Rapid Deterioration of Lens Fibers in GSH-Depleted Mouse Pups

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Lens opacities developed within 48–72 hr in mice that received a series of eight injections of L-buthionine sulfoximine, a specific inhibitor of glutathione (GSH) biosynthesis, on postnatal days 8 and 9. Initial histopathologic features consisted of swollen fibers in the central anterior cortex and displacement of cell nuclei from the bow region to the posterior cortex. These aberrations suggest early fiber cell membrane and/or cytoskeletal dysfunction. A massive wave of fiber cell lysis then engulfed the entire lens cortex and nucleus within 24 hr and left only epithelial cells intact, suggesting a concerted mechanism of cataract generation. The acellular core of the mature cataract seen on postnatal day 16 consisted of a granular matrix in which pycnotic and fragmented cell nuclei were located near the terminus of the lens epithelium. The epithelium displayed increased mitotic activity and meridional row disorganization. During the next two weeks, rapid regeneration of lens fibers, displacement of the acellular necrotic cytoplasm to the center and rear of the lens, and vacuole formation were observed. As new fibers were differentiated, partial regeneration of the bow was seen. However, the cataract was irreversible. Invest Ophthalmol Vis Sci 32:1916–1924, 1991

The functions of reduced glutathione (GSH) in the lens have been investigated for many years because of its high concentration, particularly in the epithelium, the many types of cataracts that are associated with reduction in the level of lens GSH, and the potential of this molecule to inhibit or reverse oxidative changes in the lens that may lead to cataract formation. Of equal interest is the ability of GSH to deactivate a variety of harmful substances via GSH S-transferases, the levels of which are known to be substantial in the lens.

The observation that L-buthionine-S, R-sulfoximine (BSO), an inhibitor of GSH biosynthesis, can induce large, age-dependent cataracts in preweanling mice suggested a potential model for obtaining new information about the role of GSH in maintaining lens transparency. Therefore, studies were designed to characterize the early sequence of histopathologic changes in lenses of mice injected with BSO on postnatal days 8–9. This process involved disorganization, swelling and lysis of lens fibers, and led to disintegration of the entire lens cortex and nucleus within 24 hr on approximately days 10–11. The lens epithelium remained intact. The significance of these findings will be discussed in relation to a report that BSO causes damage to epithelial cell mitochondria and nuclei when administered to rats and mice on days 2–3.

Materials and Methods

BSO was obtained commercially (Schweizerhall, South Plainfield, NJ) and was >99% pure. All other reagents were of analytical grade.

Mouse pups were derived from mating of male and female random-bred Swiss–Webster mice (Dominion Laboratories, Dublin, VA) and were housed continuously as breeding pairs. The first morning that the litters were seen was designated as postnatal day 1. Subcutaneous injections of BSO (4 μmol/g body wt) were administered with a 1-ml tuberculin syringe, fitted with a 30-gauge needle, 4 times daily at 2.5–3 hr intervals for 2 consecutive days, starting on day 8 (t0). The injection solution contained 0.20 M BSO dissolved in 0.10 M NaCl. Untreated age-matched mice
were used as controls. At appropriate intervals after \( t_0 \), the animals were killed by decapitation and their eyes were removed for histologic evaluation. To characterize the initial changes, lenses were examined at \( t = 24, 36, 48, \) and 72 hr. Further cataract development was monitored at 4, 6, 8, 10, 12, 14, 20, and 30 days after \( t_0 \). All procedures were carried out in accordance with the ARVO Resolution on the Use of Animals in Research.

Enucleated globes were rinsed with 0.02 M Hepes, pH 7.4, 0.13 M NaCl. Extraneous tissue was removed and the posterior portion of the globe was incised to allow access of fixative. The tissue was then treated for 12–24 hr with ethanol–acetic acid (3:1) at ambient temperature, followed by treatment for 24 hr in 70% ethanol, and then processed for the preparation of either whole mounts\(^{16} \) or sections.\(^{17} \) Whole mounts were scored for the total number of mitoses or for meridional row disorganization, the latter according to an arbitrary graded scale of 1+ (barely detectable) to 4+ (total absence of rows).\(^{18} \)

For the preparation of paraffin-embedded sections, the posterior incision was enlarged and an additional equatorial incision was made before the tissue was dehydrated in ethanol and cleared with Histosol (National Diagnostics, Parsippany, NJ). The tissue was infiltrated briefly with 50% Histosol/50% Paraplast Plus (Fisher Scientific, Springfield, NJ) and 100% Paraplast Plus, then incubated at 58–62°C in Paraplast Plus for 16–20 hr. Final embedding was performed by positioning the globe in Ameraffin (Scientific Products, McGaw, IL) at 55–57°C, and then cooling the medium in ice. The block was trimmed and soaked in 1% Alconox (Alconox Inc., New York, NY) at 4°C for at least 24 hr to soften the lens tissue before it was sectioned. Routine staining was done with haematoxylin and eosin. Alternatively, selected sections were stained with the Feulgen reagent to preferentially stain nuclear chromatin.

**Results**

**Age and Dose Dependence of the Cataracts**

Because of the rapidity and severity of the changes that occur during early BSO-induced cataractogenesis, the approximate time of its inception could be estimated by examination of isolated lenses under the dissecting microscope. After administration of BSO to mice on days 8 and 9 of postnatal life (4 \( \mu \)mol/g, 4 times daily), large corticonuclear cataracts were induced within 72 hr in all animals. Shortly thereafter, susceptibility to the drug began to decline. Injections begun on day 11 rarely produced cataracts, even if the duration of treatment was extended to 3 full days. Cataracts induced at this later age were smaller and less dense than those obtained by earlier treatment.

Rapidity of the response varied between litters. In some litters, onset of opacification was achieved in 100% of the lenses at 48 hr if injections were started on day 8. In other experiments, all or most of the lenses examined at 48 hr were indistinguishable from those of untreated control subjects, whereas all of those examined at 72 hr had cataracts. Earlier initiation of treatment (ie, on day 6 or 7) increased the rate and synchrony of cataractogenic response to BSO in mouse pups. However, survival of pups was less consistent before day 8, and the pups were more likely to bleed when injected. Mortality among animals injected four times on days 8 and 9 did not differ from that of control pups, provided that all injected animals weighed at least 3.8 g when L-BSO administration was initiated. This condition was achieved by limiting the litter size to ten and culling runts. Such practices have also allowed initiation of injections on day 7, with the additional provision that injections on this day be limited to three times daily to ensure 100% survival rate.

The injection frequency was reduced in some cases to establish the minimum dosage that would consistently produce cataracts when treatment was begun on day 8 (\( t_0 \)). A regimen of three injections per day for 2 days was sufficient to induce early cataractous changes. These changes were visible with a dissecting microscope within 72 hr in \( \geq \)80% of the treated animals. When the injection frequency was reduced to twice daily, cataracts either did not develop or were delayed and reduced in severity. This finding suggests that the standard L-BSO regimen of four injections daily for 2 consecutive days, at \( t_0 = \) day 8, was slightly higher than the minimum dosage required to produce cataracts within 72 hr.

**Early Progression of the Cataract**

Lenses removed within 24 or 36 hr of the time of the first BSO injection (\( t_0 \)) were indistinguishable from normal lenses of approximately the same age (day 9 or 10). However, by 48 hr, a significant fraction of the lenses displayed moderate to severe pathologic features.

An example of moderate BSO-induced lens pathologic changes at 48 hr is shown (Fig. 1A). Comparison of the bow region in this field with that in an age-matched control lens (Fig. 1B) showed disruption of the normal arrangement of cell nuclei (Fig. 1A), resulting in the presence of nuclei in the posterior subcapsular cortex. Initial fiber swelling was most pronounced at the ends near the anterior suture (Figs. 1C and 1D) and the earliest foci of cell lysis were seen in this region (Fig. 1D). These foci were characterized by the formation of extracellular pools of granular cytoplasm. Within the posterior cortex (not shown), no
abnormalities were seen, other than the presence of fiber cell nuclei.

Among those lenses with detectable abnormalities at 48 hr, cytologic disruption was typically more severe than that seen in Figure 1 (A, C, D). In the sample depicted in Figure 2 (A–C), fiber cell degeneration was pervasive. Only epithelial cells and early nucleated fibers retained their integrity. Accumulations of pycnotic and fragmented nuclei, independently identified by Feulgen staining, were found near the surviving early nucleated fibers (Fig. 2A). Apparent remnants of fibers remained within the anterior cortex (Figs. 2A and 2B), indicating that plasma membrane structure had not completely disintegrated. By contrast, in the posterior cortex of the lens, destruction of the membranes resulted in a continuous cytoplasmic matrix defined by zones of varying staining intensity and granularity (Fig. 2C).

Since pathologic changes were not seen at 36 hr, the findings described above on samples removed at 48 hr indicated that deterioration of the lens was rapid between 36 and 48 hr after \( t_0 \). Between 48 and 72 hr, this
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Fig. 2. Further progression of BSO-induced lens opacification. (A) Equatorial region, showing complete disappearance of the bow at t = 48 hr, leaving only pycnotic fragments of cell nuclei scattered among the remains of lysed lens fibers. (B) Central epithelium and superficial anterior cortex of the same lens, displaying various stages of lens swelling, disarray, and rupture in the presence of an intact epithelium. A large zone of liquefaction is evident immediately beneath the epithelium. (C) Subcapsular posterior cortex of the same lens, in which fiber degeneration appears to be complete. (D) Anterior cortex and bow region of a lens removed at t = 72 hr. The fiber cells have been replaced by a mass of granular cytoplasm, which pervaded the entire cortex and nucleus of this lens. Remnants of cell nuclei are concentrated near the terminal epithelial cells. Scale bar = 100 μm.

degeneration continued in the absence of further administration of BSO. In a more advanced stage (Fig. 2D, 72 hr), the acellular zones were of reduced staining intensity, with many clear spots, suggesting further degradative changes. Pycnotic remnants of cell nuclei persisted at the border between surviving cells of the superficial bow and the acellular anterior cortical region.

Further Cataract Development

During the next 5 days of cataract development, liquefication of the central lens cortex and nucleus continued. Meanwhile, new fiber cells differentiated in the region of the lens equator in a manner suggesting an attempt at reconstitution. Figure 3A shows a lens section obtained from a treated mouse pup at t = 8 days. The lens epithelium remained reasonably intact. However, there was a considerable degree of hyperplasia, which was correlated with progressive disturbance of the normal arrangement of cell nuclei in the transitional zone of the epithelium and bow region. The new fiber cells were abnormally round and their distribution was chaotic; the pattern of nuclei in the equatorial region bore no resemblance to the normal bow configuration.

Using whole-mount procedures, we found that the total number of mitoses seen in BSO-affected lens samples peaked at t = 6 days and exceeded 200% of that seen in normal day-14 mouse lens preparations. This coincided with a 3° to 4° grade of meridional row disorganization (Fig. 3B), thus supporting the im-
Fig. 3. Mature BSO cataracts. (A) Section of a lens removed at $t = 8$ days, showing rapid, disorganized regeneration of elongated, nucleated cells. Degeneration in the interior of the lens persists. The arrangement of nucleated cells in the equatorial region is abnormally chaotic and circular. (B) Whole mount preparation of a lens removed at $t = 6$ days, showing grade 4+ meridional row disorganization, accompanied by abnormally high incidence of mitotic figures (arrowheads). (C) Atypical cataract, seen at $t = 10$ days, in which the central epithelium is interrupted by gaps (arrowheads) and multilayered arrangements of epithelial cells (arrows). The unusually high degree of vacuolization probably reflects less complete disintegration of fiber cells than in (A). Scale bar = 100 μm.
Fig. 4. Hypermature BSO cataracts. 
(A) Anterior cortex and equatorial region (t = 14 days). The lens bow has begun to regenerate, and masses of necrotic material have accumulated beneath the epithelium. (B) In a lens removed at t = 20 days, large coalescent vacuoles have formed in the equatorial region and posterior cortex. Clefts persist between regenerating fibers, as the major mass of acellular, granular material is displaced centrally and posteriorly. (C) Central epithelium and central anterior cortex of the lens in (B), illustrating the continued presence of cell nuclei in abnormal locations (arrowheads). Scale bar = 100 μm.
pressions of hyperplasia and disorganization conveyed in Figure 3A.

In another, less typical section (Fig. 3C) obtained from a lens removed at t = 10 days, an extensive lesion in the epithelium consisted of two gaps bordered by short sequences of stratified epithelial cells. Nevertheless, the individual epithelial cells in this section appeared normal, in sharp contrast to the acellular, vacuolated cortex. Formation of extra cellular vacuoles often followed, but never preceded, lysis of lens fibers during development of the BSO cataract (compare Figs. 2D and 3A with Fig. 4B). Multiple vacuoles, such as those seen in Fig. 3C, fused to form larger ones (Fig. 4B).

Generally, observable epithelial disruption during the first 10 days of cataract development occurred after fiber cells had lysed, and was limited to disturbance of cell association during the transition from cuboidal cells to nucleated fibers (Fig. 3A) and occasional instances of gaps and stratification in the central zone (Fig. 3C).

The continued generation of new intact fiber cells during weeks 2 and 3 of cataract development led to the displacement of the necrotic material to form a central region that, in the example shown at t = 14 days (Fig. 4A), extended from the superficial anterior cortex to the posterior capsule (not shown). Although the bow configuration of fiber cell nuclei had partially disappeared in this lens, there were small clefs between the adjacent fibers. The accumulation of dense material beneath an apparently normal epithelium in this lens was noteworthy (Fig. 4A). In another lens examined at t = 20 days, pathologic features in the superficial anterior cortex included incipient vacuoles and abnormally round, swollen, occasionally nucleated fibers (Fig. 4B). Subepithelial accumulation of dense material was not seen, however. The major acellular, granular mass was displaced by new fibers to a central posterior location, and large, coalescent vacuoles were seen deep in the posterior cortex (Fig. 4B). Partial regeneration of the bow was evident in this lens; nevertheless, nuclei persisted in the central cortex (Fig. 4C). Despite the restitution of normal fibrogenesis, the BSO cataract was irreversible.

**Discussion**

Histopathologically, the BSO cataract initiated in day 8 mice involves a rapid, complete loss of differentiated lens fibers in the presence of an intact epithelial cell layer. The earliest changes (Fig. 1) are characterized by the displacement of fiber cell nuclei from their normal equatorial location and swelling of fibers in the central anterior cortex. Collectively, the observations are consistent with rapid pathologic changes that occur in the anterior cortex and bow regions. Although such aberrations may have arisen from compromised epithelial cell function, there was no obvious deterioration of the epithelial cells. The latter observation stems from the normal appearance of epithelial cells when fiber cell lysis is pervasive (Figs. 2A, 2B, and 2D), and in addition, the rapid regeneration of new fibers from the epithelial layer after the acute destruction of the lens cortex and nucleus (Fig. 3A).

Nevertheless, independent evidence supports the possibility that the epithelium is the primary site of BSO-induced pathologic changes in the lens. It has been reported that BSO induces mitochondrial and nuclear degeneration in the epithelium of rat or mouse lenses within 1 week after single injections (3 \( \mu \text{mol/g, i.p.} \)) on postnatal days 2 and 3. However, no information was given regarding the status of the lens cortex and nucleus in the neonatal cataract model or the overall pattern of opacification during the period after cessation of drug administration. Although it is difficult to relate the events generated in newborn rodents with those that we induced approximately 1 week later, certain differences are evident. In the newborn rat model, lens GSH remains \( \geq 0.2 \text{ nmol/mg} \) and lens growth is inhibited. By contrast, we found that production of cataracts with BSO after 1 week of age in the mouse involves near-total depletion of lens GSH, but little or no inhibition of lens growth during the first week of cataract development (unpublished data). Thus, the two reported BSO cataract models differ with respect to age of initiation, drug dosage, degree of GSH depletion, and early effects on lens growth. It is therefore possible that damage to epithelial cells is more significant in the neonatal cataract model than in the one described here.

Because of the rapid, almost concerted swelling and lysis of lens fibers during BSO-induced cataract development, fiber cell membrane deterioration must be an early event in the generation of BSO-induced lens opacities in 1-week-old mice. Membrane dysfunction is critically involved in the generation of most cataracts. Disulfide bond involving membrane proteins may be an important factor in such pathologic processes. Many in vitro studies have shown disturbances of ion transport or diffusion in the lens as a result of generalized oxidative stress, depletion or oxidation of GSH, disturbance of the GSH redox cycle, or reagents reacting with membrane SH groups.

At the same time, the posterior displacement of fiber cell nuclei during the initial stages of BSO-induced cataractogenesis suggests an early disturbance of cytoskeletal function in the fiber cells before rupture of their membranes and visible opacification.
Cytoskeletal abnormalities have been implicated in the generation of cataracts that originate in the lens cortex. Studies show that globular degeneration of lens fiber cells is an early event in the development of metabolic, senile, and drug-induced cortical cataracts. Perturbations of the synthesis or assembly of cytoskeletal proteins have generated cortical-based cataracts. Disturbance of GSH metabolism can produce globular degeneration of cultured lens epithelial cells, thus establishing a possible link between GSH and cytoskeletal function in the lens.

Irrespective of the locus and nature of the events leading to opacification, depletion of lens GSH is probably the prime cause of BSO cataracts. The agent used to induce the cataracts is a highly specific inhibitor of γ-glutamylcysteine synthetase, the enzyme that initiates GSH biosynthesis, and its effects on newborn mice and rats are preventable with GSH monoisopropyl ester. In addition, cataractogenesis after 1 week of age in the mouse requires near-total GSH depletion. GSH functions as a major intracellular antioxidant, either by acting as a cosubstrate in glutathione peroxidase reduction of hydroperoxides, or by direct reduction of disulfides and various free-radical species. Because most cataracts are believed to have an oxidative component, interest has been generated in the role of lens GSH in the prevention of lens opacification. The results of in vitro studies suggest that depletion of GSH in mature rat lenses results in opacification only if additional stress, eg, oxidative insult, is present. By contrast, elevation of GSH above normal levels in lens epithelial cells does not appear to provide additional protection against such oxidative injury.

On the other hand, in vivo experiments with BSO indicate that the sensitivity of the lens to GSH depletion is greater in young lenses (ie, shortly after birth) and decreases sharply during the first 2 weeks of postnatal life. This developmental change may be related to one or more of the many modifications of lens structure and function during this period, which include: decline in lens water and in the penetrability of the extracellular space, alterations of epithelial cell junctions, reduction in metabolic activity, altered patterns of protein synthesis, and regression of the tunica vasculosa. Elucidation of the mechanisms underlying BSO-induced cataractogenesis in preweanling mice and rats may provide important information relevant to both early postnatal lens development and to the functions of GSH and protein SH groups in the maintenance of normal lens physiologic features and cytoarchitecture.

The protocol described in this report induces reproducible pathologic changes that begin approximately 48 hr after the initiation of BSO treatment and always produce grossly observable opacities by 72 hr. It has been designed to maximize and synchronize the rate of cataract induction by saturating the lens with BSO. The resulting model provides an excellent system to study the temporal correlation of GSH depletion, alterations in lens proteins, mineral content and metabolic activities, and the initiation of lens cortical cataracts.

Key words: buthionine sulfoximine, glutathione (GSH), lens fibers, cataract, mouse pups

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References


