Differential Recovery of Retinal Function after Mitochondrial Inhibition by Methanol Intoxication

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Purpose. The authors' laboratory has previously documented formate-induced retinal toxicity in a rodent model of methanol intoxication. These studies determined functional, bioenergetic, and structural recovery of the retina after methanol intoxication.

Methods. Rats were intoxicated with methanol, and retinal function was assessed by electroretinography 72 hours after the initial dose of methanol and after a 72-hour recovery period. Retinal energy metabolites, glutathione (GSH) concentrations, and histology were determined at the same time points.

Results. Both rod-dominated and UV-cone–mediated electroretinogram responses were profoundly attenuated in methanol-intoxicated rats. In rats allowed to recover from methanol intoxication, there was significant, although incomplete, recovery of rod-dominated retinal function. However, there was no demonstrable improvement in UV-cone–mediated responses. Retinal adenosine triphosphate (ATP), adenosine diphosphate (ADP), and GSH concentrations were significantly reduced after intoxication. Although retinal energy metabolites returned to control values after the recovery period, retinal GSH remained significantly depleted. Histopathologic changes were apparent in the photoreceptors after methanol intoxication, with evidence of inner segment swelling and mitochondrial disruption. In animals allowed to recover from methanol intoxication, there was no evidence of histopathology at the light microscopic level; however, ultrastructural studies revealed subtle photoreceptor mitochondrial alterations.

Conclusions. These findings support the hypothesis that formate inhibits retinal mitochondrial function and increases oxidative stress. They also provide evidence for a differential sensitivity of photoreceptors to the cytotoxic actions of formic acid, with a partial recovery of rod-dominated responses and no recovery of UV-cone–mediated responses. (*Invest Ophthalmol Vis Sci.* 2001;42:834–841)

Methanol has been recognized as a human visual neurotoxin for more than a century, and the clinical features of acute human methanol toxicity have been extensively documented.[1–6] Toxic exposure to methanol typically results in an initial transient central nervous system depression, followed by an asymptomatic latent period lasting 12 to 24 hours. This latent period is then followed by the development of formic acidemia, uncompensated metabolic acidosis, visual toxicity, coma, and in extreme cases, death. Visual disturbances generally develop between 18 and 48 hours after methanol ingestion and range from mild photophobia and misty or blurred vision to markedly reduced visual acuity and complete blindness. Susceptibility among persons to the acute effects of methanol is highly variable, and the minimum lethal dose is considered to be between 300 mg/kg and 1 g/kg.[4–6] The minimum dose causing permanent visual defects is unknown, although blindness has been reported after ingestion of as little as 4 ml of methanol.[7,8]

Methanol toxicity is primarily attributable to its metabolite, formic acid. Formic acid is the toxic metabolite responsible for the metabolic acidosis and visual toxicity observed in human methanol poisoning.[5,6,9] Formate has been hypothesized to produce retinal and optic nerve toxicity by disrupting mitochondrial energy production.[10,11] In vitro studies have shown that formate inhibits the activity of cytochrome oxidase, the terminal electron acceptor of the mitochondrial electron transport chain involved in adenosine triphosphate (ATP) synthesis.[12,13] Inhibition occurs subsequent to the binding of formic acid to the ferric heme iron in cytochrome oxidase, with inhibition constants between 5 and 30 mM.[12,13] Permanent visual damage in methanol-intoxicated humans[5] and nonhuman primates[10,14] has been associated with prolonged exposures (usually longer than 24 hours) to blood formate concentrations in excess of 7 mM. However, very little information is available on the potential for recovery of retinal function after toxic exposure to methanol-derived formate.

Our laboratory has developed a rodent model of methanol intoxication in which formate oxidation is selectively inhibited by treatment with nitrous oxide (N_2O). Subanesthetic concentrations of nitrous oxide inactivate the enzyme methionine synthetase, reducing the production of tetrahydrofolate, a necessary cofactor for formate oxidation.[14–19] This allows formate to accumulate to toxic concentrations after methanol administration.[14–19] In methanol-intoxicated rats, formic acidemia, metabolic acidosis, and visual toxicity develop, analogous to the toxicity seen in methanol intoxicated humans. Previous studies in our laboratory have established this rodent model of methanol-induced visual toxicity and have documented abnormalities in the flash-evoked visual potential and electroretinogram (ERG).[16–19]

The clinical features of methanol intoxication are remarkably similar to those of Leber's hereditary optic neuropathy, nutritional amblyopia, and the recent Cuban epidemic of optic neuropathy. In each case, there is evidence that a common pathophysiological mechanism involving mitochondrial dysfunction contributes to the retinal and optic nerve dysfunction characteristic of the disease.[20,21] We hypothesize that the retinal pathophysiology of methanol intoxication is a consequence of formate-induced mitochondrial dysfunction. In this study, we examined the effect of methanol intoxication on retinal function and retinal energy metabolism and assessed the potential for recovery of retinal function after intoxication. Our findings indicate that formate accumulation after methanol intoxication inhibited retinal energy metabolism, increased oxidative stress in the retina, and profoundly attenuated retinal function. These studies also provide evidence for a complete recovery of retinal energy metabolites and a partial recovery of

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retinal glutathione (GSH) and retinal function in animals allowed to recover for 72 hours from methanol intoxication. Furthermore, our results are indicative of a differential sensitivity of photoreceptors to the cytotoxic actions of formic acid with a partial recovery of rod-dominated responses and no recovery of UV-cone–mediated responses.

METHODS

Materials

Methanol (high-performance liquid chromatography [HPLC] grade) was obtained from Sigma (St. Louis, MO). Thiobutabarbital (Inactin) was purchased from Research Biochemicals (Natick, MA), atropine sulfate from AmVet (Fort Collins, CO), hydroxypropyl methylcellulose (2.5%) drops from Iolab (Claremont, CA), and atropine sulfate ophthalmic solution drops from Phoenix (St. Joseph, MO). All other chemicals were reagent grade or better.

Animals

Adult (250–350 g) male Long–Evans rats (Harlan Sprague–Dawley, Madison, WI) were used throughout the experiments. Animals were supplied food and water ad libitum and maintained on a 12-hour light–dark schedule in a temperature- and humidity-controlled environment. All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Methanol Intoxication Protocol

Rats were placed in a thermostatically controlled plexiglas chamber (22 × 55 × 22 cm; maintained at 22–23°C) and exposed to a mixture of N_2O/O_2 (1:1; flow rate, 2 l/min) for 4 hours before the administration of methanol or saline. N_2O/O_2 exposure was continued for 72 hours after the initial dose of methanol. Two treatment protocols were used. In the first protocol (intoxication), rats were intoxicated with methanol (25% wt/vol in saline, 4 g/kg, intraperitoneally, followed by 2-g/kg supplemental doses at 24 and 48 hours) in the presence of N_2O/O_2 for 72 hours. At 72 hours, ERG analysis was performed, animals were killed, and retinal tissue was prepared for biochemical and histologic analysis. In the second protocol (recovery), rats were intoxicated with methanol (same dosage regimen as above) for 72 hours in the presence of N_2O/O_2 and allowed to recover from methanol intoxication for an additional 72 hours in the absence of N_2O/O_2. At 144 hours, ERG analysis was performed, animals were killed, and retinal tissue was prepared for biochemical and histologic analysis. Controls for these experiments included groups of rats treated with saline and exposed to N_2O/O_2 (nitrous oxide-control), rats treated with methanol, but not exposed to N_2O/O_2 (methanol-control), and untreated rats (untreated-control). Formate concentrations were determined from tail vein blood samples by fluorometric analysis as previously described.[17,18]

ERG Procedures and Analyses

ERG experiments were performed as previously described.[18] The light-stimulation apparatus consisted of a three-beam optical system.[22] All three beams were derived from tungsten-halide lamps (50 W, 12 V), and beam intensity was controlled by using neutral-density step filters. Each of the beams contained a high-speed, computer-driven shutter (Uniblitz; Vincent Associates, Rochester, NY). One beam had a wavelength computer controlled by a tunable band-pass filter (Varispec; Cambridge Research Instruments, Wilmington, DE; half-energy passband, 7 nm). The second beam was used with a short-pass UV filter (half pass, 380 nm) in experiments designed to isolate UV-cones. The third beam was used as a chromatic adapting light to suppress responses from rods and M-cones in the UV-cone isolation experiments. For this purpose, a glass long-wavelength pass filter (half pass, 590 nm) was used. The three beams were optically superimposed and focused on the lens to illuminate a 70° patch of retina in Maxwellian view. Light calibrations were made with a silicon photodiode (PIN 10 DF; United Detector Technology, Hawthorne, CA).

ERG recordings were differentially amplified and computer averaged. The amplified signal was processed through a two-stage active narrow band-pass filter, the half voltage of which was 0.2 times the center frequency. To ensure that any transients in the response that occur at the onset of the stimulus pulses were not included in the average, the initiation of signal averaging was delayed by a preset number of stimulus cycles (typically a minimum of 20). The resultant ERG is a noise-free, single-cycle, sinusoidal waveform. The averaged responses were measured (peak-to-trough amplitude) from a calibrated digital oscilloscope display.[22]

Before ERG analysis, ophthalmoscopic examination confirmed that all eyes were free of lenticular opacities or other gross anomalies. Rats were anesthetized with thiobutabarbital sodium (100 mg/kg, intraperitoneally), positioned in a stereotaxic apparatus (David Kopf, Tujunga, CA), and placed on a heating pad to maintain core body temperature at 37°C. Atropine sulfate (0.05 mg/kg, subcutaneously) was administered to inhibit respiratory tract secretions. The pupil of the eye to be tested was dilated by topical application of 1% atropine sulfate. Methylcellulose was topically applied as a lubricant and to enhance electrical conduction. A circular, silver wire recording electrode was positioned on the cornea, a reference electrode was placed above the eye, and a ground electrode was placed on the tongue. Recordings were obtained under ambient light conditions from cool white fluorescent room lights approximately 100 candelas [cd]/m^2 at the rat's eye. Flickering stimuli (light–dark ratio, 0.25:0.75) were presented. Responses to 60 successive flashes were averaged for each stimulus condition. At each test wavelength, a minimum of four stimulus intensities, spaced at intervals of 0.3 log units, were presented. The stimulus intensity yielding a 5-μV criterion response was determined by extrapolating between the two intensity points that bracketed the 5-μV response for each animal. All sensitivity measures were made in triplicate. After ERG analysis, anesthetized rats were killed by decapitation, and retinal tissue was prepared for histologic and biochemical analysis. One retina from each animal was prepared for histology, and the other retina was prepared for analysis of retinal energy metabolites and GSH concentrations.

Two experimental protocols were used to evaluate retinal function. (1) The 15-Hz/510-nm ERG response: ERGs were recorded in response to a 15-Hz flickering light at a wavelength of 510 nm over a 3-log-unit range of light intensity. For these studies the unattenuated stimulus (log relative retinal illumination [LRRI], 0) had an irradiance of 25 μW distributed over the 70° patch of illuminated retina. This can be calculated to produce retinal illumination equivalent to approximately 10^4 scotopic trolands (scot td). These recording conditions disadvantage rods; however, because at least 97% of rat photoreceptors are rods and ERGs are recorded at luminance intensities ranging from 10^1 to 10^4 scot td, it is likely that the responses to the 15-Hz/510-nm light are drawn from both rods and medium-wavelength cones (M-cones).[23–25] (2) 25-Hz/UV ERG response: Cone responses were elicited by a 25-Hz flickering UV light (380-nm cutoff) in the presence of an intense chromatic adapting light (445 μW), which eliminated responses mediated by rods and M-cones.[26] Recording conditions were the same as those used by Jacobs et al.[26] (except that the intensity of the chromatic adapting light was lower in our studies). In Jacobs et al.,[26] complete spectral sensitivity functions were measured in the rat, and it was demonstrated that UV-cone responses are separated from rod and M-cone responses. The 25-Hz/UV ERG responses were recorded over a 1-log-unit range of light intensity. For these studies, the unattenuated stimulus (LRRI, 0) had an irradiance of 12.5 μW distributed over the 70° patch of illuminated retina. By equating the effectiveness of this light to the 510-nm stimulus, we estimate that the unattenuated light produced the equivalent of $10^{2.5}$ scot td in the rat eye.

Determination of Retinal Energy Metabolites

Retinas were rapidly dissected and frozen in liquid nitrogen. Frozen retinas were extracted in 2.5% trichloroacetic acid (TCA), the suspension centrifuged, and the supernatant neutralized with 1.0 M Tris base. Protein concentrations in the pellet were determined using a modification of the dye-binding method of Bradford.[27] The neutralized supernatant was assayed for ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) by the high-performance liquid chromatography (HPLC) method of Bernocchi et al.,[28] using a system (1090L; Hewlett Packard, Palo Alto, CA) with a diode array detector and a 3-μm reversed-phase column (15 cm $\times$ 4.6 mm; Supelcosil LC-18; Supelco, Bellefonte, PA). Peak identities were confirmed by comparison of the peak retention time and peak spectral characteristics of samples with those of known standards. Quantitative measurements were made on the basis of the injection of standard solutions in known concentrations. Metabolite concentrations are expressed per milligram of protein.

Determination of Retinal GSH Concentrations

Retinas were rapidly dissected and frozen in liquid nitrogen. Frozen retinas were extracted in 2.5% TCA, the suspension centrifuged, and the supernatant assayed for GSH. Protein concentrations in the pellet were determined using a modification of the dye-binding method of Bradford.[27] GSH was assayed by the fluorometric method of Morkrasch and Teschke,[29] using *o*-phthalaldehyde. Fluorescence was measured at an excitation wavelength of 345 nm and an emission wavelength of 425 nm. GSH concentrations are expressed per milligram of protein.

Histopathologic Analysis

Retinal tissue was prepared for histology as previously described.[18,19] Briefly, eyes were enucleated, hemisected, and immersed in fixative (2.67% glutaraldehyde in 0.1 M phosphate buffer at 4°C [pH 7.3]) for 72 hours, then transferred to 4% sucrose. The anterior segment and vitreous were removed and full-thickness pieces of eye wall were dissected from the posterior pole, including the optic nerve. Tissues were postfixed in phosphate-buffered 2% osmium tetroxide (OsO_4), dehydrated in a graded ethanol series, and embedded in epoxy resin. Thick sections (1 μm) for light microscopy were stained with toluidine blue; thin sections for electron microscopy were stained with uranyl acetate-lead citrate.[18,19]

Statistical Analysis

All values are expressed as means ± SEM. A one-way analysis of variance (ANOVA) with Bonferroni's test was used to determine significant differences among groups for blood formate concentrations, energy metabolites, and GSH concentrations. For ERG studies, a one-way ANOVA with repeated measures was performed, followed by Scheffé's F procedure. In all cases, the minimum level of significance was taken as $P < 0.05$.

Results

Accumulation of Formic Acid

In the present studies, methanol was administered to N_2O/O_2-exposed rats at an initial dose of 4 g/kg, followed by supplemental doses of 2 g/kg at 24 and 48 hours. This treatment protocol has been shown to produce a state of prolonged formic acidemia with formate concentrations between 7 and 10 mM for 40 hours in methanol-intoxicated rats, resulting in visual dysfunction.[16–19] Moreover, similar concentrations of blood formate over similar periods have been shown to produce ocular toxicity experimentally in monkeys and have been associated with visual toxicity in human methanol intoxication.[5,6] As shown in Figure 1, blood formate concentrations increased linearly in both the intoxicated and recovery groups of animals during the initial 72 hours of intoxication. In the intoxicated group, formate concentrations increased from endogenous concentrations of 0.6 ± 0.3 (mean ± SEM) to 7.8 ± 0.1 mM by 72 hours. In animals allowed to recover after intoxication, there was a similar increase in blood formate concentrations during the initial 72-hour intoxication period from endogenous concentrations of 0.5 + 0.3 to 7.0 ± 0.4 mM. Formate concentrations did not differ between the intoxicated and recovery groups at any sampling point during the 72-hour intoxication period. After the 72-hour recovery period (in the absence of N_2O/O_2 and methanol), blood formate declined to endogenous concentrations in the recovery group of animals.

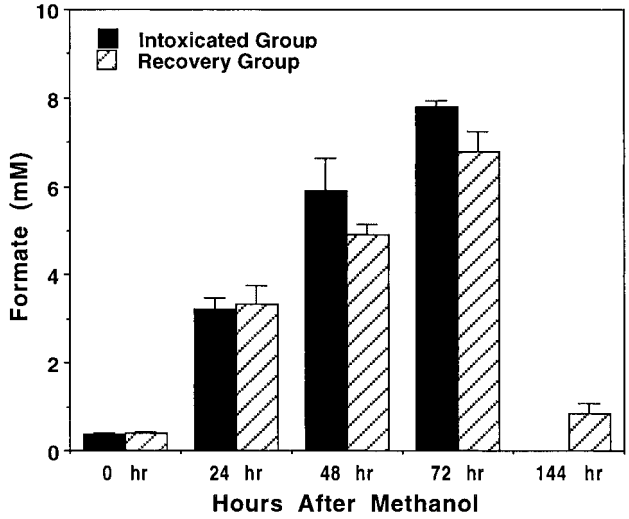

Figure 1. Effect of methanol intoxication and recovery on blood formate concentrations. Rats were exposed to a mixture of N_2O/O_2 (1:1) 4 hours before the administration of methanol and for 72 hours after the initial dose of methanol. Methanol was administered at a dose of 4 g/kg at the zero time point, followed by 2 g/kg at 24 and 48 hours. In the recovery group, the N_2O/O_2 exposure was discontinued at the 72-hour time point, and animals were allowed to recover for an additional 72 hours. Blood formate concentrations were determined before methanol administration and at 24-hour intervals after methanol administration for 72 hours. In the recovery animals, blood formate concentrations were also determined 72 hours after the N_2O/O_2 exposure was discontinued at 144 hours after the initial dose of methanol. Shown are the mean values ± SEM from five to six rats in each experimental group. Formate concentrations did not differ between the intoxicated and recovery group over the 72-hour intoxication period (ANOVA with Bonferroni's test; $P < 0.05$). Blood formate concentrations measured in N_2O-control and methanol-control rats were not significantly different from blood formate concentrations measured in untreated-control rats (data not shown).

15-Hz/510-nm ERG Response

We have previously reported that a 15-Hz/510-nm light produced a robust and reproducible ERG response in our rodent model.[18] As stated in the Methods section, these recording conditions disadvantage rods; however, because at least 97% of rat photoreceptors are rods and ERGs are recorded at luminance intensities ranging from 10^1 to 10^4 scot td, it is likely that the responses to the 15-Hz/510-nm light were drawn from both rods and M-cones.[23–25,30] Moreover, it is likely that ERG responses recorded at the lower luminance intensities ($<10^3$ scot td) reflect responses with a robust rod component, whereas, those responses recorded at the higher luminance intensities ($>10^3$ scot td) may be dominated by the activity of M-cones. We base this interpretation on flicker photometry studies in the gerbil, which show a shift from rods to cones at 10^3 scot td.[23]

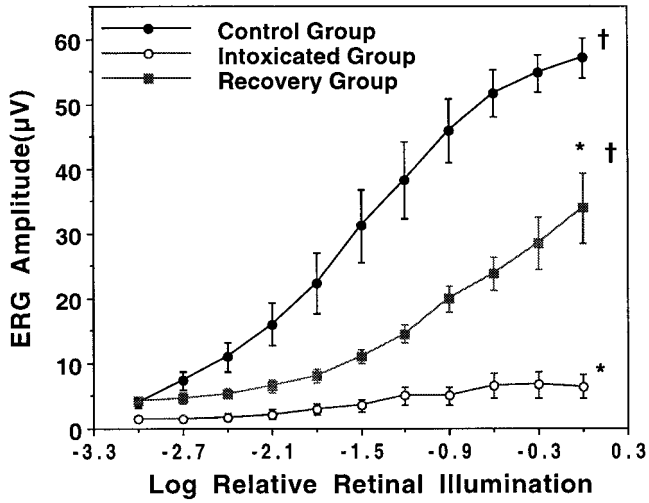

FIGURE 2. Effect of methanol intoxication and recovery on 15-Hz/510-nm ERG responses. Flicker ERG analyses were performed at 72 hours after the initial dose of methanol and 144 hours after the initial dose of methanol, after a 72-hour recovery. Shown are the mean values ± SEM from five to six rats in each treatment group. *Significant differences from values obtained in control rats; †significant differences from values obtained in intoxicated rats (repeated measures ANOVA with Scheffé's F test; $P < 0.05$). 15-Hz/510-nm luminance–response relationships measured in N_2O-control and methanol-control rats were not significantly different from 15-Hz/510-nm luminance–response relationships measured in untreated-control rats (data not shown).

The effects of methanol intoxication and recovery on the 15-Hz/510-nm ERG responses are shown in Figure 2. In the control group, 15-Hz/510-nm ERG amplitude increased linearly over the 3-log-unit range of retinal illumination intensities, achieving a maximal amplitude of 57.1 ± 3.1 μV at maximal retinal illumination (0 LRRI, equivalent to 10^4 scot td). A consistent 5-μV criterion threshold response was obtained in control animals at −2.7 ± 0.1 LRRI. We observed a decrease in retinal responsiveness and attenuation of maximal ERG amplitude in both the intoxicated and the recovery groups in comparison with untreated control animals. In intoxicated animals, ERG responses were at or below the 5-μV threshold response over the entire range of luminance intensities, indicative of a severe deficit in retinal function. In animals allowed to recover from intoxication for 72 hours, the 5-μV threshold response was not different from the control group; however, the ERG intensity response curve was significantly attenuated at all light intensities of more than −2.1 LRRI, and the maximal response to light stimulation was reduced to 33.9 ± 5.4 μV. These data are indicative of a partial recovery of this component of retinal function.

25-Hz/UV ERG Response

The function of UV-sensitive cones was examined by recording the retinal response to a 25-Hz flickering UV light (380-nm cutoff) in the presence of an intense chromatic adapting light. These conditions have been shown to isolate the UV-cone response in the rat retina.[26] The effects of methanol intoxication and recovery on 25-Hz/UV ERG responses are shown in Figure 3. In the control group, 25-Hz/UV ERG amplitude increased from a minimal value of 1.9 ± 0.4 μV to a maximal value of 19.6 ± 1.2 μV over the log unit range of retinal illumination used in these studies. A consistent 5-μV threshold criterion response was obtained in control animals at −0.4 ± 0.1 LRRI. In intoxicated animals, the 25-Hz/UV ERG responses over the entire range of luminance intensities were at or below the 5-μV threshold response. These findings are indicative of a severe deficit of cone-mediated retinal function similar to the deficit observed in the 15-Hz/510-nm response. In contrast to the partial recovery observed in the 15-Hz/510-nm ERG response, the 25-Hz/UV ERG response remained fully attenuated in animals allowed to recover from methanol intoxication, indicating that UV-cone–mediated function does not recover after methanol intoxication.

Retinal Energy Metabolites

Methanol intoxication has been hypothesized to disrupt retinal mitochondrial energy production, secondary to formate induced inhibition of mitochondrial cytochrome oxidase activity.[6,10,11] To assess the effect of formate on retinal energy metabolism in vivo, we measured concentrations of energy metabolites (ATP, ADP, and AMP) in the retinas of control, intoxicated, and recovery groups of rats (Fig. 4). In untreated control animals, concentrations of ATP, ADP, and AMP were 5.1 ± 0.6 nanomoles/mg protein, 12.5 ± 1.2 nanomoles/mg protein, and 23.7 ± 3.4 nanomoles/mg protein, respectively. After intoxication, there was a significant decrease in ATP (1.2 ± 0.2 nanomoles/mg protein) and ADP (6.2 ± 0.5 nanomoles/mg protein), and a corresponding increase in AMP (37.2 ± 4.0 nanomoles/mg protein). In animals allowed to recover from intoxication, retinal energy metabolite concentrations were not significantly different from energy metabolite concentrations measured in the retinas of control animals, consistent with a restoration of mitochondrial bioenergetics.

Retinal GSH Concentrations

GSH plays a central role in the antioxidant defenses of the cell.[31–35] Inhibition of mitochondrial function has been shown to increase oxidative stress and deplete tissue GSH.[31–35] As an index of formate-induced oxidative stress after methanol intox-

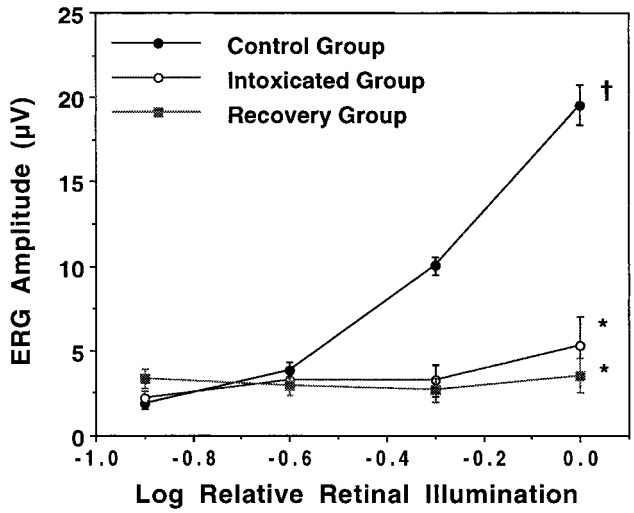

FIGURE 3. Effect of methanol intoxication and recovery on 25-Hz/UV ERG responses. ERG analyses were performed at 72 and 144 hours after the initial dose of methanol, after a 72-hour recovery. UV-cone–sensitive ERGs were recorded from the same animals in which 15-Hz/510-nm ERG responses were recorded. Shown are mean values ± SEM from five to six rats in each treatment group. *Significant differences from values obtained in control rats; †significant differences from values obtained in intoxicated rats (repeated measures ANOVA with Scheffé's F test; $P < 0.05$). UV-cone–mediated luminance–response relationships measured in N_2O-control and methanol-control rats were not significantly different from UV-cone–mediated luminance–response relationships measured in untreated-control rats (data not shown).

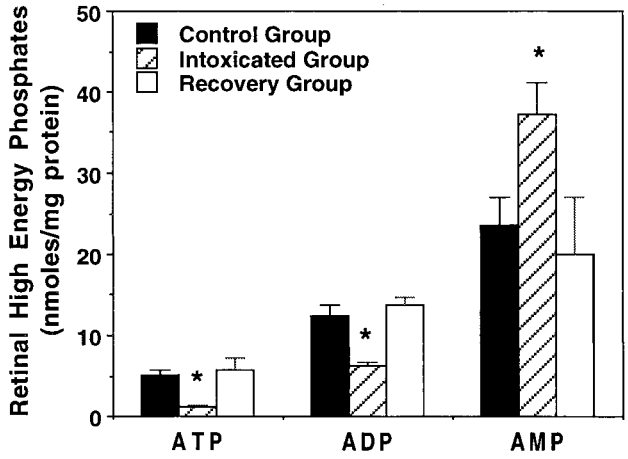

FIGURE 4. Effect of methanol intoxication and recovery on retinal energy metabolites. ATP, ADP, and AMP concentrations were measured in retinas of rats 72 and 144 hours after the initial dose of methanol, after a 72-hour recovery. Data are expressed as the mean values ± SEM from five to six rats in each treatment group. *Significant differences from values obtained in control rats (ANOVA with Bonferroni's test; $P < 0.05$). Retinal energy metabolite concentrations measured in N_2O-control and methanol-control rats were not significantly different from energy metabolite concentrations measured in untreated-control rats (data not shown).

ication and recovery, we measured GSH concentrations in the retinas of control, intoxicated, and recovery groups of rats (Fig. 5). In the control group, retinal GSH concentrations were 29.4 ± 2.0 nanomoles/mg protein. After intoxication, retinal GSH concentrations were significantly reduced to 12.7 ± 3.4 nanomoles/mg protein. After recovery, GSH concentrations were 22.3 ± 2.0 nanomoles/mg—significantly higher than the intoxicated group, but also significantly lower than control, which suggests that oxidative stress may play an important role in methanol intoxication.

Retinal Histology and Photoreceptor Ultrastructure

The effects of formate after methanol intoxication on retinal histology and ultrastructure were assessed by light and electron microscopy after intoxication and recovery. Figure 6 illustrates outer retinal morphology in representative control (Fig. 6A), intoxicated (Fig. 6B), and recovery (Fig. 6C) retinas. Retinas prepared from control animals had ordered photoreceptor inner segments with no evidence of vacuolization or swelling. The outer nuclear layer of the control retina was compact, with round and well-defined nuclei. In contrast, retinas prepared from rats intoxicated for 72 hours showed evidence of retinal edema, swelling of photoreceptor inner segments, and morphologic changes in photoreceptor nuclei. Retinal edema was evidenced by the spacing between the photoreceptor inner segments and the spacing of the nuclei in the outer nuclear layer. Photoreceptor inner segments were profoundly swollen and enlarged. Changes in the appearance of photoreceptor nuclei were also apparent in intoxicated rats. Nuclei appeared somewhat enlarged with irregularly stained chromatin. The chromatin staining pattern in photoreceptor nuclei in intoxicated animals ranged from tightly compact to dispersed and fragmented. In animals allowed to recover from methanol intoxication, retinal morphology was similar to control, with the only histologic alteration being an increased spacing between nuclei in the outer nuclear layer.

The retinas of control, intoxicated, and recovery rats were also examined by electron microscopy. We focused our ultrastructural observations on the mitochondria of the inner segments of the photoreceptors, because formate is known to act as a mitochondrial toxin, and the inner segments of the photoreceptors contain the highest density of mitochondria in the retina. Figure 7 illustrates the inner segment region in representative control (Fig. 7A), intoxicated (Fig. 7B), and recovery (Fig. 7C) retinas. Mitochondria in the photoreceptor inner segments of control animals exhibited normal morphology with well-defined cristae. The most obvious ultrastructural change observed in the outer retina of methanol-intoxicated rats was the swelling and disruption of photoreceptor mitochondria. In rats intoxicated for 72 hours, numerous photoreceptor mitochondria were profoundly swollen, with severely disrupted cristae. In intoxicated animals, there was swelling and disruption of photoreceptor mitochondria to various degrees within the retina and within the individual cells. Some mitochondria were swollen and contained expanded cristae, and other mitochondria were disrupted and showed no evidence of cristae. Photoreceptor mitochondria in animals allowed to recover from methanol intoxication showed less evidence of disruption than photoreceptor mitochondria in intoxicated animals. In recovery animals, photoreceptor mitochondrial morphology ranged from normal with well-defined cristae to rounded with expanded cristae.

DISCUSSION

We report the nature of the functional, biochemical, and structural changes produced in the retina after methanol intoxication and recovery. Several important findings are reported in this study. We provide in vivo evidence of a significant alteration in retinal energy metabolism, which supports previous in vitro studies showing that formate is a mitochondrial toxin.[12,13,36–38] These data also indicate that formate-induced mitochondrial dysfunction produced GSH depletion and increased oxidative stress. In addition, we describe the nature of the recovery of retinal function after methanol intoxication. We provide evidence for partial recovery of retinal function in rod-dominated pathways, but no recovery of UV-cone–mediated responses. These findings are indicative of a differential

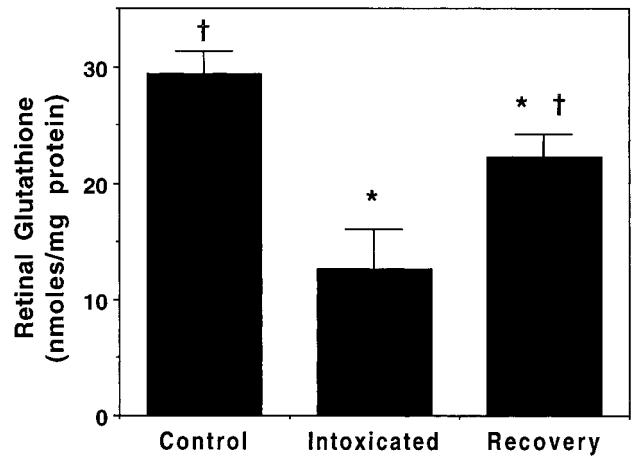

FIGURE 5. Effect of methanol intoxication and recovery on retinal GSH concentrations. GSH concentrations were measured in retinas of rats 72 and 144 hours, after a 72-hour recovery. Data are expressed as the mean values ± SEM from five to six rats in each treatment group. *Significant differences from values obtained in control rats; †significant differences from values obtained in intoxicated animals (ANOVA with Bonferroni's test; $P < 0.05$). GSH concentrations measured in N_2O-control and methanol-control rats were not significantly different from GSH concentrations measured in untreated-control rats (data not shown).

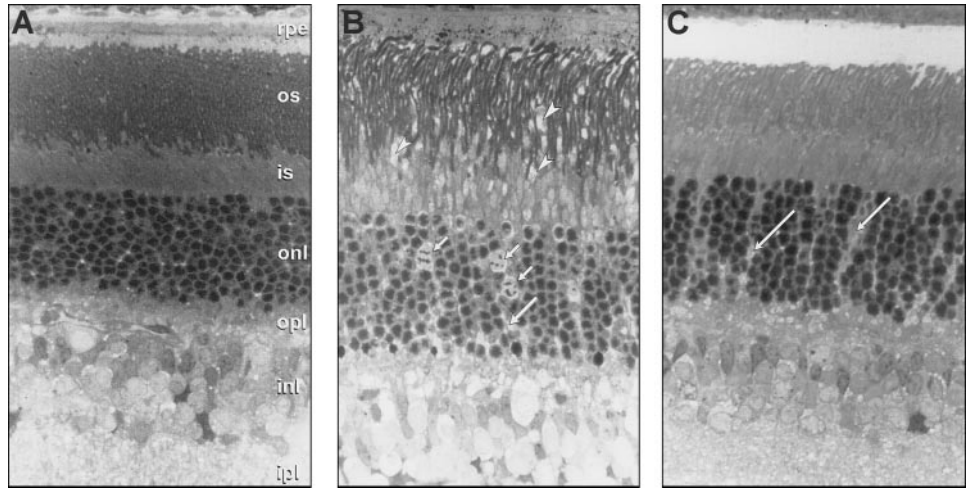

FIGURE 6. Effect of methanol intoxication and recovery on retinal histology. Outer retinal morphology in representative untreated-control (**A**), intoxicated (**B**), and recovery (**C**) rats. Sections were taken from the posterior pole of the retina within two disc diameters of the optic nerve in any direction. (**A**) rpe, retinal pigment epithelium; os, photoreceptor outer segments; is, photoreceptor inner segments; onl, outer nuclear layer; opl, outer plexiform layer; ipl, inner plexiform layer. (**B**) *Arrowheads*: Enlargement and swelling of the photoreceptor inner segments; *short arrows*: fragmented appearance of photoreceptor nuclei; *long arrow*: retinal edema, as evidenced by increased spacing between the nuclei in the outer nuclear layer. (**C**) *Arrows*: Increased spacing between the nuclei in the outer nuclear layer. No histopathologic changes were apparent at the light microscopic level in the N_2O-control or methanol-control groups (data not shown). Toluidine blue; magnification, ×450.

sensitivity of photoreceptors to the cytotoxic actions of formic acid.

Formic acid has been hypothesized to produce ocular toxicity by a disruption of mitochondrial energy production in the retina and optic nerve.[10,11,39,40] In vitro studies in our laboratory and by other investigators have shown that formate inhibits cytochrome oxidase, the terminal electron acceptor of the mitochondrial electron transport chain involved in ATP synthesis.[12,13,37,38] Inhibition occurs subsequent to the binding of formic acid with the ferric heme iron of cytochrome oxidase, and the apparent inhibition constant is between 5 to 30 mM.[12,13] Blood formate concentrations in methanol-intoxicated rats in the present study fall within this range, as do blood formate concentrations in methanol-poisoned humans and monkeys.[5,9,10] Moreover, retinal and vitreous humor formate concentrations closely parallel blood formate concentrations.[16,17] Additional in vitro studies in isolated mitochondria and cultured neuronal cells have shown that formate inhibits mitochondrial ATP synthesis and decreases cellular ATP content.[36,38] The present studies provide evidence that formate inhibits mitochondrial energy metabolism in vivo. These studies document formate-induced depletion of retinal ATP and ADP and a corresponding increase in retinal AMP after methanol intoxication. After recovery, energy metabolites returned to control concentrations, providing evidence of bioenergetic recovery. These findings strongly support the hypothesis that formate inhibits retinal mitochondrial energy metabolism and oxidative phosphorylation in methanol intoxication and are consistent with the documented actions of formate in isolated mitochondria.[12,13,38]

The vertebrate retina has several features that render it vulnerable to damage from reactive oxygen species, including abundant mitochondria and a high percentage of polyunsaturated fatty acids in photoreceptor membranes that are susceptible to lipid peroxidation.[34,35] Because of its constant exposure to irradiation and high metabolic activity, the retina has a great need for antioxidant protection.[34,35] Reduced GSH is one of the most abundant intracellular thiols in the central nervous system and acts as a major cellular antioxidant by supporting GSH peroxidase-dependent reduction of hydrogen peroxide and organic peroxides.[31–33] GSH is normally present in high concentration in the retina and has been shown to play a key role in antioxidant defenses in the retina.[34,35] Studies have shown that retinal GSH may be depleted during periods of oxidative stress.[34,35] In the present studies, we observed a significant reduction in retinal GSH concentrations after methanol intoxication. Moreover, in contrast to our findings with energy metabolites, GSH concentrations did not return to control concentrations after recovery. We hypothesize that the observed depletion of retinal GSH is a consequence of formate-induced mitochondrial inhibition. Depletion of GSH by formate could result directly from formate-induced peroxidative stress, because inhibition of mitochondrial electron transport has been shown to profoundly increase the production of

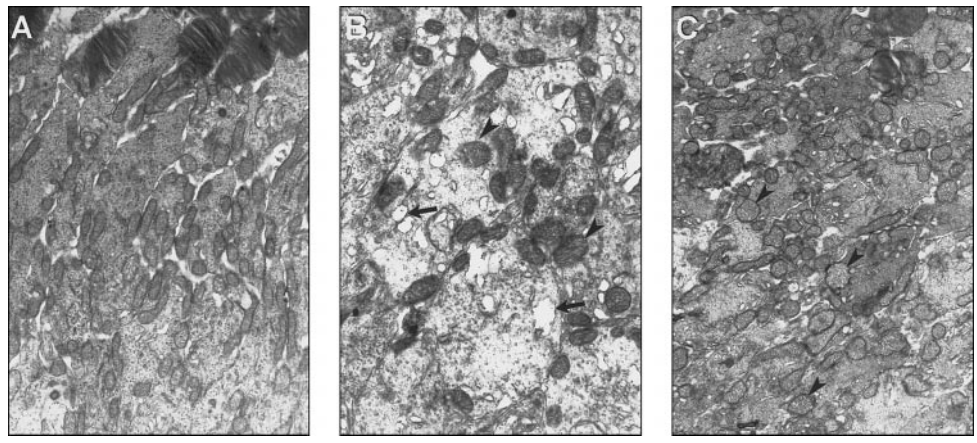

FIGURE 7. Effect of methanol intoxication and recovery on photoreceptor ultrastructure. Electron micrographs of the rod inner segment region in representative control (**A**), intoxicated (**B**), and recovery (**C**) rats. Abnormal mitochondrial morphology was present in photoreceptor inner segments. *Arrowheads*: Swollen mitochondria with expanded cristae; *arrows*: completely disrupted mitochondria with no evidence of cristae. Photoreceptor mitochondria from N_2O-control or methanol-control rats exhibited normal morphology with well-defined cristae (data not shown). Magnification, ×5000

reactive oxygen species including superoxide and hydrogen peroxide.[31–33] Alternatively, GSH depletion could occur as a consequence of formate-induced ATP depletion, because GSH synthesis is ATP dependent.[31–33] In support of the latter mechanism, studies in cultured hepatocytes have shown that inhibition of cellular energy metabolism and ATP synthesis by mitochondrial poisons lead to a rapid decline in GSH content that precedes cell death by several hours.[31]

Additional studies are under way to determine the mechanism of formate-induced GSH depletion. Because of the critical involvement of GSH in cellular defense mechanisms, depletion of intracellular GSH under conditions of mitochondrial impairment may augment the susceptibility of the retina to oxidative stress. Thus, formate-induced mitochondrial inhibition may not only increase the production of reactive oxygen species, but may also predispose the retina to increased oxidative stress through a perturbation of GSH status. A similar mechanism of tissue injury has been proposed to lead to neuronal degeneration in Parkinson's disease.[31]

The morphologic changes in the present study are also consistent with formate-induced inhibition of photoreceptor energy metabolism, GSH depletion, and increased oxidative stress. The most profound ultrastructural alterations observed in methanol-intoxicated rats were mitochondrial swelling and disruption in the inner segments of the photoreceptor cells. Similar mitochondrial changes have been associated with the production of GSH deficiency after inhibition of GSH synthesis with buthionine sulfoximine.[32] GSH plays a major role in the maintenance of mitochondrial function.[31–33] Reduction in cytosolic and mitochondrial GSH has been shown to increase mitochondrial susceptibility to oxidative stress, disrupt mitochondrial structure and function, and promote cytotoxicity.[32,33] In addition, mitochondrial disruption has also been reported in the retinas of patients with mitochondrial diseases that inhibit electron transport[41–43] and in certain forms of light-induced retinal degeneration in which inactivation of cytochrome oxidase is postulated to play a role in the disease.[44–46]

The present studies confirm and extend our previous investigations that showed that rod- and cone-mediated ERG responses were profoundly attenuated in rats intoxicated with methanol for 72 hours.[14] We also observed a differential recovery of retinal function after methanol intoxication. In rats allowed to recover for 72 hours from methanol intoxication, there was a significant, although incomplete recovery of the 15-Hz/510-nm ERG response. In contrast, there was no evidence of recovery of UV-cone–mediated function. The rat retina contains three types of photoreceptors, rods, M-cones, and UV-cones.[24–26] Rods comprise approximately 97% of rat photoreceptors.[24–26] Although our recording conditions can clearly discriminate UV-cone function, the 15-Hz/510-nm ERG measurements cannot discriminate between rod and M-cone function. In the absence of a definitive assessment of M-cone function, it is unknown whether the observed sensitivity to the cytotoxic actions of formate is specific for UV-cones or reflects a general property of cones. However, it is clear that UV-cone responses are more severely affected after methanol intoxication than rod and M-cone responses.

Previous studies have shown that rod-dominated retinal responses are affected earlier in the course of intoxication and at lower formate concentrations than the UV-cone–mediated responses. Taken together, these data suggest that although UV-cones may be resistant to the initial cytotoxic actions of formate,[18] once poisoned, they do not recover, or their recovery is delayed. These findings have important implications. In cases of human methanol intoxication, the most common outcome is loss of central, but not peripheral, vision. This has been attributed to the loss of central fibers in the optic nerve.[10,11] However, the present findings raise the possibility that the loss of central vision may also involve the loss of cone function, because the density of cones is greatest in the central retina. One potential explanation for the initial resistance and subsequent vulnerability of UV-cone photoreceptors to the toxic actions of formate may be the greater numbers of mitochondria present in cones in comparison with rods.[47] We have previously postulated that cones have a greater metabolic reserve, allowing them to continue to function for a longer period in the presence of a metabolic toxin, to explain the delayed attenuation of UV-cone function relative to rod-dominated function.[18] It is also likely that prolonged metabolic inhibition in cells containing many mitochondria could generate excessive amounts of reactive oxygen species, overwhelming antioxidant protection systems and resulting in cell death.[48] Of importance, because decrements in retinal energy production and oxidative stress have been postulated to be involved in the pathogenesis of numerous retinal diseases including age-related macular degeneration and diabetic retinopathy, studies of formate-induced retinal dysfunction may provide valuable insight into the pathogenesis of other acquired and genetic retinal disorders.

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References

1. Wood CA, Buller F. Poisoning by wood alcohol: cases of death and blindness from Colombian spirits and other methylated preparations. *JAMA.* 1904;43:972–977.
2. Benton CD, Calhoun FP. The ocular effects of methyl alcohol poisoning: report of a catastrophe involving three hundred and twenty persons. *Trans Am Acad Ophthalmol Otolaryngol.* 1952; 56:875–883.
3. Roe O. The metabolism and toxicity of methanol. *Pharmacol Rev.* 1955;7:399–412.
4. Roe O. Species differences in methanol poisoning. *Crit Rev Toxicol.* 1982;10:275–286.
5. Jacobsen D, McMartin KE. Methanol and ethylene glycol poisonings: mechanism of toxicity, clinical course diagnosis and treatment. *Med Toxicol.* 1986;1:309–334.
6. Eells JT. Methanol. In: Thurman, RG, Kaufmann FC, eds. *Browning's Toxicity and Metabolism of Industrial Solvents.*. Amsterdam: Elsevier; 1992. *Alcohols and Esters*; vol 4:3–15.
7. Bennett I, Cary F, Mitchell GJ, Cooper M. Acute methyl alcohol poisoning: a review based on experience in an outbreak of 323 cases. *Medicine.* 1953;56:875–883.
8. Tong T. The alcohols. *Crit Care Q.* 1982;4:75–85.
9. McMartin KE, Ambre JJ, Tephly TR. Methanol poisoning in human subjects: role of formic acid accumulation in the metabolic acidosis. *Am J Med.* 1980;68:414–418.
10. Hayreh MM, Hayreh SS, Baumbach GL, Cancilla P, Martin–Amat G, Tephly TR. In: Merigan W, Weiss B, eds. *Neurotoxicity of the Visual System*. New York: Raven Press; 1980:35–53.
11. Sharpe J, Hostovsky M, Bilbao J, Rewcastle NB. Methanol optic neuropathy: a histopathological study. *Neurology.* 1982;32:1093–1100.
12. Nicholls P. Formate as an inhibitor of cytochrome c oxidase. *Biochem Biophys Res Commun.* 1975;67:610–616.
13. Nicholls P. The effects of formate on cytochrome aa3 and on electron transport in the intact respiratory chain. *Biochim Biophys Acta.* 1976;430:13–29.
14. Hayreh MS, Hayreh SS, Baumbach P, Cancila P, Martin–Amat G, Tephly, TR. Methyl alcohol poisoning, III: ocular toxicity. *Arch Ophthalmol.* 1977;95:1851–1858.
15. Eells JT, Makar AB, Noker PE, Tephly TR. Methanol poisoning and formate oxidation in nitrous oxide treated rats. *J Pharmacol Exp Ther.* 1981;217:57–61.

16. Eells JT, Salzman MM, Lewandowski MF, Murray TG. Formate-induced alterations in retinal function in methanol-intoxicated rats. *Toxicol Appl Pharmacol.* 1996;140:58–69.
17. Eells JT. Methanol-induced visual toxicity in the rat. *J Pharmacol Exp Ther.* 1991;257:56–63.
18. Seme MT, Summerfelt P, Henry MM, Neitz J, Eells JT. Formate-induced inhibition of photoreceptor function in methanol intoxication. *J Pharmacol Exp Ther.* 1999;289:361–370.
19. Murray TG, Burton TC, Rajani C, Lewandowski MF, Burke JM, Eells JT. Methanol poisoning: a rodent model with structural and functional evidence for retinal involvement. *Arch Ophthalmol.* 1991; 109:1012–1016.
20. Sadun AA, Martone JF, Muci–Mendoza, R. Epidemic optic neuropathy in Cuba: eye findings. *Arch Ophthalmol.* 1994;112:691–699.
21. Rizzo JF. Adenosine triphosphate deficiency: a genre of optic neuropathy. *Neurology.* 1995;45:11–16.
22. Neitz J, Jacobs G. Reexamination of spectral mechanisms in the rat. *J Comp Psychol.* 1986;100:21–29.
23. Jacobs G, Neitz J. Cone monochromacy and a reversed Purkinje shift in the gerbil. *Exp Eye Res.* 1989;45:317–403.
24. Szel A, Rohlich P. Two cone types of rat retina detected by anti-visual pigment antibodies. *Exp Eye Res.* 1992;55:47–52.
25. Deegan JF, Jacobs GH. On the identity of the cone types of the rat retina. *Exp Eye Res.* 1993;56:375–377.
26. Jacobs G, Neitz J, Deegan J. Retinal receptors in rodents maximally sensitive to ultraviolet light. *Nature.* 1991;353:655–656.
27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248–252.
28. Bernocchi P, Ceconi C, Cargnoni A, Pedersini P, Curello S, Ferrari R. Extraction and assay of creatine phosphate, purine and pyridine nucleotides in cardiac tissue by reversed-phase high-performance liquid chromatography. *Anal Biochem.* 1994;222:374–379.
29. Mokrasch LC, Teschke EJ. Glutathione content of cultured cells and rodent brain regions: a specific fluorometric assay. *Anal Biochem.* 1984;140:506–509.
30. LaVail MM. Survival of some photoreceptor cells in albino rats following long-term exposure to continuous light. *Invest Ophthalmol Vis Sci.* 1976;15:64–70.
31. DiMonte DA, Chan P, Sandy MS. Glutathione in Parkinson's disease: a link between oxidative stress and mitochondrial damage? *Ann Neurol.* 1992;32:S111–S115.
32. Meister A. Mitochondrial changes associated with glutathione deficiency. *Biochim Biophys Acta.* 1995;127:35–42.
33. Garcia–Ruiz C, Colell A, Morales A, Kaplowitz N, Fernandez–Checa JC. Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcriptional factor nf-κB: studies with isolated mitochondria and rat hepatocytes. *Mol Pharmacol.* 1995;48:825–834.
34. Schutte M, Werner P. Redistribution of glutathione in the ischemic rat retina. *Neurosci Lett.* 1998;246:53–56.
35. Winkler BS, Giblin FJ. Glutathione oxidation in retina: effects on biochemical and electrical activities. *Exp Eye Res.* 1983;36:287–297.
36. Dorman DC, Bolon B, Morgan KT. The toxic effects of formate in dissociated primary mouse neural cell cultures. *Toxicol Appl Pharmacol.* 1993;122:265–272.
37. Erecinska M, Wilson DF. Inhibitors of cytochrome c oxidase. *Pharmacol Ther.* 1980;8:1–20.
38. Eells JT, Salzman MM, Milligan SN. Selective inhibition of ATP synthesis in retinal mitochondria by formic acid (Abstract). *Fundam Appl Toxicol.* 1997;36:72.
39. Martin–Amat G, Tephly TR, McMartin KE, et al. Methyl alcohol poisoning, II: development of a model for ocular toxicity in methyl alcohol poisoning using the Rhesus monkey. *Arch Ophthalmol.* 1977;95:1847–1850.
40. Martin–Amat G, McMartin KE, Hayreh SS, Hayreh MS, Tephly TR. Methanol poisoning: ocular toxicity produced by formate. *Toxicol Appl Pharmacol.* 1978;45:201–208.
41. McKechnie NM, King M, Lee WR. Retinal pathology in Kearns–Sayre syndrome. *Br J Ophthalmol.* 1985;69:63–69.
42. Runge P, Calver D, Marshall J, Taylor D. Histopathology of mitochondrial cytopathy and the Laurence–Moon–Biedl syndrome. *Br J Ophthalmol.* 1986;70:782–796.
43. McKelvie PA, Morley JB, Byrne E, Marzuki S. Mitochondrial encephalomyopathies: A correlation between neuropathological findings and defects in mitochondrial DNA. *J Neurol Sci.* 1991; 102:51–60.
44. Lawwill T. Three major physiological processes caused by light in the primate retina: a search for mechanisms. *Trans Am Ophthalmol Soc.* 1982;80:517–520.
45. Rapp LM, Tolman BL, Dhindsa HS. Separate mechanisms for retinal damage by ultraviolet-A and mid-visible light. *Invest Ophthalmol Vis Sci.* 1990;31:1186–1190.
46. Pautler EL, Morita M, Beezley D. Hemoprotein(s) mediate blue light damage in the retinal pigment epithelium. *Photochem Photobiol.* 1990;51:599–605.
47. Kageyama GH, Wong–Riley MTT. The histochemical localization of cytochrome oxidase in the retina and lateral geniculate nucleus of the ferret, cat, and monkey, with particular reference to retinal mosaics and on/off-center visual channels. *J Neurosci.* 1984;4: 2445–2459.
48. Cassarino DS, Bennett JP. An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses and cell death in neurodegeneration. *Brain Res Brain Res Rev.* 1999; 29:1–25.