as shown in Table 3 is that right to left differences are smaller when the depot is placed in the upper cornea.

Key words: Endothelial permeability, aqueous flow rate, fluorophotometry, corticosteroids

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References


An Improved Fixation Technique for Maintaining the Fine Structure of the Nuclear Zone of Neonatal Mouse Lens

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The fine structure of the nuclear zone of neonatal mouse lenses can vary considerably according to the fixation used. When normal neonatal mouse lenses are fixed in a commonly used chilled glutaraldehyde solution, the nuclear zone develops a grossly visible opacity, and irregular sized protein granules appear in the subsequent sections. Similar artifacts of aggregated irregular sized protein granules appear when cataractous mouse lens sections are conventionally processed. These artifacts can be avoided by soaking the lens in 0.15 M reduced glutathione solution for 10–15 min before fixation in a phosphate buffered 2% glutaraldehyde solution (pH 7.4) at 27–35 C. Normal lenses treated in this manner maintain transparency in the nuclear zone throughout the fixation-embedding procedure, and the resulting sections show finely uniform granularity with the cell membrane well preserved. Similarly processed nuclear portions of cataractous lenses of Nakano mice show uniformly aggregated protein granules, measuring about 350 A in diameter. The cell membranes in the cataractous zone are also not interrupted. Invest Ophthalmol Vis Sci 24:1311–1316, 1983

The nuclear zone of normal neonatal mouse lenses invariably become opaque after cooling or fixation with glutaraldehyde-containing fixatives. This lens opacity formation due to cooling has been termed cold cataract. Although physiologic and physical studies on this phenomenon have been extensively reported, the mechanism for opacity formation upon glutaraldehyde fixation has not been described. Upon comparing various fixation techniques we have found that the fine structure of the nuclear zone of neonatal mouse lens can considerably vary depending upon the technique used. It is particularly difficult to maintain the fine homogeneous granular appearance of crystallin substance in the cytoplasm.

Here we present an improved fixation method for the preservation of lens cell fine structure. This method is of particular interest for the adult cataractous mouse lens.
Materials and Methods. Normal Swiss Webster albino mice and Nakano cataractous mice were used in this study. Normal animals were killed with an overdose injection of sodium pentobarbital on the 2nd and 5th postnatal day, and cataractous mice were killed on day 90.

To develop an optimal procedure for the preservation of the fine structure in neonatal mouse lens, several different concentrations of fixatives (e.g., glutaraldehyde, paraformaldehyde, formalin, and OsO₄) in 0.1 M phosphate buffer were used. Fixation was carried out at several different pH ranges (5.5–9.0) and temperatures (0–55 °C). Fixation times were also varied from 1 hour to 5 days. The addition of additives such as picric acid or sucrose and pretreatment in reduced glutathione (GSH), oxidized glutathione (GSSG), mercaptoethanol, dithiothreitol (DTT) and amino acid (glutamic acid, cysteine, glycine and lysine) were also tried in conjunction with fixation in 2% glutaraldehyde.

Best maintenance of lens cell fine structure was obtained by the following methods:

**Method 1:** for young mouse lens

1. The mouse was placed at 36 °C for 30 min before death.
2. Whole lens was fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 27–35 °C for 5 min.*
3. The lens was placed in 0.15 M GSH in distilled water and cut into two or three pieces with a sharp razor blade.
4. These pieces were then soaked in the same GSH solution for an additional 10–15 min at 27–35 °C.
5. After soaking, tissues were fixed with 2% glutaraldehyde solution at 27–35 °C for 12 hrs and postfixed in 1% OsO₄ at 4 °C for 5 hrs.

**Method 2:** for adult mouse lens

1. After steps 1–3 of method 1, the cortical zone of the lens was removed with a razor blade. The remaining nuclear zone measured approximately 0.5 mm. This procedure was performed while the tissue was immersed in 0.15 M GSH in distilled water.
2. Proceed as in steps 4 and 5 of method 1.

During fixation, the nuclear region of the lens was trimmed into small pieces. The pieces were postfixed in a 1% osmium tetroxide solution of 0.1 M phosphate buffer (pH 7.4) at 4 °C for 5 hrs, then dehydrated in ethanol and embedded in a hard epoxy resin.

Results. Neonatal mouse lens: Glutaraldehyde fixation of a normal neonatal mouse lens invariably results in formation of an opaque mass in the center of the lens. The superficial anterior cortical region of the lens, particularly the bow cortex, turns yellow during the fixation, presumably due to Schiff’s base formation between glutaraldehyde and an amino acid group. The size and density of the opaque mass in the nuclear zone does not appear to be a function of pH, osmolarity or temperature. Moreover, a similar opacity occurs with other fixatives (paraformaldehyde, formalin, and OsO₄). The location of opacity induced by glutaraldehyde in the neonatal mouse appears identical to that of the cold induced cataract.

Translucency of the nuclear zone of the lens is well maintained upon fixation with glutaraldehyde following pretreatment in GSH or GSSG at 27–35 °C or prefixation of a whole lens in a glutaraldehyde-GSH mixture at 27–30 °C. The lenses were soaked in GSSG solution three times longer than in GSH solution. Pretreatment with amino acids such as glutamic acid, cysteine, glycine or lysine and mercaptoethanol or DTT (which is used for retarding the oxidation of sulphydryl groups) did not preserve the lens translucency. Prefixation of the lens in a mixture of glutaraldehyde and amino acids was also not effective.

Light microscopy: When neonatal mouse lens is exposed to low temperature, a commonly accepted procedure, a cold cataract immediately forms in the nuclear zone. The opaque nuclear zone of these cold-treated lenses histologically demonstrated a large number of aggregated masses. The aggregations were generally round in shape and variable in size. While present throughout the deep cortical region, their highest concentration was in nuclear region (Fig. 1A). These round aggregations were not observed when the lenses were fixed at temperatures ranging from room temperature to 35 °C. However, micrographs of these lenses showed many tiny dots in the cytoplasm, especially in the paramembranous zone (Fig. 1B). Micrographs of the nuclear zone of neonatal lens pretreated in GSH at 30 °C reveal uniform cytoplasmics (Fig. 1C). No intense staining was noted in the paramembranous zone of the lens cell following this new fixation technique.

Electron microscopy: The lenses fixed with glutaraldehyde at 4 °C showed many dense aggregated materials in the cytoplasmics of lens cells. The aggregations appeared round in shape and ranged from 0.2 to 2.0 μm in size. Between each aggregations the cytoplasm was relatively electron lucent (Fig. 2A). Although protein granules became considerably uniform in size upon pretreatment in GSH, lenses fixed at 4 °C still showed marked formation of dense aggregations (Fig. 3A). When lenses were fixed with

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* This step can be omitted for neonatal mouse lens.
Fig. 1. A, Neonatal lens fixed with glutaraldehyde at 4°C showing many dense aggregated materials (×1040). B, Neonatal lens fixed with glutaraldehyde at room temperature to 35°C. Many tiny dots can be observed in the cytoplasm (×1040). C, Neonatal lens pretreated in GSH at 30°C reveal lens cells with amorphous cytoplasms (×1040).

Fig. 2. A, Neonatal lens fixed with glutaraldehyde at 4°C showing many dense aggregated materials (×7800). B, Neonatal lens fixed with glutaraldehyde at room temperature at 35°C. The cells show coagulations of the lens crystallins specifically along the cell membrane (×7800).
Fig. 3. A, Neonatal lens pretreated in GSH at 4°C show many dense aggregations, but there were homogenous crystallin granules (×7800). B, Neonatal lens pretreated in GSH at 30°C demonstrate uniform granularity of the crystallin substance (×12000). C, The size of the individual granules measure from 100Å to 200Å in diameter (×62400).

Fig. 4. A, Nakano cataractous lens fixed with a chilled solution of glutaraldehyde contain many aggregations (×7800). B, Nakano cataractous lens fixed at room temperature to 35°C with glutaraldehyde showing coagulations of crystallin substance along the cell membrane (×7800).
Fig. 5. A, Nakano cataractous lens pretreated in GSH showing uniformly aggregated crystallin granules (×7800) and at higher magnification. B, Well-preserved cell membranes (×62400).

glutaraldehyde at room temperature to 35°C, the cells showed coagulations of the lens crystallins specifically along the cell membrane (Fig. 2B).

Electron micrographs of the nuclear zone of neonatal mouse lenses pretreated in GSH at 30°C demonstrated strikingly uniform granularity of the crystallin substance and good preservation of the cell membrane (Fig. 3B). The size of the individual granules measured from 100Å to 200Å in diameter (Fig. 3C).

**Adult cataractous mouse lenses:** Advanced stage cataractous lenses of Nakano mouse were used in this study. The cataractous nuclear zone of these lenses behaved similar to the nuclear zone of neonatal lens following fixation in glutaraldehyde. Lens cells fixed in a chilled solution of glutaraldehyde produced many aggregations, with marked interruptions of the cell membranes (Fig. 4A). Lenses fixed at higher than room temperature showed coagulations of crystallin substances along the cell membrane (Fig. 4B). When pretreated in GSH, the nuclear zone of Nakano cataractous lens showed uniformly fixed crystallin granules. However, these granules were considerably coarser than normal and measured 300Å to 500Å in diameter (Fig. 5B). The cell membranes in these cataractous lenses were well preserved with no breakage noted (Fig. 5A). The number of gap junctions appeared to be somewhat smaller than normal.

**Discussion.** When the lens is exposed to temperature below 20°C, free crystallin granules appear to aggregate prior to infiltration of glutaraldehyde. Low-temperature crystallin aggregation is believed to occur as a result of the protein-water phase separation phenomenon. The occurrence of this phenomenon suggests that crystallin proteins are flexible, monomeric, and polymeric forms in the cytoplasm.

It is known that glutaraldehyde reacts with proteins to form both intra- and inter-molecular crosslinks, to link numerous molecules together as a heteropolymer, and to cause large aggregations to crosslink with free proteins. Consequently, it is conceivable that flexible crystallin proteins in neonatal mouse lens cells convert into large membrane associated aggregations following the infiltration of glutaraldehyde.

GSH in lens tissue is known to maintain protein sulphydryls in reduced forms; and to cleave protein-protein disulfide bonds. But these properties do not explain its ability to maintain lens cell fine structure during fixation as mercaptoethanol and DTT, which have similar properties, do not facilitate good preservation. GSH may not be permeable to all types of cells. Rather, it is thought that when mixed with glutaraldehyde, the some portion of the GSH amino acid sequence binds with the fixative. It is known that glutaraldehyde can react with α-amino acids, the N-terminal groups of some peptides, and the sulphydryl group of cysteine. It is, therefore, reasonable to presume that GSH prevents crystallin aggregation by inhibiting glutaraldehyde's ability to form crosslinks.
pretreatment with GSH-glutaraldehyde mixture, additional fixation with glutaraldehyde will not create large crystallin aggregation.

In adult mice, cold cataract formation or glutaraldehyde induced aggregation is not common. This may be due to the presence of oxidized sulfhydryl groups in the lens nucleus which have been demonstrated as early as the 17th postnatal day.11 Also, an increase of masked N-terminal amino groups by \( \alpha,\beta \)-crystallins during the normal aging process12 may effectively reduce the number of sites able to react with glutaraldehyde in the cytoplasm.

The fixation technique used in this study can also be used to fix adult cataractous mouse lens. Soaking cataractous lenses in GSH seems to facilitate preservation of fine structure. No large cytoplasmic aggregations were observed and cell membranes were not broken.

By removing the aggregation artifact, this fixation technique facilitates a clearer delineation of the morphological differences between normal and pathological conditions in the lens. However, further studies are required to elucidate the details of the correlation between GSH in living cells, and GSH and glutaraldehyde.

Key Words: reduced glutathione, glutaraldehyde, temperature, neonatal mouse lens, nuclear zone, fine structure

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References


Ganglion Cell Topography in Human Fetal Retinae

Jan M. Provis, Frank A. Billson, and Peter Russell*

Whole-mounted human fetal retinae of gestational ages 14–40 weeks have been studied. These preparations clearly show the distribution of retinal ganglion cells or their precursors across the retina, and the pattern of the retinal vessels and vessel primordia. The ganglion cell layer is present at 14 weeks of gestation and distribution of cells in this layer (ganglion cell precursors) is at first uniform. Ganglion cell density gradients that foreshadow those seen in the adult retina become evident by about 20 weeks gestation. Both mature ganglion cells and precursor cells are present in retinae at about 24 weeks and precursor cells are still seen in the peripheral parts of the retina at about 30 weeks of gestation. The development of mature ganglion cells would appear to coincide with the establishment of retinal circulation, and proceeds in centrifugal sequence from central retinal. Invest Ophthalmol Vis Sci 24:1316–1320, 1983

Previous studies of embryonic and fetal human retinae have established the temporal sequence of its laminar development and described the early maturation of neural and non-neural elements during the first 3 to 4 months of gestation. By the end of the first trimester, in posterior retina, both the outer and inner plexiform layers are clearly discernable, and synapticogenesis in these layers is underway.1 Retinal ganglion cells and their axons become evident during the