Diabetic-Like Retinopathy: Early and Late Intervention Therapies in Galactose-Fed Rats

W. Gerald Robison, Jr.,1 Jorge L Jacot,1 Joel P. Glover,1 Michael D. Basso,2 and Thomas C. Hohman2

PURPOSE. To determine whether the diabetic-like thickening of retinal capillary basement membrane (RCBM) that develops in the galactose-fed rat model of diabetic ocular complications could be halted or ameliorated after 4 or 8 months of galactosemia by treatment with ARI-509, a potent new aldose reductase inhibitor (ARI), or by withdrawal of the galactose diet.

METHODS. Weanling female Sprague-Dawley rats were randomized into eight groups and fed laboratory chow plus 50% starch, control group (CON); 50% D-galactose, galactose-fed group (GAL); 50% D-galactose with ARI-509 at 25 mg/kg or 10 mg/kg body wt per day, high-dose prevention group (HDP) and low-dose prevention group (LDP), respectively; 50% D-galactose for 4 or 8 months and then intervention by addition of ARI-509 (25 mg/kg body wt per day), 4-month intervention group (4IN) and 8-month intervention group (8IN), respectively; or 50% D-galactose for 4 or 8 months and then intervention by withdrawing galactose and replacing it with the 50% starch diet, 4-month galactose withdrawal group (4GW) and 8-month galactose withdrawal group (8GW), respectively. After 4, 8, 16, and 24 months of the experimental diets, the levels of carbohydrates in tissues and the extent of RCBM thickening in capillaries of the outer plexiform layer were determined in all groups.

RESULTS. Retinal polyol was reduced by 95% in all ARI-treated groups and by 100% in the 4GW and 8GW groups after withdrawal of the galactose. The mean RCBM thickness increased rapidly in GAL rats, becoming almost two times greater (189 ± 9.4 nm) than in CON rats (103 ± 3.4 nm) by 24 months. Treatment with ARI-509 in high and low doses (HDP, LDP) initiated with the introduction of the galactose diet significantly prevented RCBM thickening at all time points (P < 0.05). In contrast, intervention by withdrawing galactose from the diet or by adding the high dose of ARI-509 had no significant effect (P < 0.05) on RCBM thickening until the 24-month time point (4IN, 166 ± 10.3 nm; 8IN, 161 ± 8.2 nm; 4GW, 136 ± 51 nm; 8GW, 163 ± 9.6 nm).

CONCLUSIONS. Both early and late interventions decreased RCBM thickening compared with that in untreated GAL rats. The decreased thickening, however, was not evident until 16 to 20 months after the intervention. Because RCBM thickening is one of the earliest changes in diabetic and galactosemic retinopathy, the findings suggest that RCBM thickening and possibly subsequent retinal lesions are caused by early biochemical alterations induced by the galactose diet that are not readily reversed. The delayed response to therapy is consistent with that observed in the Diabetes Control and Complications Trial. The cumulative evidence indicates that intervention should begin as early after onset of diabetes as possible, and long follow-up periods should be used to evaluate efficacy.

spectrum of diabetic-like retinal vascular lesions develops, as does the specific type of retinopathy otherwise found only in diabetes. Thus, the galactose-fed rat has become one of the established models for the study of diabetic retinopathy.

Several studies using galactose-fed animals have shown that these diabetic-like retinal lesions are prevented or at least are delayed when treatment with an aldose reductase inhibitor (ARI) is initiated at the same time as the galactose diet. However, the progression of retinal microangiopathy is not halted when ARI treatment is initiated after measurable retinal lesions have developed. Even withdrawal of the galactose diet does not interrupt the process. These experimental results may have important implications for studies of new treatments for retinopathy in patients with diabetes.

The clinical trials conducted to date have not been initiated or at near the time of diagnosis of diabetes. Even though fundus lesions were not detected in some of the subjects included, histologic findings from studies of retinas of diabetic donor eyes suggest that microscopic lesions below the clinical levels of detection were likely present. Therefore, the extent of preexisting damage and how it affects intervention remain unknown.

The purpose of the present study was to determine whether diabetic-like retinopathy would be delayed, halted, or reversed by early (4-month) and/or late (8-month) intervention in galactose-fed rats. The interventions involved treatment with a potent, new ARI (ARI-509; Wyeth-Ayerst Laboratories Research, Princeton, NJ) or withdrawal of the galactose diet at the 4- and 8-month time points. Retinal capillary basement membrane (RCBM) thickening was used to evaluate the effects of the interventions, because such thickening is a hallmark of diabetic retinopathy, can be precisely quantified, and correlated well with other retinal lesions in an intervention and prevention study on galactosemic rats. The progressive increase in RCBM thickness exhibited with untreated hyperglycemia and galactosemia provides the advantage of offering one of the most objective means of assessing the degree of retinal vessel damage. In the present study, either a high (25 mg/kg per day) or a low (10 mg/kg per day) dose of ARI-509-initiated at the same time as the galactose diet prevented RCBM thickening at all time points. However, the effectiveness of the 4- and 8-month intervention treatments did not become evident until 16 and 20 months after the interventions.

**Materials and Methods**

**Animals**

Weanling female Sprague-Dawley rats were fed a control diet for two weeks that consisted of a basic laboratory chow diet (Purina laboratory chow #5001: Bio-Serve, Frenchtown, NJ) mixed evenly with 50% starch. The rats were then randomized into eight treatment groups and fed diets as follows: 50% starch, control group (CON); 50% d-galactose, galactose-fed group (GAL); 50% d-galactose with the inhibitor ARI-509 mixed in the diet to render a dose of 25 mg/kg or 10 mg/kg of body weight daily, high-dose prevention group (HDP) and low-dose prevention group (LDP), respectively; 50% d-galactose for 4 or 8 months and then intervention by addition of ARI-509 (25 mg/kg of body weight daily), 4-month intervention group (4IN) and 8-month intervention group (8IN), respectively; or 50% d-galactose for 4 or 8 months and then intervention by withdrawing galactose and replacing the galactose diet with the 50% starch diet, 4-month galactose withdrawal group (4GW) and 8-month galactose withdrawal group (8GW), respectively. Mixing of diet components and adjustment of ingredients and body weights to maintain a daily dose of 25 mg/kg (HDP) or 10 mg/kg (LDP) of body weight throughout the experiment were performed as described. All animals were given water and food ad libitum and were maintained under a 12-hours-on/12-hours-off light cycle with cage-level illumination of less than 25 foot-candles. Ten rats in each group were killed by an overdose of halothane at the 4- (except the LDP group), 8-, and 16-month time points and 16 rats in each group were killed at the 24-month time point. Eyes from five rats of each group at each time point were chosen randomly for retinal sugar and polyol determinations. One eye (randomly preselected) from each of the remaining animals was prepared for electron microscopy of transected capillaries. Animal care and handling conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to the Guide for the Care and Use of Laboratory Animals.

**Carbohydrate Levels in Blood and Retina**

At each time point, the plasma glucose levels, the percentage of glycated (nonenzymatically galactosylated) hemoglobin, the erythrocyte and retinal galactose and galactitol levels, and the retinal myoinositol levels were measured in rats of each group, as described. Because there were no significant \( P < 0.05 \) differences in these biochemical parameters at the individual time points for each treatment group, the data for all of the time points were combined. For the CON, GAL, and HDP groups, the mean values presented in Table 1 represent data from the 4-, 8-, 16-, and 24-month time points. For the LDP group, data from the 8-, 16-, and 24-month time points were combined. For the 4IN and 4GW groups and the 8IN and 8GW groups, the mean values represent data from the time points after the diet change (8, 16, and 24 months for 16 and 24 months, respectively). The \( n \) numbers in Table 1 indicate the number of animals measured for all time points for each experimental group.

**Computer-Assisted Morphometric Measurements**

Retinal capillary basement membrane thickness was determined from electron micrographs (10 per animal) of transected capillaries of the outer plexiform layer from the temporal region of the central retina within 1 mm of the optic nerve. The images were selected, traced, and digitized as described. All images were analyzed using an image analysis system with 640 X 480 X 24-bit resolution (C-Imaging 1280, Compix Imaging Systems, Cranberry Township, PA).

**Data Collection and Analysis**

All data were collected and evaluations were performed by investigators who were masked to the identity of the treatment groups. The mean and SEM were calculated for all of the time points from each treatment group. Exploratory data analysis was performed on raw data, independently for each month, to check for nonnormal and nonhomogeneous variance. The maximum likelihood Box-Cox transformation, which maximizes the normality, homogeneity of variance, and the good-
### Table 1. Mean Carbohydrate Levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma glucose (mM)</th>
<th>Glycated hemoglobin (%)</th>
<th>Erythrocyte galactose (nmol/mg hemoglobin)</th>
<th>Erythrocyte galactitol (nmol/mg hemoglobin)</th>
<th>Retinal galactose (nmol/mg wet weight)</th>
<th>Retinal galactitol (nmol/mg wet weight)</th>
<th>Retinal myoinositol (nmol/mg wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON (n = 20)</td>
<td>8.32 ± 0.91</td>
<td>2.61 ± 0.10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td>GAL (n = 20)</td>
<td>6.86 ± 0.68</td>
<td>10.95 ± 0.93†</td>
<td>10.38 ± 0.67†</td>
<td>10.65 ± 1.07†</td>
<td>10.20 ± 1.48†</td>
<td>9.72 ± 0.80†</td>
<td>2.57 ± 0.70§</td>
</tr>
<tr>
<td>HDP (n = 20)</td>
<td>6.53 ± 0.33</td>
<td>17.46 ± 0.74§</td>
<td>17.30 ± 2.06§</td>
<td>16.40 ± 1.35§</td>
<td>19.37 ± 1.39‡</td>
<td>18.00 ± 0.30‡</td>
<td>ND</td>
</tr>
<tr>
<td>LDP (n = 15)</td>
<td>6.40 ± 0.48</td>
<td>2.95 ± 0.10‡</td>
<td>0.29 ± 0.04§</td>
<td>0.23 ± 0.05§</td>
<td>0.25 ± 0.02§</td>
<td>0.31 ± 0.06§</td>
<td>ND</td>
</tr>
<tr>
<td>4IN* (n = 15)</td>
<td>6.76 ± 0.44</td>
<td>5.35 ± 0.47‡</td>
<td>4.62 ± 0.65‡</td>
<td>4.36 ± 0.84‡</td>
<td>3.57 ± 0.36‡</td>
<td>4.32 ± 0.72‡</td>
<td>ND</td>
</tr>
<tr>
<td>8IN* (n = 10)</td>
<td>5.96 ± 1.33</td>
<td>5.33 ± 0.48‡</td>
<td>0.22 ± 0.04§</td>
<td>0.23 ± 0.01§</td>
<td>0.23 ± 0.02§</td>
<td>0.21 ± 0.01§</td>
<td>ND</td>
</tr>
<tr>
<td>4GW† (n = 15)</td>
<td>7.75 ± 0.84</td>
<td>1.28 ± 0.06</td>
<td>1.03 ± 0.13</td>
<td>1.56 ± 0.13</td>
<td>1.45 ± 0.06</td>
<td>1.65 ± 0.05</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are averages for animals in each group at all time points of necropsy: only after intervention or withdrawal for those groups alone. Values (means ± SEM) for each group included in that group, because there were no significant differences (P < 0.05) in the biochemical measurements performed at each time point. ND, below the limit of quantification.

CON, control: fed a basic laboratory chow diet plus 50% starch. Data averaged for all time points: 4, 8, 16, and 24 months; GAL, untreated galactose: fed basic diet plus 50% D-galactose at 8, 16, and 24 months; HDP, high dose prevention: fed basic diet plus 50% D-galactose with ARI-509 at 25 mg/kg per day. Data averaged for all time points: 4, 8, 16, and 24 months; LDP, low dose prevention: fed basic diet plus 50% D-galactose with ARI-509 at 10 mg/kg per day. Data averaged for three time points: 8, 16, and 24 months; 4IN, 4-month intervention: fed basic diet plus 50% D-galactose with ARI-509 at 10 mg/kg per day. Data averaged for three time points: 8, 16, and 24 months; 8IN, 8-month intervention: fed basic diet plus 50% D-galactose with ARI-509 at 25 mg/kg per day. Data averaged for two time points: 16 and 24 months; 4GW, 4-month galactose withdrawal: fed basic diet plus 50% D-galactose for 4 months and then intervention by galactose withdrawal. Data averaged for three time points: 8, 16, and 24 months; 8GW, 8-month galactose withdrawal: fed basic diet plus 50% D-galactose for 8 months and then intervention by galactose withdrawal. Data averaged for two time points: 16 and 24 months.

* Mean values in animals sampled from these groups after intervention with an aldose reductase inhibitor.

† Mean values in animals sampled from these groups after galactose withdrawal.

‡ Significantly increased (P < 0.01) compared with control subjects.

§ Significantly decreased (P < 0.05) compared with galactose-fed untreated rats.
The carbohydrate levels measured in plasma, erythrocyte, and retinal samples collected throughout the experiment are summarized in Table 1. The mean plasma glucose ($\pm$SEM) and mean retinal myoinositol ($\pm$SEM) levels remained within normal ranges (5.96 ± 1.33–8.32 ± 0.91 mM and 1.03 ± 0.13–1.75 ± 0.07 nmol/mg wet weight, respectively), regardless of the treatment group. Neither erythrocyte nor retinal galactose was detectable in the CON group at any time point. However, the levels of both were elevated significantly ($P < 0.05$) in all of the rats fed the 50% galactose diet (GAL, HDP, LDP, 4IN, 8IN, 4GW, and 8GW), compared with CON rats, regardless of treatment with the ARI. The levels of galactose were nondetectable after the galactose had been withdrawn from the diet (4GW and 8GW). The mean level of glycated (galactosylated) hemoglobin ($\pm$SEM) was significantly increased ($P < 0.05$; 9.72% ± 0.80%–10.95% ± 0.95%) in all of the groups that were fed a 50% galactose diet (GAL, HDP, LDP, 4IN, 8IN, 4GW, and 8GW) compared with the CON group (2.61% ± 0.10%). After the galactose withdrawal groups (4GW and 8GW) were switched from the galactose to the CON diet, their levels of glycohemoglobin (2.57% ± 0.7% and 1.62% ± 0.27%, respectively) were not significantly different ($P < 0.05$) from those of the CON group.

Galactitol was not detected in the erythrocytes or retinas of CON rats. The mean erythrocyte galactitol ($\pm$SEM) was 2.95 ± 0.1 mmol/mg hemoglobin in the GAL group and was decreased by 90.2% (0.29 ± 0.04 mmol/mg hemoglobin) and 92.2% (0.23 ± 0.05 mmol/mg hemoglobin) in the prevention groups (HDP and LDP, respectively). The mean retinal galactitol ($\pm$SEM) was 5.33 ± 0.48 mmol/mg wet weight in the GAL group and was decreased in the prevention groups by 95.9% (0.22 ± 0.04 mmol/mg wet weight) and 95.7% (0.23 ± 0.01 mmol/mg wet weight), HDP and LDP, respectively. After ARI treatment in the intervention groups (4IN and 8IN), erythrocyte and retinal galactitol were decreased to levels not significantly different ($P < 0.05$) from those of the prevention groups. After withdrawal of the galactose diet (4GW and 8GW), galactitol levels became nondetectable in the retina and erythrocytes.

The mean thickness of retinal capillary basement membranes ($\pm$SEM) was significantly ($P < 0.05$) greater in the GAL group compared with that of control rats at all time points (Fig. 1), becoming almost two times thicker (188.95 ± 9.39 nm) than that of CON rats (102.56 ± 3.36 nm) at the 24-month time
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**Figure 2.** Percent glycated (galactosylated) hemoglobin. Mean percentage (±SEM) for the durations and animal numbers (n) indicated in the following diet groups: □ control (CON); □ 50% galactose (GAL); □ 50% galactose with high-dose aldose reductase inhibitor (25 mg/kg per day ARI-509) prevention (HDP) treatment; and □ 50% galactose with low-dose aldose reductase inhibitor (10 mg/kg per day ARI-509) prevention (LDP) treatment.

The thickness in control rats was significantly greater (P < 0.05) at the 8-month than at the 4-month time point. The mean thickness of retinal capillary basement membranes remained at control levels in both prevention groups (HDP and LDP) at all time points (Fig. 1). The prevention of RCBM thickening correlated with the prevention of erythrocyte and retinal galactitol accumulations by ARI treatment (Table 1), but not with the levels of glycated (galactosylated) hemoglobin (Fig. 2).

In the 4-month intervention and withdrawal groups (4IN and 4GW), the RCBM thickness at the 8-month and 16-month time points (4 and 12 months after the interventions, respectively) was not significantly different (P < 0.05) from those of the GAL groups measured at the same time points (Fig. 3). However, at the 24-month time point (20 months after the interventions) the thickness of retinal capillary basement membranes was significantly less (P < 0.05) than that in the GAL group, but was significantly more (P < 0.05) than that in the CON group. Figure 4 shows the relative thickness of retinal capillary basement membranes (24-month time point) typical of rats that were inhibitor-treated from the onset of galactosemia (HDP) and rats that received intervention treatment with the ARI after 4 months of galactose feeding (4IN) compared with galactosemic rats that were untreated (GAL) and control rats (CON) fed a 50% starch diet throughout the experiment.

When the interventions were initiated after 8 months of galactose feeding (8IN and 8GW), the RCBM thickness was not significantly (P < 0.05) different from that of the GAL group at the 16-month time point (8 months after intervention), but was significantly thinner (P < 0.05) than that of the GAL group at the 24-month time point (16 months after intervention; Fig. 5).

**Discussion**

Aldose reductase inhibitor treatment initiated at the onset of galactosemia prevented diabetic-like RCBM thickening in the galactose-fed rat model of diabetic ocular complications. The lower dose (10 mg/kg per day) of the inhibitor ARI-509 was as effective as the higher dose (25 mg/kg per day) in reducing the galactose-induced accumulations of retinal galactitol and in preventing RCBM thickening. These observa-
FIGURE 3. Retinal capillary basement membrane thickness with 4-month intervention and withdrawal. Mean thickness (in nanometers ± SEM) for the durations and animal numbers (n) indicated after intervention or withdrawal in the following diet groups: ■ control (CON); □ 50% galactose (GAL); ▪ 50% galactose with intervention at 4 months (4IN) by addition of an aldose reductase inhibitor (25 mg/kg per day ARI-509); and • 50% galactose for 4 months, then a 50% starch diet (galactose withdrawal: 4GW). *Significantly less (P < 0.05) than GAL rats.

In the 4- and 8-month intervention groups, in which rats with galactosemia either had their galactose diet withdrawn (4GW and 8GW) or were treated with ARI-509 but remained galactosemic (4IN and 8IN), RCBM thickness continued to increase. Retinal capillary basement membrane thickness was significantly greater than that of the controls at 16 and 24 months and, compared with RCBM thickness in the GAL rats, no significant intervention effect on RCBM thickness was observed until the 24-month time point.

The fact that intervention was not as effective as the prevention therapy suggests that certain slowly reversible changes occurred early in the disease process and delayed the observable effectiveness of treatments. The findings are consistent with previous intervention studies in diabetic rats, diabetic dogs, galactosemic rats, and galactosemic dogs, which also show little or no effectiveness with delayed interventions.

Cumulative evidence suggests that retinal microvascular changes persist and progress for some time after intervention, whether by withdrawal of galactose, tight glycemic control, or addition of an ARI. The nature of the early changes and factors that make diabetic retinopathy refractive to treatment are unknown. However, the importance of having a long follow-up period for the demonstration of beneficial treatment effects was shown clearly in the Diabetes Control and Complications Trial. The reduced effectiveness of intervention ob-
served in the present study is also consistent with the results of the trial in which the risk of a sustained progression of retinopathy was reduced with intensive insulin treatment by 78.5% in the primary prevention cohort, but only by 64.5% in the secondary intervention cohort, despite similar levels of baseline glycemic control.3

The present findings suggest that if it were possible to begin treatment with an ARI at the onset of diabetes, diabetic retinopathy could be prevented in patients with diabetes, provided an adequate dose of a nontoxic compound could be determined. However, such an early treatment is not yet feasible in the clinic, and neither the length of time that intervention can be delayed and still render a beneficial effect nor an ideal aldose reductase therapy has been identified. The fact that withdrawal from galactose (termination of galactosemia) had an effect no better than that of ARI intervention suggests that the extent of preexisting damage at the time of intervention is a primary factor in determining the outcome of therapy. A caveat should be considered with respect to the interpretation of the data in this study. Although RCBM thickening is considered to be a hallmark of diabetic retinopathy18 and its occurrence and prevention correlate with those of other diabetic-like microangiopathies of galactose-fed rats,15 its validity as a surrogate measure for these other vascular lesions remains to be fully established.

In summary, although therapy initiated at the onset of elevated plasma hexose concentrations completely prevented RCBM thickening, neither early nor late intervention therapy, regardless of the type, was as effective. The findings suggest that therapy in humans that is designed for prevention, such as the currently proposed treatments with antioxidants, antglycation agents, or ARIs,12 probably should begin early after the onset of diabetes to have the greatest beneficial effect on the developing retinopathy. This suggestion is supported by the findings of the Diabetes Control and Complications Trial,1,3 which indicated that early intervention improves the outcome significantly.

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References

1. Diabetes Control and Complications Trial (DCCT) Research Group. The effect of intensive treatment of diabetes on the devel-

Figure 5. Retinal capillary basement membrane thickness with 8-month intervention and withdrawal. Mean thickness (in nanometers ± SEM) for the durations and animal numbers (n) indicated after intervention or withdrawal in the following diet groups: control (CON); 50% galactose (GAL); 50% galactose with intervention at 8 months (8IN) by addition of an aldose reductase inhibitor (25 mg/kg per day ARI-509); and 50% galactose for 8 months, then a 50% starch diet (galactose withdrawal: 8GW). *Significantly less (P < 0.05) than GAL rats.

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