Effects of topical administration of an aldose reductase inhibitor on cataract formation in dogs fed a diet high in galactose

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Objective—To determine effects of a topical formulation of an aldose reductase inhibitor (ARI) on the development of sugar cataracts in dogs fed a diet high in galactose.

Animals—Ten 6-month old Beagles.

Procedures—Dogs were fed a diet containing 30% galactose, and after 16 weeks, 6 dogs were treated topically with a proprietary ARI formulation and 4 dogs were treated with a placebo. Cataract formation was monitored by means of slit-lamp biomicroscopy and fundus photography. Dogs were euthanized after 10 weeks of treatment, and lenses were evaluated for degree of opacity, myo-inositol and galactitol concentrations, and concentration of the ARI.

Results—All dogs developed bilateral cortical opacities dense enough to result in a decrease in the tapetal reflex after being fed the galactose-containing diet for 16 weeks. Administration of the ARI arrested further development of cataract formation. In contrast, cataracts in the vehicle-treated dogs progressed over the 10-week period to the mature stage. Evaluation of the isolated lenses after 26 weeks of galactose feeding indicated that lenses from treated dogs were significantly less optically dense than lenses from control dogs. Lenticular myo-inositol concentration was significantly higher in the treated than in the control dogs.

Conclusions and Clinical Relevance—Results suggest that topical application of a proprietary ARI formulation may arrest or reverse the development of sugar cataracts in dogs fed a diet high in galactose. This suggests that this ARI formulation may be beneficial in maintaining or improving functional vision in diabetic dogs with early lens opacities. (*Am J Vet Res* 2006;67:1783–1787)

Abbreviations

ARI	Aldose reductase inhibitor
HPLC	High-pressure liquid chromatography

In dogs, diabetes mellitus is characterized by the rapid appearance of bilateral sugar cataracts. Over the past 30 years, the incidence of canine diabetes mellitus has increased 3-fold.[1,2] Currently, nearly 1 in 3 dogs with cataracts is also diabetic. Since cataracts lead to vision loss that can currently only be treated by surgery, a medical treatment that preserves vision and prevents the need for surgery in diabetic dogs would be beneficial.

Cataracts can be experimentally produced in animals by inducing diabetes mellitus or feeding a diet high in lactose or galactose, with the rate of cataract formation proportional to the blood glucose or galactose concentration.[3-5] Studies in rats suggest that galactosemic "sugar" cataracts undergo histologic and biochemical changes similar to those seen with diabetic "sugar" cataracts[3,6-8] and that clinical progression is similar for galactosemic and diabetic cataracts.[9,10]

Oxidative stress, redox changes, altered membrane permeability, glycation, and production of advanced glycation end products all contribute to the formation of diabetic cataracts,[11] but extensive studies[5,12,13] have shown that lenticular aldose reductase activity is the primary factor in cataract development. Specifically, biochemical changes in the lens that ultimately lead to cataract formation are initiated by the intracellular accumulation of sorbitol or galactitol. Aldose reductase reduces glucose to sorbitol and reduces galactose to galactitol. Sorbitol, in turn, is oxidized by sorbitol dehydrogenase to fructose, but galactitol is not further metabolized.

The critical role of aldose reductase in cataract formation has been confirmed in diabetic animals and animals fed a diet high in galactose. For example, hyperglycemic mice and mice fed a diet high in galactose do not develop cataracts because they have low aldose reductase concentrations in their lenses. In contrast, transgenic mice that express high lenticular aldose reductase concentrations rapidly form diabetic and galactosemic cataracts.[14] Furthermore, diabetic cataract formation is enhanced when sorbitol dehydrogenase activity is also deleted in transgenic mice with high lenticular aldose reductase concentrations. Similarly, diabetic cataracts are uncommon in cats, compared with dogs, even though incidences of diabetes mellitus in cats and dogs are similar because concentrations of aldose reductase are much lower in the lenses of cats than in the lenses of age-matched dogs.[15,16] Further proof of the role of aldose reductase in the development of cataracts comes from studies[17,18] showing that ARIs can arrest cataract formation, even

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though they do not decrease lens glycosylation or formation of advanced glycation end products, and arrest the biochemical changes associated with oxidative stress in the lenses of diabetic animals and animals fed a diet high in galactose.[13,19,20]

Several studies[6,21,22] have shown that ARIs can prevent the development of cataracts in animals when given at the onset of galactosemia or diabetes. In addition, studies[23-26] have shown that ARIs can reverse the formation of cataracts in rats, but only if given during the early vacuolar stage of cataract formation. In many dogs, however, diabetes mellitus is diagnosed only after the owner has brought the dog to a veterinarian because of apparent lens changes. Thus, in most diabetic dogs, it is likely that substantial biochemical changes have already occurred in the lens at the time diabetes mellitus is diagnosed. To be useful clinically, therefore, ARIs must be able to arrest the progression of cataracts or reverse their development in dogs that already have clinical evidence of cataract formation. The purpose of the study reported here, therefore, was to determine whether a new topical formulation of an ARI would arrest or reverse the development of cataracts in dogs fed a diet high in galactose.

Materials and Methods

Dogs—Ten 6-month-old purpose-bred male Beagles[a] were used in the study. Dogs were housed in individual runs for the duration of the study. For all dogs, results of complete physical and ophthalmologic examinations performed prior to the study were normal. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*.

Experimental protocol—To induce cataract formation, dogs were fed a diet containing 30% galactose[b] for the duration of the study, with each dog receiving 450 g of the diet at approximately 8 AM each day. After this diet had been fed for 16 weeks, 6 dogs were randomly assigned to the treatment group and the remaining 4 dogs were assigned to the control group. Dogs in the treatment group were treated with a proprietary topical formulation of an ARI,[c] whereas dogs in the control group were treated with a placebo consisting of vehicle alone. For both groups, treatment consisted of topical application of 2 drops of the drug formulation or placebo in each eye, administered 10 minutes apart, at 8 AM and 4 PM. Treatments were administered for 10 weeks.

At the end of the study (ie, week 26), dogs were euthanized. Both eyes were enucleated from each dog, and the lenses were carefully removed by means of a posterior approach. All lenses were evaluated for density. Myo-inositol and galactitol concentrations were determined in the lens from 1 eye of each dog, and ARI concentration was determined in the lens of the contralateral eye.

Ophthalmic evaluations of lens changes—Ophthalmic examinations were conducted at the onset of the study (ie, week 0) to establish that all dogs were free from lens opacities and retinal lesions. Subsequent follow-up ophthalmic examinations, including indirect ophthalmoscopy and slit-lamp biomicroscopy, were performed at approximately 4-week intervals by veterinary ophthalmologists blinded to treatment group assignments of the dogs. Dogs were not anesthetized during these examinations; mydriasis was induced prior to examination by means of topical administration of 1% tropicamide hydrochloride. Lens changes were documented by means of fundus photography[d] at the onset of the study and during weeks 16 and 25.

Clinicopathologic testing—For all dogs, a CBC and serum biochemical profile were performed at the onset of the study (ie, week 0) and at the end of the 10 weeks of treatment (ie, week 26). The serum biochemical profiles include measurement of serum glucose, urea nitrogen, creatinine, sodium, potassium, chloride, calcium, albumin, globulin, and total bilirubin concentrations and serum aspartate transaminase, alanine transaminase, and alkaline phosphatase activities. Tests were performed by a commercial laboratory.[e]

Glycosylated hemoglobin concentration was measured at the onset of the study and during week 26 by means of HPLC. Testing was performed by a commercial laboratory.[f]

Concentrations of galactose and galactitol in RBCs and serum were measured at the onset of the study and during weeks 12 and 25. For these analyses, venous blood samples were collected into evacuated tubes containing EDTA. Blood samples were washed twice with 2 mL of saline (0.9% NaCl) solution and centrifuged at 1,800 × *g* for 15 minutes. Supernatants obtained after each centrifugation were combined and mixed with 2 mL of HPLC-grade water containing 3 µmol of xylose as an internal standard. The mixture was then deproteinized with 1 mL of 0.3N zinc sulfate and 1 mL of 0.3N barium hydroxide. Similarly, the precipitate containing RBCs was transferred to a glass homogenizer tube with 2 mL of HPLC-grade water containing 3 µmol of xylose as an internal standard, and the homogenate was deproteinized with 1 mL of 0.3N zinc sulfate and 1 mL of 0.3N barium hydroxide. Deproteinized RBC and plasma samples were centrifuged at 10,000 × *g* for 15 minutes, and a 400-µL aliquot of each sample was evaporated.[g] The dried residue was dissolved in 900 µL of pyridine and then derivatized with 900 µL of phenyl isocyanate at 55°C for 60 minutes. After cooling in an ice bath, the reaction was halted with cold methanol, and the sample was again heated for 5 minutes. Samples were then evaluated by means of HPLC, as described.[27]

Lens density—Density of the individual lenses was determined by placing the lenses on the lit surface of a digitizing slide scanner.[h] Color digital images of each lens were obtained with and without a grid. Lens opacity was determined from the images obtained without a grid by use of standard software.[i] Briefly, each lens image was inverted, and color ranges for the red, green, and blue spectrum were set from 0 to 255. The intensity of each photograph was calibrated by means of pixels at the lens equator, where the black ciliary processes attached. Next, the incident concentration of each photograph was calibrated by selecting pixels outside of the lens and away from the ciliary process. Multiple rectangular areas of interest were constructed until most (approx 80%) of the lens area was covered. The weighted average of the integrated optical density for all areas of interest was then obtained. Total lens clarity was defined as an integrated optical density of 0.0.

Lenticular myo-inositol and galactitol concentrations—In the lens, myo-inositol concentration decreases as sorbitol or galactitol accumulates. For determination of lens myo-inositol and galactitol concentrations, each lens was homogenized in a glass homogenizer with 2 mL of HPLC-grade water containing 3 µmol of xylose as an internal standard. Homogenate proteins were removed by centrifugation[j] overnight at 8°C. Filtrates were dried,[g] and dried residues were dissolved in 900 µL of pyridine and then derivatized with 900 µL of phenyl isocyanate at 55°C for 60 minutes. After cooling in an ice bath, the reaction was halted with cold methanol, and the sample was again heated for 5 minutes. Samples were then evaluated by means of HPLC, as described.[27] Briefly, samples were analyzed with an automated instrument[k] equipped with a diode array detector. Samples (5 µL) were injected onto a 150 × 4.6-mm column[l] containing a

3.2 × 15-mm guard column at 35°C and were eluted isocratically with 20mM potassium phosphate–acetonitrile (35:65 vol%; pH, 7.0) at a flow rate of 1.0 mL/min and detection at 235 nm. Sample concentrations were quantified by comparison with standard curves for glucose, galactose, sorbitol, galactitol, myo-inositol, and xylose (0.008 to 6.0 µmol).

Lenticular ARI concentration—For determination of lens ARI concentration, lenses were homogenized with HPLC-grade water containing sorbinil, an ARI, as an internal standard. The homogenates were deproteinated by treatment with sodium fluoride, then acidified with hydrochloric acid and extracted with diethyl ether. The ether layer was washed with 0.25M phosphate buffer (pH, 7.0). The ether layer was then evaporated, and the residue was dissolved in HPLC-grade methanol. Concentrations of ARI were measured on a reverse-phase, 5-µm C18 column[m] equipped with a 3.2 × 15-mm guard column by eluting with an isocratic mixture of 55% HPLC-grade methanol and 45% HPLC-grade water at a flow rate of 0.5 mL/min. Chromatography was conducted at room temperature, and the compound was detected at 220 nm over a linear range of 0 to 70 µg.

Statistical analysis—Data are given as mean ± SD. For analysis of lens density data, the mean value for the 2 lenses from each dog was used. The independent 2-sample *t* test was used to compare values between the treatment and control group. All analyses were performed with standard software.[n] Values of $P < 0.05$ were considered significant.

Results

For all dogs, results of CBCs and serum biochemical profiles performed at the onset (ie, week 0) and termination (week 26) of the study were unremarkable, and no clinically important abnormalities were found. All dogs were equally galactosemic as demonstrated by the 25- to 26-week blood samples, in which no significant differences between the treatment and control group were found in regard to plasma galactose concentrations (436 ± 106µM vs 544 ± 63.4µM), plasma galactitol concentrations (9.9 ± 3.2µM vs 16.4 ± 10.4µM), RBC galactose concentrations (108 ± 42.4µM vs 95.7 ± 30.2µM), and glycosylated hemoglobin concentrations (4.9 ± 0.1% vs 5.0 ± 0.1%). However, week 26 RBC galactitol concentrations were significantly lower among dogs in the treatment group (17.0 ± 8.50µM), compared with dogs in the control group (112.7 ± 36.3µM).

As the study progressed, suture accentuation was the earliest observed lens change and was apparent after dogs had been fed the high-galactose diet for 4 weeks. After the diet was fed for 8 weeks, vacuoles were apparent, and by 12 weeks, superficial cortical opacities were apparent in all dogs. At 16 weeks, bilateral cortical opacities were present in all dogs, and minimal tapetal reflex of the flash of the fundus camera was evident because of the density of the lens opacities (**Figure 1**). Among dogs in the treatment group, the amount of tapetal reflex appeared subjectively to increase over time following the initiation of treatment with the ARI formulation, suggesting that the density of lens opacities had decreased or lens clearing had occurred. In contrast, among dogs in the control group, the amount of tapetal reflex subjectively appeared to decrease further as the study progressed, and at the termination of the study, all control dogs had dense, mature cataracts.

When images of the individual lenses were obtained with a grid, it appeared subjectively that more of the grid was visible through the lenses obtained from treatment group dogs than from control dogs. Analysis of digitized images obtained without a grid indicated that integrated optical density of lenses from control dogs ($3.9 \pm 2.2 \times 10^{-3}$ pixel density × mm^2) was significantly greater than density of lenses from treatment group dogs ($0.47 \pm 0.34 \times 10^{-3}$ pixel density × mm^2), even though the percentage of lens area analyzed for control dogs (81.6 ± 3.8%) was not significantly different from the percentage for treatment group dogs (80.3 ± 2.8%).

Lenticular myo-inositol concentration was significantly higher in treatment group dogs (311.0 ± 89.4µM) than in control dogs (105.0 ± 18.0µM; **Figure 2**). In contrast, lenticular galactitol concentration in treatment group dogs (34.7 ± 14.2mM) was not significantly different from concentration in control dogs (50.9 ± 7.0mM). For dogs in the treatment group,

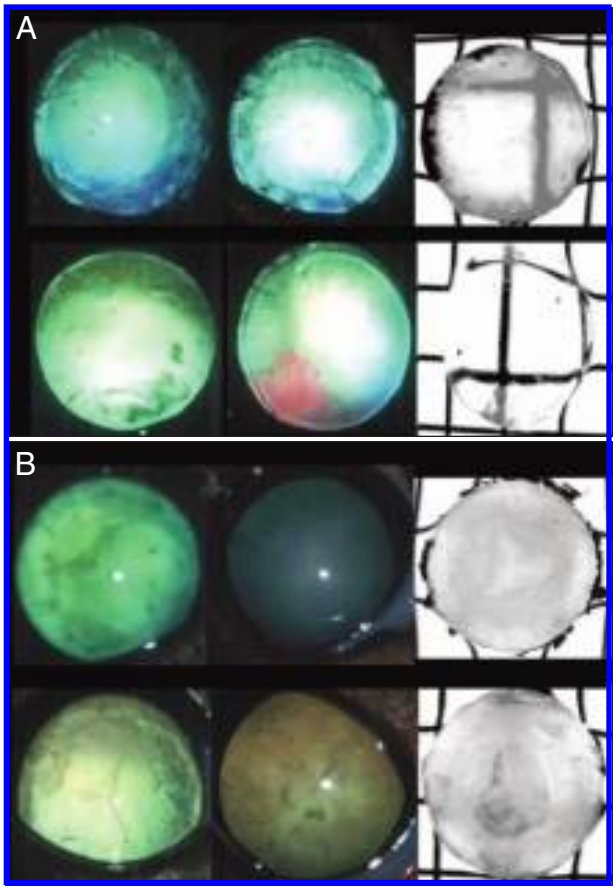

Figure 1—Appearance of the lenses in 2 dogs fed a diet containing 30% galactose that were treated with a topical formulation of an ARI (A) and in 2 dogs fed the same diet that were treated with a placebo (B). In each series, the image on the far left represents the appearance of the eye after the high-galactose diet had been fed for 15 weeks (1 week prior to the start of ARI or control treatment); the image in the center represents the appearance of the same eye after 9 weeks of treatment (ie, week 25); and the image on the far right represents the appearance of the isolated lens, obtained after euthanasia of the dog 1 week later, photographed over a grid. Notice the absence of a tapetal reflex of the flash of the fundus camera in the eyes of the control dogs, particularly after the high-galactose diet had been fed for 25 weeks.

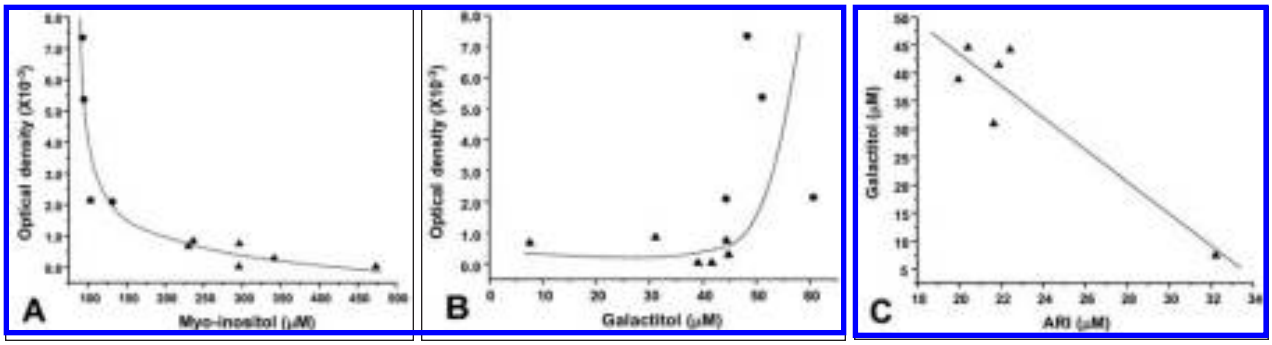

Figure 2—Scatterplots of weighted average integrated optical density versus lenticular myo-inositol (A) and lenticular galactitol (B) concentrations in 10 dogs fed a high-galactose diet that were treated with a topical ARI formulation (n = 6; triangles) or a placebo (4; circles) and of lenticular galactitol concentration versus lenticular ARI concentration in treated dogs (C). The dotted line in part C represents the least squares fit (r^2 = 0.84).

there was a significant negative relationship (r^2 = 0.84) between lenticular ARI concentration and lenticular galactitol concentration.

Discussion

Results of the present study suggest that topical application of a proprietary ARI formulation may arrest or reverse the development of sugar cataracts in dogs fed a diet high in galactose. This suggests that this ARI formulation may be beneficial in maintaining or improving functional vision in diabetic dogs with early lens opacities.

The initial lens changes associated with cataract formation in dogs fed a diet high in galactose are similar to those that occur in diabetic dogs,[28] with cataract formation directly linked to aldose reductase-catalyzed accumulation of galactitol and sorbitol, respectively.[9,15,16,22] In dogs with diabetes, glucose is converted to sorbitol, which, in turn, is oxidized to fructose by sorbitol dehydrogenase. Therefore, lenticular sorbitol accumulation is regulated both by inhibition of aldose reductase activity and by sorbitol dehydrogenase activity. In contrast, in dogs fed a diet high in galactose, galactitol accumulation can only be controlled by inhibition of aldose reductase activity because galactitol is not further metabolized, with the net result being that galactitol accumulates more rapidly and at higher concentrations in dogs fed a diet high in galactose than sorbitol accumulates in diabetic dogs. As a result, cataract formation is more rapid and more severe in dogs fed a diet high in galactose than in diabetic dogs. Therefore, dogs fed a diet high in galactose are often used to evaluate the efficacy of ARIs in cataract prevention because more robust inhibition is required.[3]

In dogs, lenticular aldose reductase activity is age dependent, with activity decreasing to plateau concentrations in adult animals. As a result, the onset and severity of cataract formation are also age dependent.[29] In the present study, we elected to use 6-month-old dogs because results of a previous study[29] indicated that cortical opacities develop by 12 weeks and mature cataracts develop by 26 weeks when dogs of this age group are fed a diet containing 30% galactose.

Previous studies[3,4,6] of the efficacy of ARIs have focused on prevention, with the drugs administered at the onset of diabetes or galactosemia, and these studies have established that ARIs are effective in preventing cataract formation in diabetic and galactosemic rats. Similarly, a previous study[22] demonstrated that the onset and progression of cataracts in 9-month-old dogs fed a diet high in galactose can be inhibited in a dose-dependent manner with ARIs. In contrast, studies[3,23-26] investigating whether ARIs can arrest or reverse the progression of cataract development once cataracts have formed have been limited. It has generally been thought that opacities resulting from lens fiber degeneration are irreversible and that only those opacities resulting from early vacuolation can be reversed. In the present study, however, we found that opacities resulting from fiber degeneration could be reversed, with overall density of the lens significantly reduced, by use of a topical ARI formulation. This decrease in lens density was associated with a subjective improvement in the apparent tapetal reflex. Taken together, these results suggest that, compared with control dogs, functional vision in dogs treated with the ARI improved or was maintained.

Results of biochemical analyses in the present study also supported the conclusion that ARI treatment reduced cataract formation. In general, lenticular concentrations of sorbitol and galactitol initially increase during cataract formation, but then decrease because of increased lens permeability as the cataract becomes more severe. In contrast, lenticular myo-inositol concentrations are inversely proportional to lenticular concentrations of sorbitol and galactitol. In the present study, lenticular myo-inositol concentrations were significantly higher in dogs treated with the ARI than in control dogs, suggesting that aldose reductase activity was indeed being inhibited in the treated dogs.

Although no adverse effects resulting from long-term oral administration of ARIs have been published, it has been suggested that some ARIs may modify select hepatic enzymes associated with oxidative defense or P_{450} induction.[30,31] In contrast, this has not been observed with topical administration.[32] Thus, topical administration may be preferred, particularly because hepatic function may already be compromised in diabetic dogs. However, the finding that RBC galactitol concentration was significantly decreased in treatment group dogs, compared with control dogs, in the present study indicates that there were some minor systemic effects following topical application of this ARI.

a. Marshall Farms USA Inc, North Rose, NY.

b. Bio-Serve, Frenchtown, NJ.
c. Kinostat, Therapeutic Vision Inc, Omaha, Neb, and the University of Nebraska Medical Center, Omaha, Neb.
d. FS-3 fundus camera, Nikon, Tokyo, Japan.
e. Regional Pathology Service, Nebraska Medical Center, Omaha, Neb.
f. Associated Regional and University Pathologists Inc, Salt Lake City, Utah.
g. Savant SpeedVac, Thermo Electron Corp, Waltham, Mass.
h. FOTOVIX, Tamron USA Inc, Commack, NY.
i. Image-Pro Plus, Media Cybernetics, Silver Spring, Md.
j. Microcon YM-10 centrifugal filter device, Millipore Corp, Burlington, Mass.
k. ChemStation 1100, Hewlett Packard, Agilent Technologies, Palo Alto, Calif.
l. TSKgel ODS-80Tm, Tosoh Bioscience LLC, Montgomeryville, Pa.
m. LUNA, Phenomenex, Torrance, Calif.
n. Origin, version 7.0, OriginLab Corp, Northampton, Mass.

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