Prevention of oxidative damage to rat lens by pyruvate *in vitro*: possible attenuation *in vivo*

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Abstract

Studies have been conducted to assess the possible preventive effect of pyruvate against lens protein oxidation and consequent denaturation and insolubilization. Rat lens organ culture system was used for these studies. The content of water insoluble proteins (urea soluble) increased if the lenses were cultured in medium containing hydrogen peroxide. Incorporation of pyruvate in the medium prevented such insolubilization. The insolubilization was associated primarily with loss of gamma crystallin fraction of the soluble proteins. PAGE analysis demonstrated that insolubilization is related to –S–S– bond formation which was preventable by pyruvate. Since pyruvate is a normal tissue metabolite the findings are considered pathophysiologically significant against cataract formation. This was apparent by the prevention of selenite cataract *in vivo* by intraperitoneal administration of pyruvate. Curr. Eye Res. 14: 643–649, 1995.

Key words: cataract; crystallins; oxidative stress; peroxide; pyruvate; rat lens

Introduction

The conversion of molecular oxygen to its more reactive species (ROS) has been suggested to be involved in the genesis of a variety of aging diseases (1). The pathological changes associated with these diseases are supposedly initiated by the ROS triggered unwanted reactions such as the oxidation of enzymatic and nonenzymatic soluble and structural proteins, membrane and cytosolic lipids, degradation of nucleic acids and mucopolysaccharides, and depletion of several smaller molecular weight redox regulators such as glutathione and ascorbate. The overall biochemical and subsequent functional aberrations initiated by ROS reactivity are commonly referred to as oxidative stress. Such a stress has also been suggested to contribute towards the genesis of cataracts as well (2–9), a highly prevalent multifactorial aging disease. This hypothesis is supported primarily by studies demonstrating certain biochemical aberrations in the lenses cultured in media generating oxyradicals. Previous studies have demonstrated that some of the aberrations such as the oxyradical dependent damage to the lens cation transport can be prevented by pyruvate—a compound known to act as a peroxide scavenger (10). However, crystallin modification has been shown to be one of the hallmarks of the appearance of opacity in a cataractous lens (11–13). The appearance of opacity is primarily due to aggregation and precipitation of the initially soluble lens crystallins. Hydrogen peroxide is considered to be one of the primary agents involved in initiating protein damage reflected in its oxidation and insolubilization. Its concentration has been found to be higher in the cataractous lenses as well as in the corresponding aqueous (14). Hence, the present investigations were designed to study the effect of pyruvate against peroxide–induced protein oxidation and insolubilization in rat lenses under organ culture. Such changes were found to be preventable by this keto acid of metabolic origin. The *in vivo* significance of these findings was examined by studying the effect of pyruvate against cataract formation in animals given sodium selenite. This compound has previously been shown to induce cataractogenesis via oxidative stress (15, 16).

Materials and methods

All chemicals used were procured from Sigma Chemical Company (St Louis, MO, USA). The Sprague–Dawley rats weighing between 35–60 g were derived from breeding stocks provided by Harlan Farms (Indianapolis) through the Animal Facilities, and used in accordance with the ARVO guidelines. Organ culture of the lenses was conducted by the techniques as previously described (13). Briefly, animals were sacrificed by decapitation, eyes enucleated, lenses dissected out by posterior approach and cultured in medium-199 at 37°C in

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a humidified incubator, gassed with 95% air:5% CO_2 mixture. Various additions to the medium have been described in the legends. The post incubation lenses were then studied as follows.

The lens was homogenized in a nitrogen atmosphere in 1 ml of nitrogen saturated phosphate buffered saline (PBS) and centrifuged promptly at 6000×*g* to separate soluble and insoluble fractions. The soluble fraction was aspirated out and used immediately for the determination of ATP, GSH and protein. A portion was also frozen for subsequent studies. The insoluble pellet was washed with nitrogen saturated PBS to get rid of the adherent soluble proteins and nonprotein constituents. This was accomplished by repeated vortexing the pellet with 1 ml PBS, centrifugation and aspiration of the supernatant. The washed pellet was then vortexed with 1 ml of freshly prepared 7 M urea in 0.05 M Tris buffer pH 7.4, incubated for 30 min at 37°C and centrifuged to obtain the supernatant fraction containing the urea soluble proteins. The tube containing this fraction was again nitrogenized and stored at 4°C.

Aliquots of the urea soluble fraction were mixed with equal volume of a denaturing solution (sample buffer) containing 0.0625 M Tris (pH 6.8), glycerol (10%), sodium dodecyl sulfate (4%), and bromophenol blue (0.0025%) and heated at 100°C for 5 min with or without β–mercaptoethanol (2%). The mixture was then cooled and subjected to polyacrylamide gel electrophoresis following the Laemmli's procedure (17). The concentrations of polyacrylamide in stacking and resolving gels were 4 and 12 percent respectively. The size of the gel casted was 7.3×10.2×0.1 cms. The voltage used was 200 volts. Following electrophoresis, the gels were submerged in a fixative solution containing methanol, acetic acid and water (40:10:50 v/v) for a least 30 min, and silver stained following the procedure of Merril *et al.* (1982) (18) using Biorad reagents (Cat #161-0443 to 161-0447). Molecular weight standards were also run simultaneously.

Total protein in the absence of urea was determined by Lowry's procedure. In the presence of urea, however, the Bradford's procedure was considered more convenient (Biorad Cat. #500-0001).

The percentage distribution of the crystallins in the soluble fraction was determined by gel filtration technique. Eight post incubated lenses were homogenized in 2 ml of PBS and centrifuged at 10,000×*g*. The supernatant fraction containing the soluble crystallins was dialyzed against 100 vols of 0.025 M phosphate buffer (containing 1 mM EDTA) pH 7.4 for 2 h at 4°C, changing the buffer every 30 min. 1.5 ml of the dialyzed sample (≈50 mg protein) was then loaded on a Sephadex G-200 column (72×1.5 cm) equilibrated with the phosphate buffer and maintained in a cold room. The column was eluted with the same buffer in 5 ml fractions using a Bio–Rad Fraction Collector. The optical absorption of the eluents was measured at 280 nm. Approximately 40 fractions were required for complete elution.

For GSH determination, 0.5 ml of the freshly prepared aqueous extract prepared in 50 mM EDTA was mixed with equal volume of 10% trichloroacetic and centrifuged to get an acid soluble fraction. 0.1 ml of this extract was mixed with 1.0 ml of 0.3 M Na_2HPO_4 and 0.5 ml of 5,5′–dithiobis (2–nitrobenzoic acid, DTNB) (19). The resulting yellow color was then measured at 410 nm against a standard run simultaneously. The DTNB reagent consisted of 4 mg of DTNB dissolved in 10 ml of 1% trisodium citrate.

ATP was determined by measuring the luminescence produced with Sigma-50 firefly lantern extract (Luciferin–Luciferase mixture) (20). Typically, 200 µl of the aqueous extract was mixed with 50 µl of the firefly extract in a Turner design (Model 20) photometer and the chemiluminescence produced measured against appropriate ATP standards.

For *in vivo* studies, cataract was induced by intraperitoneal injection of 0.5 micromoles of sodium selente (in 0.1 ml saline) per pup on the 10th postnatal day. Cataract appearance was followed by pen light illumination after the eyes were open. In the control group animals were given 0.1 ml of normal saline starting on the 7th day postnatally. Cataract was apparent when the eyes opened around the 14th day. In the experimental group, physiological saline was replaced by 55 mg sodium pyruvate dissolved in normal saline. Treatments in both the groups were terminated on the 25th day when the eyes were photographed. Experiments were also under taken to determine the elevation of pyruvate in the aqueous after its intraperitoneal injection. Aqueous samples were hence pooled from 4 to 6 eyes and pyruvate determined enzymatically by allowing to react with NADH in the presence of LDH and measuring the formation of NAD spectrometrically (21).

Results

Previous studies have demonstrated that lenses when organ cultured in media generating active oxygen undergo physio-

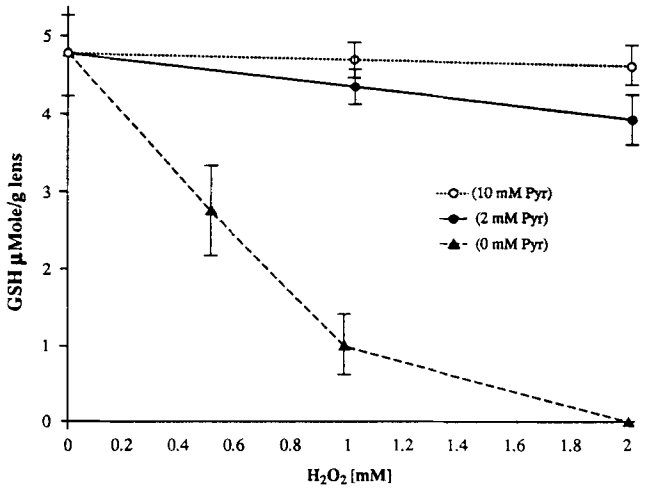

Figure 1. Glutathione level in the lens: the post-incubation lenses were homogenized in 0.05 M EDTA (1 ml/lens) to obtain an aqueous extract and GSH determined after acid precipitation of proteins as described in the text. The I bars indicated the standard deviations. (n = 6)

logical damage as indexed by the loss in their ability to transport rubidium ions against a concentration gradient as well as the loss of ATP and glutathione. The present experiments were designed to study the preventive effect, if any, of pyruvate against oxidation induced protein denaturation and insolubilization. GSH and ATP were measured as positive controls of oxidative stress.

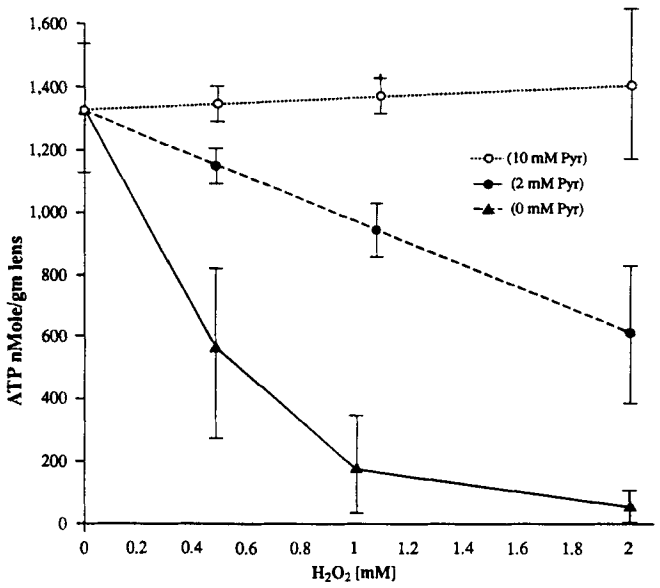

Figure 2. ATP levels in the lens: an aqueous extract of the lens prepared by homogenization and centrifugation of the post incubation lenses in 1 ml of 0.05 M EDTA (1 lens/ml) and was used for ATP determination as follows: 0.2 ml of the aqueous extract was introduced into a test tube (6×50 mm) and placed in a Turner Design photometer model 20. 50 µl of Sigma FLE-50 firefly extract reconstituted with 5 ml of water was then added and the emitted luminescence recorded. A standard was run simultaneously. The results are expressed as mean ± SD (I). n = 6

As described in Figure 1, there is a significant decline in the levels of lens GSH by 17 h of incubation with H_2O_2, the decline being more substantial with 1 to 2 mM levels of H_2O_2. There was a similar decline in the levels of ATP (Fig. 2). These levels of H_2O_2 were therefore considered suitable for studying oxidative insolubilization of protein. These experiments also served as positive controls. Since the decline in the levels of GSH and ATP by H_2O_2 was preventable by 10 mM pyruvate, possible prevention of the protein insolubilization was studied with pyruvate concentrations ranging from 5 to 15 mM.

Data on the contents of water soluble and urea soluble proteins in lenses incubated with various levels of peroxide and pyruvate have been summarized in Table 1. As indicated, the level of water soluble proteins decreased significantly by 2 mM H_2O_2, a less significant but noticeable change was observed at 1 mM. The insoluble (urea soluble) protein content also increased maximally in lenses incubated with 2.0 mM H_2O_2. Hence, the effect of pyruvate against protein insolubilization was studied in lenses incubated with 2 mM H_2O_2. As also described in Table 1, addition of pyruvate to the medium at 10 mM offered almost complete protection against the decrease in the water soluble protein fraction and against the concomitant increase in the urea soluble protein fraction. The protective effect was relatively less with 5 mM pyruvate. The protective effect was also reflected from the ratio of the water soluble to urea soluble fraction. The ratio decreased significantly in the lenses incubated with H_2O_2, the decrease being five fold by 2.0 mM H_2O_2. This decrease was completely abolished by 10 mM pyruvate. The distributions of total proteins between the water and urea soluble fractions in the control lenses incubated without hydrogen peroxide, in the absence and presence of pyruvate were identical. Hence the preventive pyruvate effect

Table 1. Insoluble protein generation by H_2O_2: effect of pyruvate

	(A) H_2O_2 concentration (mM)	(B) Pyruvate (mM)	(C) Water soluble protein (mg/g wet wt.)	(D) Urea soluble protein (mg/g wet wt.)	(E) Water soluble /Urea soluble
1	0	–	373* ± 6	24* ± 4	15.5
2	0.5	–	368 ± 8	28 ± 6	13.5
3	1.0	–	346 ± 12	41 ± 8	8.5
4	2.0	–	281* ± 17	92* ± 8	3.05
5	2.0	5.0	348 ± 17	38 ± 13	9.15
6	2.0	10.0	370* ± 12	26* ± 8	14.2
7	0	10.0	372 ± 5	23 ± 5	16.2

Rat lenses weighing 16 mg were used. Two lenses were incubated in 4 ml of TC 199 medium at 37°C in an atmosphere of 95:5 air/CO_2 mixture for 16 h, with additions described above. Subsequently, the lenses were rinsed with normal saline and homogenized as described in the text. Soluble and insoluble fractions were separated by centrifugation at 6000×*g*. The insoluble pellet was washed twice with phosphate buffered saline (PBS), mixed in 300 µl of 7 M urea containing 0.05 M Tris, pH 7.4 and microfuged to obtain the supernatant representing the urea soluble fraction. Protein was then determined on both the fractions as described in the text. The values are described as mean ± S.D. The n ≥ 4, P values between 1 & 4 and 6 & 4, columns C & D are <0.001. These values are marked with asterisk.

appears intimately related to oxidative stress. As shown by the gel filtration profile of the soluble proteins (Fig. 3), the content of gamma crystallin decreased substantially in the lenses incubated with hydrogen peroxide. This was prevented by the presence of pyruvate. As more clearly described in Table 2, the aberrations in the percentage of crystallin distribution affected by H_2O_2 is significantly normalized by pyruvate.

To ascertain further the possible protective effect of pyruvate against oxidative denaturation and insolubilization of proteins, the water insoluble fraction was dissolved in 7 M urea and fingerprinted by PAGE. In order to get clear differences in the PAGE profiles of proteins of lenses incubated with hydrogen peroxide in the absence and presence of pyruvate, homogenization of lens in 7 M urea had to be done aerobically. Differences in PAGE patterns observed if the lenses were homogenized in 7 M urea anaerobically were quantitatively less striking, but apparent (gel picture not given). As shown by the SDS–PAGE run without treatment with β–mercaptoethanol (Fig. 4), the stain intensity in the stacking gel is greater in Lane 3 representing hydrogen peroxide treatment, as compared to basal (Lane 1), basal + pyruvate (Lane 2), and basal + H_2O_2 + pyruvate (Lane 4). However, the difference between Lane 3 & 1 is less pronounced as compared to Lanes 3 & 2 and 3 & 4. The 97 K region was only minimally different between the different groups except in Lane 4 where H_2O_2 and pyruvate were present together during incubation. The region between 30 to 66 K was also less deeply stained in the pyruvate derived samples, particularly in Lane 4.

Treatment of the sample with β–mercaptoethanol prior to electrophoresis demonstrated a more striking difference (Fig. 5). The bands in the stacking gel, as well as in the 97 K and 30 to 66 K dalton regions, except 43 K protein, disappeared

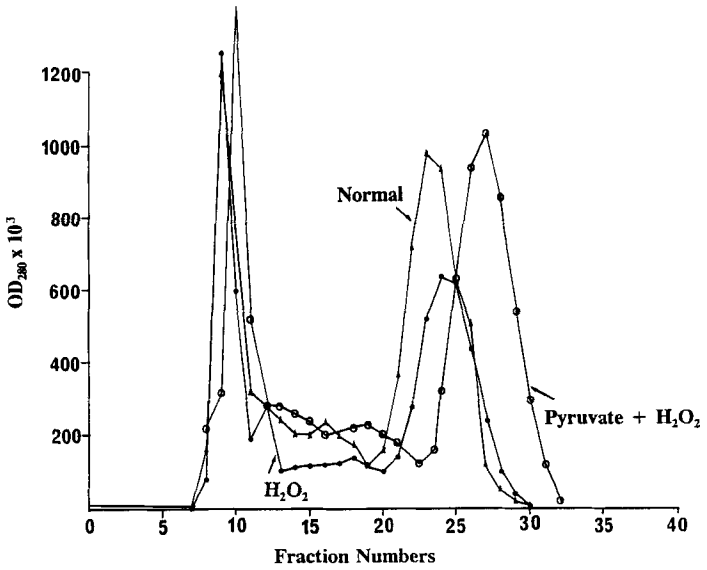

Figure 3. Chromatogram of the soluble lens proteins: the PBS extract of the lens was chromatographed as described in the text. 5 ml fractions were collected and OD_{280} measured on a Gilford 2400 spectrophotometer. The chromatographs have been superimposed with displacement for comparison.

Table 2. Relative distribution of crystallins in rat lenses: effect of H_2O_2 and pyruvate

Incubation medium and additions	mg/gm wet weight				
	Water Soluble Fraction			Urea soluble fraction	Total
	Alpha	Beta	Gamma	HMW	
1) Basal	96 (24.5)	69 (17.6)	208 (53.1)	18 (4.6)	391
2) Basal + H_2O_2 (2 mM)	87 (22.4)	55 (14.1)	148 (38.1)	98 (25.2)	388
2 + Pyruvate (10 mM)	95 (24.3)	69 (17.6)	205 (52.5)	21 (5.3)	390

Data were generated by quantitation of the water soluble proteins from fractions shown in Figure 1 and of the corresponding urea soluble fraction prepared from the PBS insoluble material as described in the text. Figures in parenthesis represent the relative percentages of various fractions.

significantly from Lanes 1, 2, & 4. In lane 3, representing hydrogen peroxide treatment, these proteins still existed, indicating that perhaps the proteins in hydrogen peroxide treated lenses are more susceptible to further oxidation and crosslinking on homogenization in air. These results are, therefore, significant from the point of view of susceptibility to structural damage to the tissue protein associated with oxyradical induced aberrations.

Figure 6 is a representative photograph of the eyes of the rats taken on the 25th day in different groups. It is apparent that administration of pyruvate prevented the cataractogenic effect of sodium selenite. The concentration of pyruvate in the normal aqueous was 0.8 ± 0.1 mM. 15 min after pyruvate administration the level increased to 5 ± 0.25 mM, the number of experiments in each case was 3. Further investigations on the transport of pyruvate across the blood aqueous barrier as well as through the cornea are in progress. The availability of enough aqueous from the individual pups required for such investigations has been a limiting factor for a more desirable pharmacokinetic study. The physiological effectivenes of pyruvate in cataract prevention, however is very apparent.

Discussion

The primary intent of these investigations was to examine further the potential antioxidant and anticataractogenic effects of pyruvate, a normal tissue metabolite.

Decrease in GSH is one of early hallmarks of oxidative stress as well as cataract formation (8). This tripeptide, among its other functions, is considered to be an important antioxidant reserve in the lens. Exposure of lenses to oxidative stress *in vitro* as well as *in vivo* leads to a very perceptible decrease of GSH even before the development of frank opacity occurs. Prevention of its depletion by pyruvate, therefore, serves as a preliminary indication of the anticataract potential of pyruvate (13). However, the actual opacification consists of insolubilized and aggregated proteins. Hence the crucial test of an anticataract function of a metabolite lies with its ability to prevent the latter from taking place. The present investigations indicate that pyruvate is an effective agent in this regard. As described in the preceding paragraphs, incubation of lenses with H_2O_2 leads to significant increase in the amount of urea soluble (water insoluble) protein. There was a simultaneous decrease in the water soluble fraction leading to an ultimate decrease in the water soluble/urea soluble ratio. These changes are reversed significantly by pyruvate. The content of the water soluble as well as the urea soluble proteins were minimally affected by hydrogen peroxide in the presence of pyruvate. The ratio of water soluble to urea soluble (water insoluble) proteins was also normalized. The protective effect of pyruvate

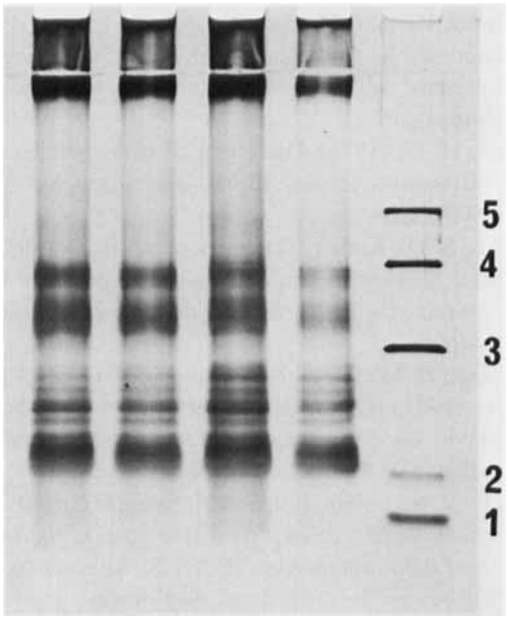

Figure 4. SDS–PAGE profile of urea soluble proteins run without the use of mercaptoethanol in the denaturing buffer. The urea soluble proteins were isolated and chromatographed as described in the text. Lane 1 = Freshly dissected lens. Lane 2 = Lens incubated in the basal medium. Lane 3 = Incubated in medium containing hydrogen peroxide (2 mM). Lane 4 = Lenses incubated as in Lane 3 + sodium pyruvate (10 mM). Molecular weight standards 1 = 14 Kd, 2 = 18 Kd, 3 = 29 Kd, 4 = 48 Kd, 5 = 66 Kd.

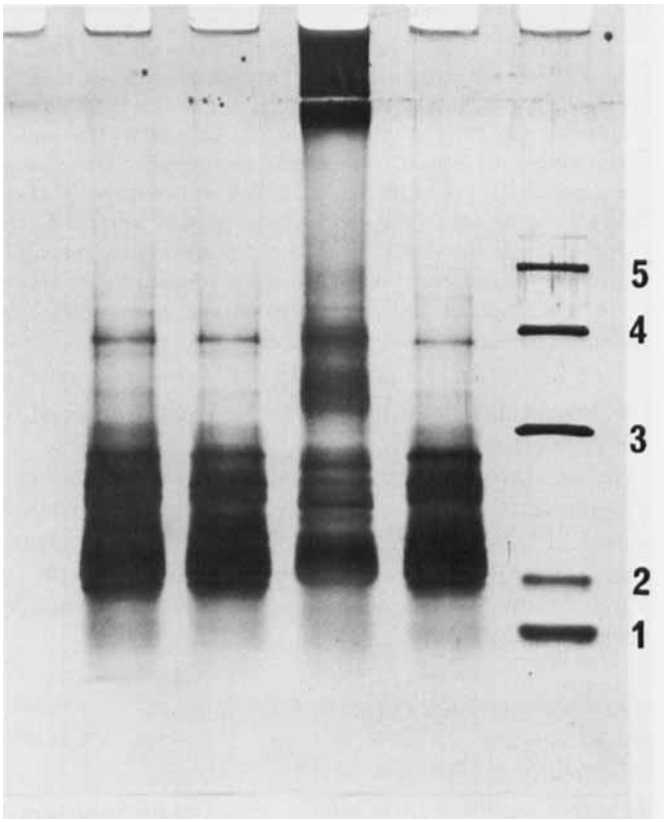

Figure 5. SDS–PAGE profile of urea soluble proteins run after treating the sample with mercaptoethanol as described in the text. Molecular weights standards: 1 = 14 Kd, 2 = 18 Kd, 3 = 29 Kd, 4 = 48 Kd, 5 = 66 Kd.

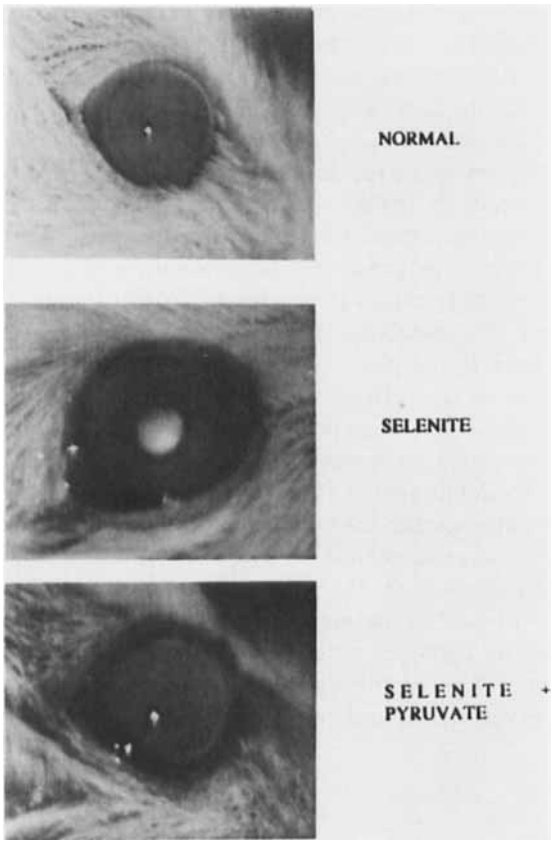

Figure 6. Photographs of the eyes of representative normal, selenite, and selenite plus pyruvare treated rat pups. The weight of the animals at this stage was 35 + 5 gm. Photographs were taken with mideo digital system and copied with normal photography. The pictures were taken 25 days postnatally and 15 days after selenite injection. The total number of animals in each group was at least 8. The experiments were repeated 3 times. 85 + 5 percent of the animals in the pyruvate treated group did not develop central opacity against similar percentage of animals in the selenite alone group who developed cataracts as shown in the photograph.

was also apparent from the protein fingerprints obtained by SDS Page electrophoresis.

The mechanism of the beneficial effects of pyruvate may be multimodal. It is a well known scavenger of hydrogen peroxide (22, 23). More recent experiments from our laboratory suggest that it is capable of scavenging $O_2^{\cdot -}$ (unpublished). As indicated below, both the scavenging reactions are thermodynamically feasible.

	$\Delta G^{o\prime}$	K′eq
$CH_3COCOOH + H_2O_2 \rightarrow CH_3COOH + H_2O + CO_2$	−78	1.5×10^{57}
$CH_3COCOOH + 2O_2^{\cdot -} + 2H^+ \rightarrow CH_3COOH + H_2O + CO_2 + O_2$	−107	4.5×10^{78}

The protective effect could also be related to the reduction of pyruvate to lactate by lactate dehydrogenase. The enzyme, while primarily using NADH for the conversion of pyruvate to lactate can also use NADPH if the pyruvate concentration is increased (24). In support of this theory, we have observed that pyruvate can competitively inhibit aldose reductase catalyzed NADPH dependent reduction of aldehyde sugars to polyols (25). Such a utilization of reducing equivalents from NADPH by pyruvate when administered exogenously would stimulate NADP dependent diversion of glucose to HMP shunt (26), with the consequence of regenerating and maintaining appropriate GSH/GSSG ratio and consequently protecting the tissue against peroxide dependent damage. The beneficial effect of pyruvate against oxidative stress can hence be exerted through several means, including the protection against mitochondrial damage by peroxide. Hence, further studies on the use of this compound as a therapeutic lens antioxidant may prove interesting.

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