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Attenuation of Galactose-Induced Cataract by Pyruvate

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Data in the present paper demonstrate a significant inhibition in the progress of sugar cataract formation by systemic administration of pyruvate. The formation of the cataract was induced by feeding young rats a diet containing 30% galactose. All animals fed this diet developed nuclear lens opacity by the end of 30 days. This was delayed if the diet and water contained, in addition, 2% sodium pyruvate. The incidence of cataract in the latter group was 0% at day 30 and only 25% at day 55. Physiologically, the inhibition was associated with the prevention of lens membrane damage as reflected by its ability to maintain transport of rubidium ions against a concentration gradient; decreased tissue hydration as indexed by the lens wet weight; inhibition of protein glycation, and higher levels of ATP. Since pyruvate, being a normal tissue metabolite, is likely to be non-toxic, the findings are considered useful for further pharmacological studies with this and other similar metabolites, relevant to protection against various secondary complications of diabetes and galactosemia.

Keywords: Sugar cataract, galactose, pyruvate, antioxidant, prevention

INTRODUCTION

Cataract is one of the most noticeable manifestations of galactosemia.[1] Its manifestation is also accelerated in diabetes.[2,3] Earlier studies with experimental animals suggest that an excessive synthesis of sugar alcohols in the lens plays a significant role in the pathogenesis of such cataracts.[4–9] A role of such synthesis in the case of humans, however, is limited due to the very low level of lens aldose reductase, the enzyme responsible for the synthesis of the sugar alcohol.[10] A number of studies suggest that an increased generation of the reactive species of oxygen (ROS) by auto-oxidation of the excessive amounts of the sugars prevalent under the hyperglycemic conditions and consequent oxidative stress could be involved in the onset of various diabetic complications, including those in the eye.[11–13] In addition, a direct glycation of the proteins and their consequent denaturation has also been suggested to be involved.[14–16] The latter process is again dependent upon the availability of ROS.

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The auto-oxidation products of the sugars containing additional carbonyl groups are by themselves more potent glycating agents than the parent sugars. That oxidation might play a significant role in the pathogenesis of sugar cataracts is more directly indicated by the preventive effects of vitamins C, E, and of BHT.[17–21]

We have recently reported that oxyradical induced damage to the lens under organ culture conditions can be effectively prevented by pyruvate,[22–24] a substance previously known to scavenge peroxide[25,26] and other ROS.[27] We have also observed that it inhibits glycation and subsequent denaturation of the lens proteins in aqueous solutions.[28] Additionally, it has been demonstrated to prevent sugar induced deactivation of certain enzymes.[29] Such deactivation by the sugars is attributable to an oxidative denaturation as well as glycation of the enzyme protein.[30]

Based on these biochemical and organ culture studies, we hypothesized the possibility of an actual prevention of the sugar cataract by oral pyruvate supplementation. The results presented in this communication are consistent with this possibility. The progress of cataract formation in animals fed galactose was found to be delayed significantly by incorporating this substance in the diet and water at a 2% level. The glycation of the lens proteins, which was significantly elevated by galactose feeding, was also inhibited. An overall favorable physiological effect was apparent by the higher levels of tissue ATP and prevention against the deterioration of the sodium pump.

MATERIALS AND METHODS

All the chemicals used were obtained from Sigma Chemical Company, St. Louis, MO. Sprague-Dawley male rats were used as experimental animals in accordance with the ARVO guidelines.

Rats weighing 55 ± 5 g were randomly divided into two groups, consisting of 24 animals in each. Group I received a 30% galactose diet, prepared by mixing galactose with the powdered basal Harlan-Teklad rat chow. Water was given *ad libitum*. Group II received the above diet containing 200 mg of sodium pyruvate per 10 g of the diet and drinking water. The diet and the drinking water were prepared freshly every day. Food consumption in the two groups was similar, as apparent by blood galactose measurements (5.5 ± 1.0 mM) using Boehringer Mannheim enzymatic kit #176303.

That systemically administered pyruvate reaches the aqueous humor was ascertained by administering through a gastric tube a bolus of 300 mg of sodium pyruvate dissolved in 1 ml of water and determining its level in the aqueous humor at 0, 15, 30, 45 and 60 min. The levels were determined enzymatically using the Sigma reagent #726. Separate animals were used at each time point. These experiments were performed prior to starting the actual experiments on cataract formation. Levels of aqueous humor pyruvate were subsequently determined in the animals maintained on the two diets.

Damage to the lens caused by galactose feeding was assessed by measuring the active uptake of rubidium ions by the lenses isolated from the two groups, using the techniques previously described.[22] Briefly, freshly isolated lenses were incubated overnight (17 h) in 4 ml of Tyrode medium containing ^{86}RbCl in trace amounts. Incubation was done at 37°C in an incubator gassed with 95% air and 5% carbon dioxide mixture. Subsequently, the lenses were removed from the medium, rinsed with 200 µl of physiological saline, and their radioactivity determined by gamma counting. Medium radioactivity was also determined. The uptake of the ion was expressed as its distribution ratio between the lens water and the medium of incubation. Lens water was taken to be 60% of the wet tissue weight.

For efflux measurements, the lenses were first loaded with ^{86}Rb+ by incubating them with

a higher amount of radioactive RbCl as compared to that in the above experiments meant to measure the uptake. The lenses so loaded were then rinsed with physiological saline and their radioactivity determined as described above. They were then transferred to the Tyrode medium containing 10 mM non-radioactive RbCl and 0.1 mM ouabain and incubated as described above. The efflux of the rubidium ion from the lens was then estimated by determining the percentage of the initial lens radioactivity diffusing out into the medium as a function of time.

ATP was determined by the reactivity of the tissue's aqueous extract with luciferin in the presence of luciferase and determination of the luminescence produced. Freshly isolated lenses were homogenized in 1 ml of ice cold distilled water and centrifuged. Fifty µl of the supernatant was promptly mixed with 200 µl of an arsenate buffered reagent containing luciferin and luciferase (Sigma FLE-50) and the luminescence measured in a Turner Designs photometer. A standard was simultaneously run.

Dulcitol was determined by HPLC using the method of Miwa *et al.*[31] Briefly, the individual lenses were deproteinized by homogenization with 70% aqueous alcohol (1 ml/lens) and centrifugation. An aliquot of the supernatant was then lyophilized. The dried residue was then derivatized by treating it with 70 µl of pyridine and 20 µl of phenyl isocyanate. The derivatized samples were analyzed by HPLC using TSK-gel ODS-80TM column. Acetonitrile : ethanol : water (5 : 2 : 3) mixture was used as the eluting solvent. The peaks were quantified using Beckman system Gold software.

The level of the glycated protein was also determined in the aqueous extracts of the tissue. Fifty µl of the supernatant was used for analysis using the boronate affinity column method of Abraham *et al.*[15] Sigma kit #442-B was used for most of the reagents and the pre-packed column. Total protein was quantitated using the Biorad Bradford reagent.

The formation of cataract was followed by visual inspection and by ophthalmoscopic examination using a 1% mydriacyl drop for dilating the pupil. Photographic documentation was made by Topcon SL-45 photo slit lamp based on the Scheimpflug principle.[32]

RESULTS

Since the inhibition of cataract formation by an orally administered agent is most likely dependent on a direct availability of the compound in the aqueous humor, initial experiments were conducted to ascertain this, by giving an intragastric bolus of sodium pyruvate and determining the aqueous levels at 15, 30, 45 and 60 min. The control animals were given 1 ml of water. The aqueous humors from both the eyes of the animals were pooled for analysis. The basal level of pyruvate in the control aqueous was 170 ± 10 µM. As shown in Figure 1, it rose to about 5 times (910 ± 100 µM) in 15 min after the administration of pyruvate. It remained consistently elevated till 60 min (650 ± 260 µM), falling nearly to the basal level by the end of the second hour. Random determination of pyruvate in the aqueous was also done in the animals maintained on the two diets. As shown in Figure 2, the level in the pyruvate group was again significantly higher than in the galactose group not given any pyruvate.

The results on the effect of galactose feeding with or without pyruvate on the uptake of rubidium ion by the lenses are summarized in Figure 3. The distribution ratio of the ion (CL/CM) between the lens water (CL) and the medium (CM) attained by the end of the incubation was approximately 45 in the basal controls. As expected, the ratio was much lower (10 ± 5) in the case of lenses isolated from the galactose fed animals. In comparison, the ratio was substantially higher in the case of lenses from the animals fed the galactose diet containing sodium pyruvate.

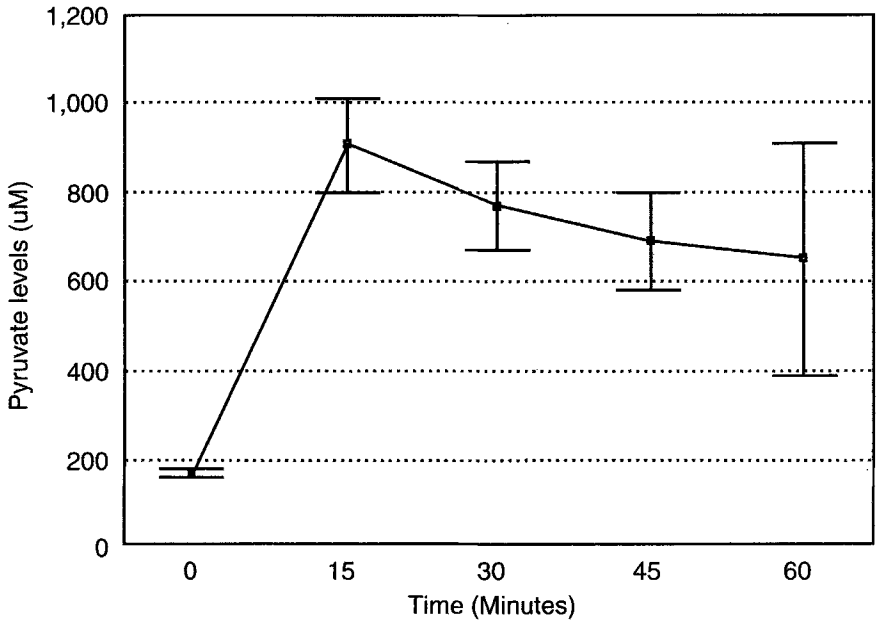


FIGURE 1 Pyruvate levels in the aqueous humor after an intra-gastric administration: Animals were anesthetized with 1% ketamine–xylazine (0.2 ml) given intra-muscularly. Ocular anesthesia was re-enforced with 1% pontocaine drops. Aqueous humor was withdrawn with a 28-gauge needle. Samples from both the eyes were pooled for analysis. The results are summarized as Mean ± S.D. $n = 3$ in each case.

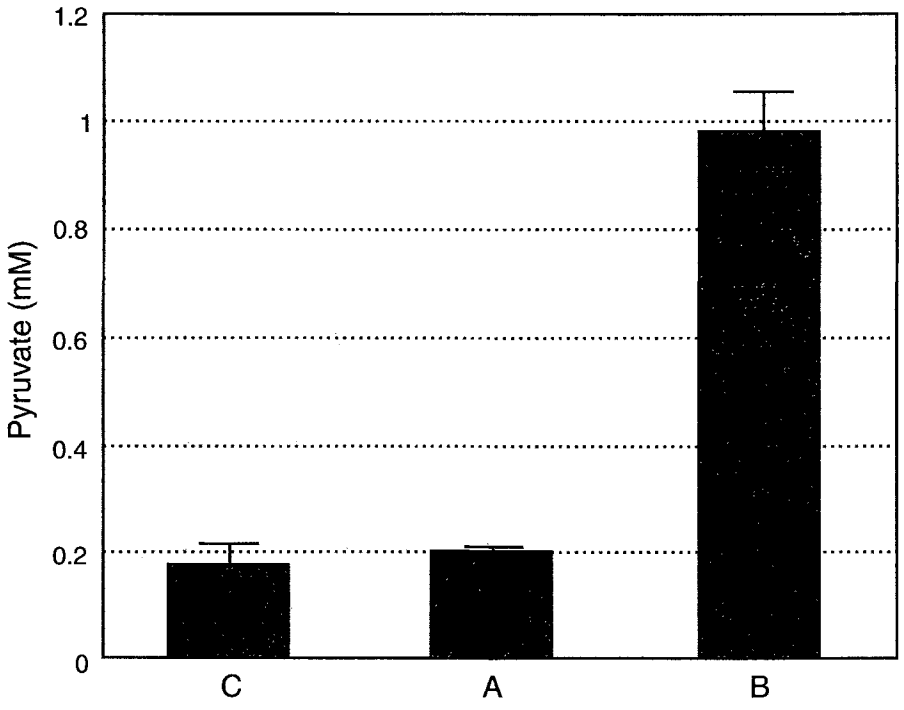


FIGURE 2 Pyruvate levels in the aqueous humor of rats maintained on normal, galactose and galactose + pyruvate diets: C = basal diet. A = galactose diet. B = galactose diet containing pyruvate. Each point represents analysis from three animals. The samples from both the eyes were pooled for analysis. The results are expressed as Mean ± S.D. $n = 3$.

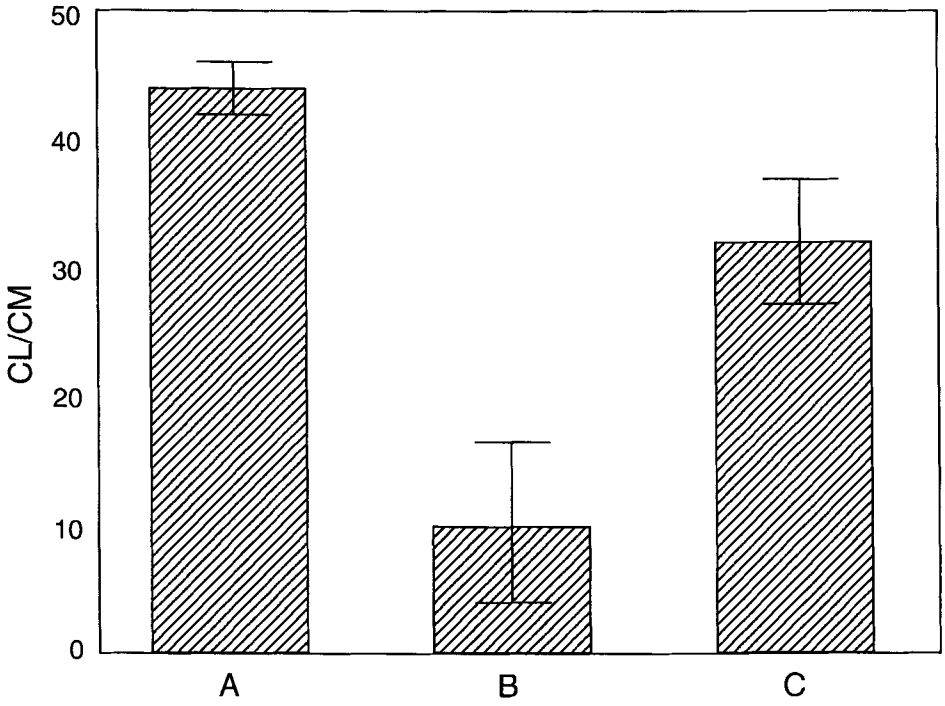


FIGURE 3 Uptake of rubidium by rat lenses maintained on normal, galactose and galactose + pyruvate diets: The results are expressed as the distribution ratio of the ion between the lens water (CL) and the medium of incubation (CM). The T-bars represent S.D. $n = 4$ in each case. A = normal control. B = lenses from the galactose fed rats. C = lenses from animals fed galactose + pyruvate. The lenses used were from animals maintained on the diets for six days.

As shown in Figure 4, the leakage of the ion is significantly greater from the lenses of the galactose fed animals as compared to the controls. The leakage from the lenses of animals fed pyruvate along with galactose was similar to that of the controls, suggesting again a beneficial effect of pyruvate in preventing membrane damage.

The increase in lens weight is also one of the early signs of lens damage by galactose.[5] As shown in Table I, the lens weights in the pyruvate group are also lower, towards the normal, as compared to the lenses of the animals fed galactose without pyruvate.

The beneficial effect of pyruvate was apparent also from the ATP levels. It was significantly lower in the galactosemic lens as compared to the basal control (Table II). In the pyruvate group, the level was significantly higher than the galactose alone group, remaining closer to the levels in the basal controls.

Results on the levels of glycosylated proteins in different groups have been summarized in Figure 5. As indicated, the basal level of the soluble lens proteins in a glycosylated state is approximately 6% of the total. In the galactose fed group, it increased to approximately 12%, double that of the basal control. Hence significant glycation took place during the cataract formation. More interestingly, it did not increase to any noticeable extent in the galactose + pyruvate fed animals. Table III summarizes data on the dulcitol levels in different groups of animals. The level of this polyol was significantly lower in the pyruvate group. But the decrease, although statistically significant, was not great enough to account for the maintenance of the lens weight, which was nearly normal. This could be

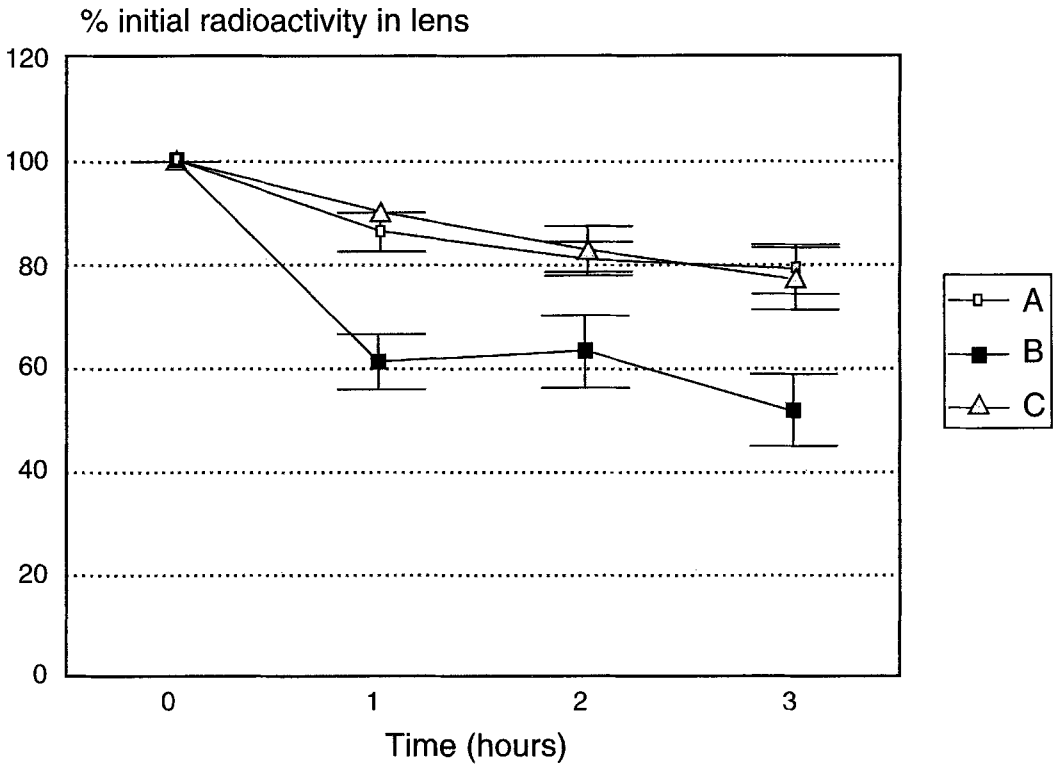


FIGURE 4 Efflux of rubidium by rat lenses maintained on normal, galactose and galactose + pyruvate diets: The results are expressed as the percentage of rubidium diffused out of the lenses loaded with ^{86}Rb. The T-bars represent S.D. $n = 4$ in each case. A = normal controls. B = lenses from galactose fed animals. C = lenses from animals fed galactose + pyruvate. The lenses used were from animals kept on the respective diets for six days.

TABLE I Lens weights (mg)

Days on the diet	C	A	B
3	21.3 ± 04	25.0 ± 0.74*	21.4 ± 0.45
6	24.5 ± 0.7	28.3 ± 0.90*	25.5 ± 1.0

C = normal control. A = galactose diet. B = galactose + pyruvate diet. The weights are expressed as Mean ± S.D. $n = 6$. *Values described under A are significantly different than those under B and C; $P < 0.05$. The differences between B and C are not significant.

TABLE II Lens ATP levels (nmol/g)

Days	C	A	B
6	2020 ± 240	1078 ± 187*	1999 ± 213
8	2020 ± 240	775 ± 225*	1862 ± 176

Values are expressed as Mean ± S.D. $n = 6$ in each case. C = control diet (basal). A = galactose diet. B = galactose + pyruvate diet.
*Values under A are significantly different from the appropriate controls; $P < 0.001$.

accounted for by the nearly normal status of the membrane transport function as apparent by the measurements on rubidium uptake.

In view of the observed effectiveness of pyruvate in preventing membrane damage and consequent hydration, glycation of the proteins and in maintaining a higher level of ATP, the dietary regimens were continued and appearance of actual cataracts followed up to 60 days. At least 12 animals were followed in each group.

In conformity with several earlier studies, a nuclear cataract developed by the end of the fourth week in the animals given the basal galactose diet. This end point is easily visible

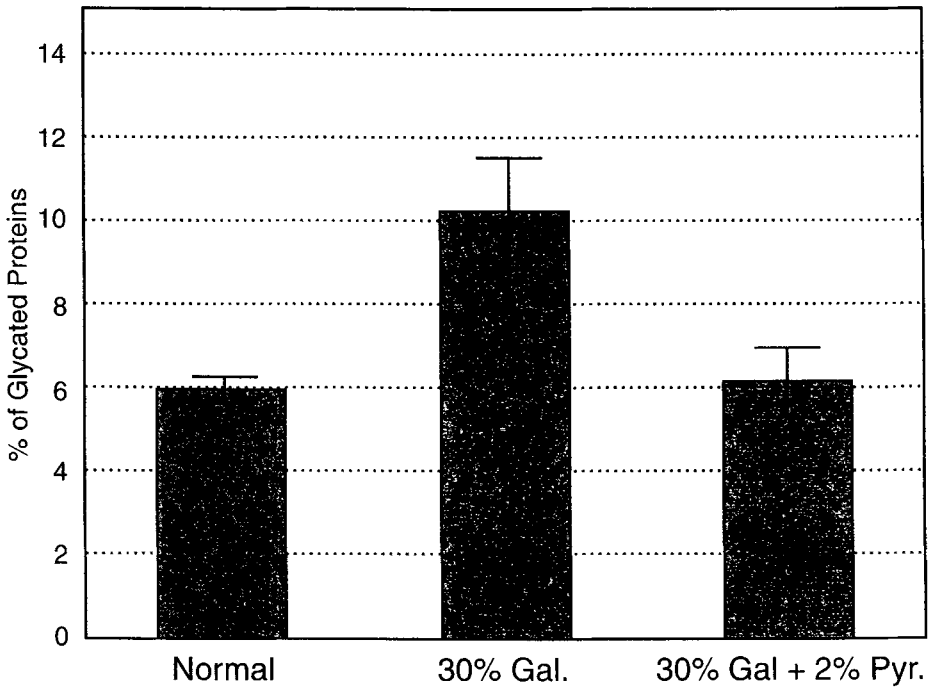


FIGURE 5 Levels of glycated proteins in lens: The values are expressed as the percentage of the total soluble proteins. The results are expressed as Mean ± S.D. $n = 6$. The lenses were analyzed after the animals were on the diets for 3 weeks.

TABLE III Lens dulcitol content in galactose diet fed rats (μmoles/g). Effect of pyruvate treatment

Days	Control	Experimental
1	48 ± 10.4	30 ± 7.4*
2	152 ± 3.0	53.4 ± 18.0*
3	166 ± 31.0	93.5 ± 17.8*
4	154 ± 10.0	104 ± 12.5*

The values are expressed as Mean ± S.D. $n = 6$ in each case. Control: animals fed the 30% galactose diet. Experimental: The diet and water contained, in addition, 2% sodium pyruvate.
*All values in experimental group are significantly lower than the corresponding day values in the control group; $P < 0.001$.

to the naked eye as shown in Figure 6. In contrast, such a cataract was absent in the pyruvate group. As shown in Figure 7, the process of cataract maturation was delayed even further. While all the animals in the control group has a full blown cataract by the end of the month, only about 25% of the animals in the pyruvate group developed a comparable opacity by the end of 55 days. Hence the delaying effect of pyruvate was considered highly significant.

Figure 8 is a representative of the slit lamp (Topcon SL-45) pictures of the eyes in the two groups. As apparent, the opacity of the lens in the control (galactose alone group) is fairly advanced. On the contrary, such opacity is not apparent in the pyruvate group except in 25% of the animals in this group. These experiments were terminated on the 60th day.

DISCUSSION

The development of cataract is a multifactorial process. However, it is commonly believed that oxidative stress, as in several other aging diseases, is one of the important risk factors. The risk is magnified in diabetes or other hyperglycemic conditions due to the ROS generated by auto-oxidation of the sugar aldehydes

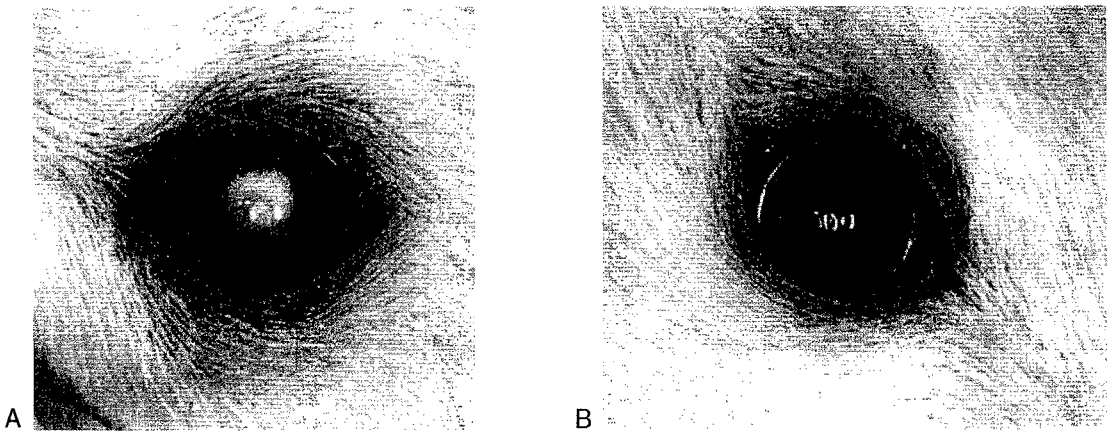


FIGURE 6 Appearance of nuclear cataracts. A = 30% galactose. B = A + 2% pyruvate. Pictures were taken after 30 days on the above diets. (See Color plate I at the end of this issue.)

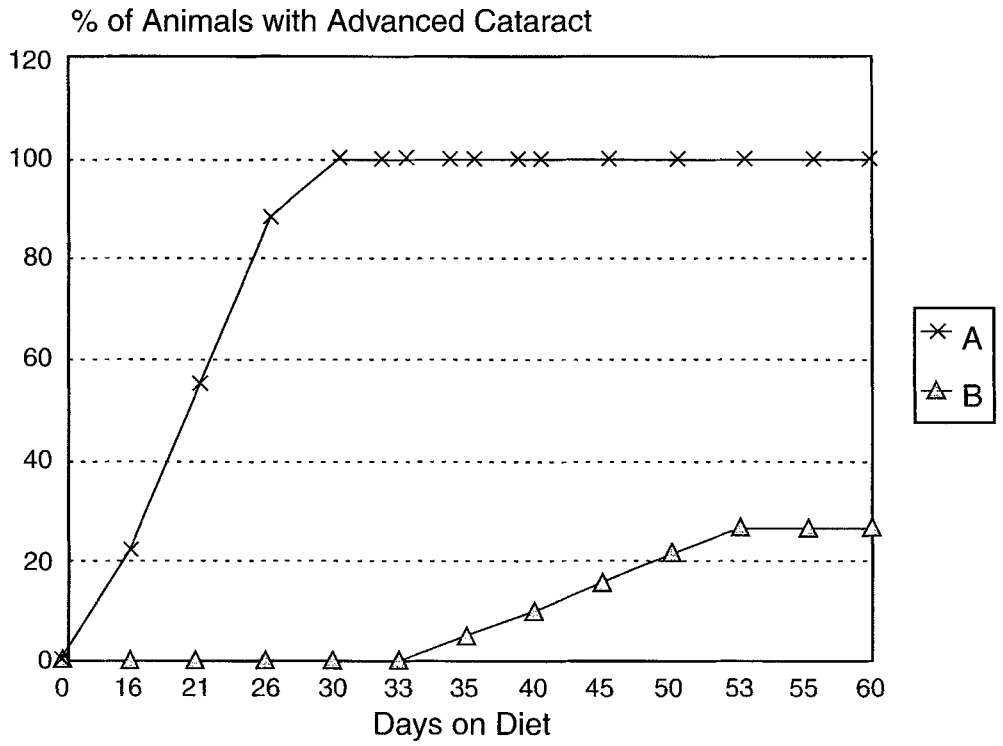
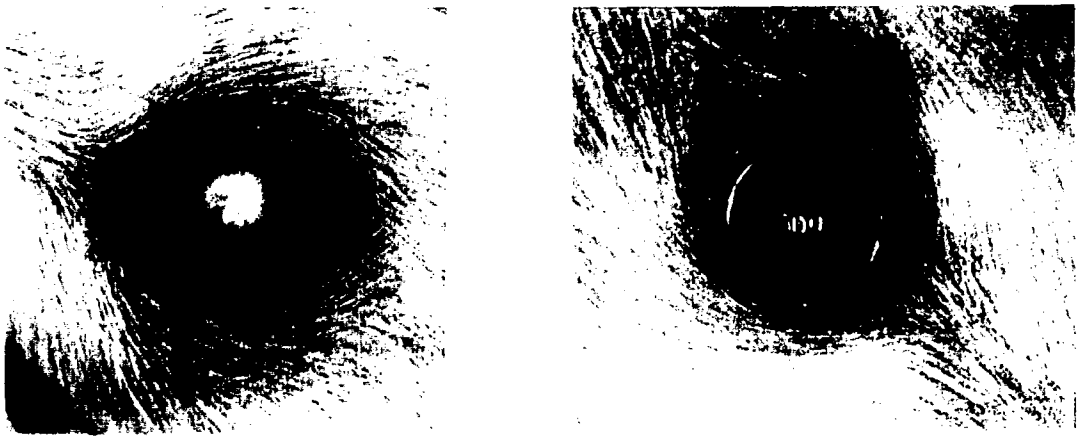


FIGURE 7 Progress of cataract formation in rats: A = animals maintained on a 30% galactose diet. B = A + 2% pyruvate in diet and water.

generating simultaneously dicarbonyl derivatives. These derivatives are highly potent protein glycating agents. Such glycation renders the proteins further susceptible to ROS dependent damage. Functional changes in proteins can take place by ROS even prior to glycation, by loss of their –SH groups. That an oxidative stress is involved in hyperglycemic manifestations



Color Plate I (see page 260, figure 6) Appearance of nuclear cataracts. A = 30% galactose. B = A + 2% pyruvate. Pictures were taken after 30 days on the above diets.

Color Plate II (see page 261, figure 8) Slit lamp pictures of the eyes of animals maintained on galactose or galactose + pyruvate diet for sixty days. A is a representative of the eyes of the 30% galactose diet fed rats. B represents the eyes of the animals maintained on a 2% pyruvate present in the galactose diet and water.

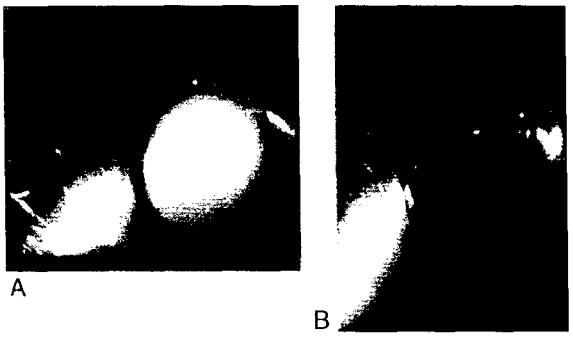


FIGURE 8 Slit lamp pictures of the eyes of animals maintained on galactose or galactose + pyruvate diet for sixty days. A is a representative of the eyes of the 30% galactose diet fed rats. B represents the eyes of the animals maintained on a 2% pyruvate present in the galactose diet and water. (See Color plate II at the end of this issue.)

including cataracts is apparent from several studies referred to earlier. It may hence be feasible to attenuate the formation of cataracts and other sugar related complications by use of appropriate antioxidants. This study is concerned with the possible attenuation of sugar cataracts by use of pyruvate.

Earlier lens organ culture studies have demonstrated that pyruvate can prevent oxyradical induced physiological damage.[22–24] Its preventive effect is easily demonstrable by its ability to protect the lens against damage to its active transport functions when it is cultured either with pure hydrogen peroxide or with the xanthine/xanthine oxidase system. As also demonstrated, the preventive effect depends upon its ability to directly scavenge the peroxide, as well as the free radical species derived from oxygen, such as superoxide and hydroxyl radicals. In addition, pyruvate provides metabolic support to the tissue via facilitating glycolysis. It has also been shown to prevent hyperglycemia induced enzyme deactivation as well glycation of the lens proteins in solutions.[28,29] The latter phenomenon leads to crystallin denaturation and its eventual aggregation to form high molecular weight species, causing light scattering. Since ROS damage to the tissue under culture, as well as glycation of isolated lens proteins could be prevented by pyruvate, the primary objective of the present investigations was to determine if it can also attenuate, if not prevent, the formation of sugar cataracts *in vivo*. The galactose model has been used for convenience. The possibility of such an effect was indicated in an earlier preliminary report[33] wherein frequent topical application of a high concentration of sodium pyruvate (1.5 M) seemed partially preventive. Lower concentrations were not effective. However, repeated application of such a concentrated eye drop on the cornea causes ocular discomfort and osmotic withdrawal of the aqueous constituents, making interpretation of the results more difficult. Nocturnal eating habits of the animal and consequent desirability of instilling the drops in the night is an additional difficulty. Hence further studies on the possible anti-cataractogenic effect of pyruvate were considered desirable, especially in view of the recent findings showing its ability to prevent protein glycation and consequent formation of the denatured lens proteins[28] associated with cataract formation. In view of the difficulties with the topical administration, the present studies were done by administering the compound orally, along with the diet and water, ensuring its consistent supply to the body, simultaneously with the cataractogenic agent.

As described under Results, its oral administration leads to a significant increase in its level in the aqueous humor, assuring its availability to the lens. The physiological alterations in the lens associated with galactose feeding were also noticeably prevented. Most importantly, the time required for cataract maturation was substantially prolonged in the pyruvate group. Such a prolongation, as well as a near total inhibition, has also been observed previously by administration of some experimental drugs acting as inhibitors of aldose reductase.[34] However, many of these drugs turn out to be toxic and unfit for further clinical trials. Hence the use of nutritional and metabolic antioxidants is

preferable. Some of the retinal changes associated with hyperglycemia and claimed to be prevented by certain inhibitors of aldose reductase are also now known to be attenuated by certain antioxidants.[35] In addition to its action as a direct antioxidant, pyruvate also supports the tissue metabolically, as directly reflected by the higher levels of lens ATP. Pyruvate also offers some advantages over the exogenous use of ascorbate and tochopherols. Both these compounds can become pro-oxidant by generating ROS in the presence of excessive amounts of trace metals released during tissue degradation. Generation of such ROS from pyruvate is very unlikely. In addition, pyruvate can help in the maintenance of the tissue redox system as has been found in studies with cardiac ischemia. It can do so also in the lens by its small but significant inhibitory effect against the NADPH dependent polyol synthesis.

Since cataract development in the model used progresses at a rapid pace, it is likely that pyruvate or other alpha keto acids might be more effective against the diabetic manifestations which manifest much more slowly, especially in humans. The present studies demonstrating an *in vivo* effectiveness of pyruvate are hence considered useful for further pharmacological and therapeutic examination.

Acknowledgements

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