-induced oxidative stress. In accordance with these observations, higher levels of lipid peroxidation and hypomagnesaemia have been reported in patients with type 2 diabetes [41]. Free radicals in diabetes mellitus (DM) cause the peroxidative breakdown of phospholipids that leads to accumulation of MDA [42]. Ribeiro et al. [43] demonstrated a negative correlation between magnesium and glucose levels, as well as between magnesium and oxidative stress. In the current study, the MDA concentration in the vehicle-treated group was significantly higher than that in the other groups, indicating increased lipid peroxidation induced by galactosemia. Treatment with MgT prevented lipid peroxidation as indicated by a decrease in the MDA level in the lens.

Galactosemia also induces mRNA expression of iNOS followed by a high level of NO production. Reaction of NO with superoxide anions produces peroxynitrite ($ONOO^-$), which is a highly oxidative species and is capable of nitrating tyrosine residues of crystalline proteins, leading to the formation of nitrotyrosine [32,44-46]. Nitrosative stress due to increased endogenous NO production can potentiate lens opacification [47,48]. Magnesium deficiency has been shown to enhance the production of NO by iNOS induction in the lens. Production of excess NO leads to the decreased ATP level that causes lens ionic imbalance by impairing the ATPase functions [49]. In the current study, treatment with MgT caused a significant decrease in iNOS and nitrotyrosine indicating a significant reduction in lenticular nitrosative stress.

In this study, treatment with taurine also caused reduced oxidative stress and nitrosative stress and consequently delayed cataractogenesis. Taurine is involved in several physiologic reactions, such as osmoregulation, calcium transport regulation, and cell membrane stabilization, and protects tissues against oxidative damage by scavenging hypochlorous acid (HOCl) [50,51]. Taurine has previously been shown to significantly preserve the level of GSH in galactosemic animals [52]. The levels of taurine and GSH have been shown to be significantly reduced in the diabetic cataractous lens [53]. Mahmoud et al. [54] demonstrated that the administration of taurine to galactose-fed rats attenuates the increased lipid peroxidation, enhances the levels of antioxidants, inhibits the activity of the aldose reductase enzyme, and improves the crystallin profile. The MDA content of lenses incubated with galactose plus taurine in media supplemented with glucose was found to be lower than that of the control lenses incubated without taurine [55]. Dietary taurine supplementation also ameliorates MDA levels, GSSG/GSH, and NAD^+/NADH but failed to prevent the osmotically mediated depletion of GSH and the decrease in glucose utilization and ATP levels in the diabetic precataractous lens [56]. In accordance with these findings, we observed that the effects of MgT were more prominent than those of taurine alone. Thus, the addition of magnesium to taurine seems to play a significant role in preventing lenticular oxidative stress.

Oxidative stress–induced lipid peroxidative damage of lenticular membranes leads to inactivation of membrane proteins such as Ca^{2+}-ATPase. Inhibition of the function of Ca^{2+}-ATPase results in the loss of calcium homeostasis and calcium accumulation in the lens. As a consequence of calcium influx in rodent lenses, calpains, a family of well-characterized calcium-dependent proteases, are activated. Calpain activation induces rapid proteolysis of water-soluble proteins, that is, lens crystallins, and cytoskeletal proteins. Proteolysis exposes the hydrophobic regions of the lens proteins, which then interact to form insoluble aggregates [57]. Insolubilization results in precipitation and aggregation of fragmented proteins and, finally, loss of lens transparency [58].

Importantly, the present study showed restoration of the Ca/Mg ratio particularly in the MgT treatment group.

Magnesium is a cofactor for several enzymes such as Na^+/K^+ ATPase and Ca^{2+}-ATPase in the lens that maintain the lenticular ionic balance [59,60] (Figure 4). Both pumps are magnesium dependent, and magnesium deficiency can impair ATPase functions [61,62]. Furthermore, magnesium deficiency enhances oxidative stress leading to damage to ATPases [38]. Lenticular ionic imbalance leads to increased oxidative stress [3] as is shown in the vehicle-treated group in the present study. Na^+/K^+-ATPase dysfunction leads to cellular swelling by osmotic stress, and interruption of the Ca^{2+}-ATPase function leads to increased intracellular calcium ions and that causes activation of calpain II and consequently proteolysis of crystalline, the major protein in the lens. Proteolysis converts crystalline into an insoluble form that causes the development of cataract [58,63-65]. In the current study, we observed normalization of the soluble/insoluble protein ratio in both treatment groups, particularly the MgT group, indicating less activation of Ca-dependent calpain. Correction of the ionic imbalance and reduction in lenticular oxidative stress appear to contribute to reduced calpain activation and, therefore, normalization of the soluble/insoluble protein ratio. Reduced oxidative stress by itself also prevents the formation of protein aggregates by protecting against the oxidation of sulfhydryl groups on proteins [66]. In the present study, treatment with MgT caused restoration of the Ca/Mg ratio thus preventing oxidative stress, preserving the ATPase functions, and delaying the development of cataract. Similar effects in the taurine treatment group, however, were less pronounced due to decreased correction of the ionic imbalance. In conclusion, topical liposomal MgT delays the onset and progression of galactose-induced cataract in rats by maintaining mineral homeostasis in the lens and reducing lenticular oxidative and nitrosative stress in galactose-fed rats.

ACKNOWLEDGMENTS

Funding statement: This work was supported in part by the DANA Kecemerlangan Penyelidikan under the project 600-RMI/ST/DANA 5/3/Dst (354/2011) in part by the MOH (Malaysia) under the project 600-RMI/RAGS 5/3 (103/2013).

Contributor: All authors have substantially contributed to conception, designing, drafting the article and in final approval of the manuscript version to be submitted. All authors have jointly decided to designate Assoc Prof Dr Renu Agarwal to be responsible for taking decision regarding the presence of authors and the order of their presence in the manuscript. Assoc Prof Dr Renu Agarwal has also been selected by all authors to be responsible for all future communication with the journal regarding this manuscript.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 10 July 2016. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.