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Tauroursodeoxycholic Acid Preservation of Photoreceptor Structure and Function in the rd10 Mouse through Postnatal Day 30


PURPOSE. Retinitis pigmentosa (RP) is a progressive neurodegenerative disease resulting in blindness for which there is no current treatment. Although the members of the family of RP diseases differ in etiology, their outcomes are the same: apoptosis of rods and then by cones. Recently, the bile acid tauroursodeoxycholic acid (TUDCA) has been shown to have antiapoptotic properties in neurodegenerative diseases, including those of the retina. In this study the authors examined the efficacy of TUDCA on preserving rod and cone function and morphology at postnatal day 30 (P30) in the rd10 mouse, a model of RP.

METHODS. Wild-type C57BL/6J and rd10 mice were systemically injected with TUDCA (500 mg/kg) every 3 days from P6 to P30 and were compared with vehicle (0.15 M NaHCO₃). At P30, retinal function was measured with electoretinography, and morphologic preservation of the rods and cones was assessed with immunohistochemistry.

RESULTS. Dark-adapted electoretinographic (ERG) responses were twofold greater in rd10 mice treated with TUDCA than with vehicle, likewise light-adapted responses were twofold larger in TUDCA-treated mice than in controls at the brightest ERG flash intensities. TUDCA-treated rd10 retinas had fivefold more photoreceptors than vehicle-treated retinas. TUDCA treatments did not alter retinal function or morphology of wild-type mice when administered to age-matched mice.

CONCLUSIONS. TUDCA is efficacious and safe in preserving vision in the rd10 mouse model of RP when treated between P6 and P30. At P30, a developmental stage at which nearly all rods are absent in the rd10 mouse model of RP, TUDCA treatment preserved rod and cone function and greatly preserved overall photoreceptor numbers. (Invest Ophthalmol Vis Sci. 2008;49: 2148–2155) DOI:10.1167/iovs.07-1012
The present study tests the hypothesis that TUDCA preserves rods and cones to P30, the stage at which photoreceptor cell loss peaks. At this stage of degeneration in the rd10 model, most rods have degenerated, and only some cones remain. This stage of degeneration represents the end stage of RP for most patients because it is estimated that only 0.5% of RP patients develop complete blindness (no light perception). Thus, these experiments test the efficacy of TUDCA at a critical stage of degeneration. To test this hypothesis, rd10 mice were treated with TUDCA or vehicle from P6 to P30, and rod and cone function and morphology were assessed by electroretinography, histology, immunohistochemistry, and TUNEL labeling. Although TUDCA and UDCA have been shown to be very well tolerated in animals and humans, these studies have all been performed in adult animals. Thus, in this study, we also sought to determine whether the antiapoptotic affects of TUDCA had an effect on early retinal development by treating age-matched C57BL/6J wild-type (WT) mice at the same time points. These studies demonstrate that TUDCA is effective in preserving photoreceptors in the rd10 mice at P30.

**Materials and Methods**

**Animals**

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Atlanta VA Medical Center and conform to the standards of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To establish a breeding colony, Pde6b(rdo) mice on a C57BL/6J background (or rd10) were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed under controlled lighting conditions on a 14-hour light/10-hour dark cycle (25–200 lux). Each litter was randomly divided at P6 to receive TUDCA treatment (500 mg/kg; Calbiochem, San Diego, CA; n = 17) or vehicle (0.15 M NaHCO3, 1 mL/kg; Sigma, St. Louis, MO; n = 14). A breeding colony of WT C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) received the same treatments as the rd10 mice (n = 5 for TUDCA, n = 2 for vehicle treatment) starting at P6. TUDCA and vehicle solutions were made up fresh before every injection and were pH adjusted to 7.4. Mice were weighed and injected once every 3 days beginning at P6 and ending at P30, resulting in eight total administrations per animal for the treated and vehicle groups. All injections were made subcutaneously at the nape of the neck.

**Electroretinographic Methods**

Mice were dark adapted overnight, and electroretinography was performed at P30 with a commercial recording system (UTAS 3000; LKC Technologies, Gaithersburg, MD). After anesthesia induction (ketamine; 80 mg/kg; xylazine, 16 mg/kg), the cornea was anesthetized (1% tetracaine), and the pupils were dilated (1% tropicamide, 1% cyclopentolate). Body temperature was maintained at 37°C by placing the mice on a heating pad inside a Faraday cage. The active electrode consisted of a silver wire loop that was positioned on the cornea using 1% methylcellulose. Needle electrodes placed in the cheek and tail served as reference and ground, respectively. A desktop Ganzfeld was used to administer a series of increasingly intense light flashes ranging from −3.0 to 2.1 log cd m−2. Dark-adapted ERG recordings were averaged over 5 to 10 separate flashes per light intensity, with the interstimulus time increasing from 10 to 60 seconds as the flash intensity increased. Animals were then light adapted for 10 minutes using a steady background light (30 cd/m2). Cone-isolating responses were recorded to a seven-step intensity series (−0.82 to 1.88 log cd · s/m2) presented at 3 Hz in the presence of the same light-adapting background. Light-adapted ERGs were averaged over 25 separate flashes per intensity.

**Histologic Methods**

After ERG recordings, deeply anesthetized mice were killed by cervical dislocation. Eyes were immediately enucleated. Fixative was injected into the superior limbus to mark orientation and to aid in the rapid fixation of the retina. All left eyes were immersion fixed in 4% paraformaldehyde for 30 minutes for TUNEL labeling, and all right eyes were fixed overnight in 2.5% glutaraldehyde/2% paraformaldehyde for light microscopy. After fixation, right eyes were dehydrated through a graded alcohol series, infiltrated with propylene oxide, and embedded in resin (Epon 812/Des 756; Electron Microscopy Sciences, Hatfield, PA). Sections (0.5 μm) bisecting the optic disc superiorly to inferiorly were then cut on an ultramicrotome (Ultrotome; Leica, Chicago) using a histodiamond knife and collected on glass slides. Slides were stained with 1% aqueous toluidine blue (Sigma, St. Louis, MO).

Left eyes were processed through a graded series of alcohols and embedded in paraffin. Sections (5-μm thick) were cut on a rotary microtome, bisecting the optic disc superiorly to inferiorly. Paraffin sections were used for cone opsin immunolabeling and TUNEL stain.

**Total Photoreceptor Cell Counts**

Plastic sections were analyzed using light microscopy (DMRB; Leica, Bannockburn, IL) to determine photoreceptor cell counts. Four retinal regions in the vertical meridian (0.5 mm in width) were photographed at 20× magnification. Locations in reference to the optic nerve head were 2.0 to 1.5 mm superior, 1.0 to 0.5 mm superior, 0.5 to 1.0 mm inferior, and 1.5 to 2.0 mm inferior. Photoreceptor nuclei counts were performed using image software (Plus 5.0; ImagePro, Silver Spring, MD). Three sections were counted for each of the four retinal areas in each eye. These values were then averaged, and analysis of variance (ANOVA; SPSS 8.0; SPSS, Inc., Chicago, IL) was performed.

**Cone Photoreceptor Labeling**

Paraffin sections were deparaffinized with xylene, followed by a graded series of alcohol rinses. After an initial blocking step with 5% goat serum (Chemicon, Temecula, CA) made with blocking buffer (Superblock; Pierce, Rockford, IL), sections were incubated in antipsin green/red and blue (1:500; Chemicon) for 48 hours at room temperature. The primary antibody was visualized by labeling with goat anti-rabbit IgG secondary antibody (1:500; Alexa-Fluor 488; Abcam, Cambridge, MA) for 1 hour, after optimization with a titrated series for both the primary and the secondary antibody. Each slide contained a negative control by eliminating primary antibody from one section per slide. Sections were then coverslipped with an aqueous mounting medium (Gel/Mount; Biomedex, Foster City, CA). Digital micrographs were captured of images at 20× magnification using a confocal microscope. All micrographs were taken from sections stained the same day with the same camera settings.

**Cone Photoreceptor Nuclei Counts**

Rod and cone photoreceptor nuclei have differently shaped heterochromatin such that rods have a dense central clump and cones have irregularly shaped heterochromatin that can appear as one to three clumps in tissue sections. Thus, cone photoreceptor nuclei were quantified by counting all photoreceptor nuclei with two or more clumps of heterochromatin in toluidine blue-stained plastic sections (see Fig. 5 for examples of cone vs. rod photoreceptor nuclei). Using the same plastic sections and retina regions described, three indepen-
RESULTS

Results are broken into two broad sets of experiments, those dealing with the efficacy of TUDCA in the rd10 mouse model of RP and those testing TUDCA in age-matched WT mice with the same doses.

Efficacy of TUDCA in the rd10 Mouse

TUDCA Preserves Retinal Function at P30. To determine whether TUDCA treatments preserved rod and cone function in rd10 mice, we performed dark- and light-adapted electroretinography at P30. We found that TUDCA treatment significantly preserved both rod and cone function in rd10 mice. Comparing TUDCA- and vehicle-treated mice, dark-adapted ERGs from TUDCA-treated animals showed significantly larger waveforms (Fig. 1A). At the highest flash intensity, the a-wave amplitude was approximately five times larger in TUDCA-treated mice than in the control treatment groups, whereas the b-wave amplitude was twice as large as were the other treatment groups (Fig. 1). Furthermore, mice treated with TUDCA showed significant preservation of mean a- and b-wave amplitudes (Fig. 1B) over a range of ERG flash intensities, as assessed by repeated-measures ANOVA (a-wave: F(6, 138) = 2.66, P = 0.038; b-wave: F(4, 96) = 2.55, P = 0.044; n = 16 for TUDCA-treated group; n = 10 for vehicle-treated group). Cone-mediated, light-adapted ERG waveforms were significantly larger in TUDCA-treated rd10 mice than in vehicle (Fig. 2A). In the representative waveforms shown in Figure 2A, the electroretinogram from the TUDCA-treated animal was clearly larger in amplitude at all intensities, and the b-wave threshold was measurable at −0.8 log cd·s/m² compared with −0.4 log cd·s/m² for the vehicle-treated animal. When amplitude was plotted across flash intensity, it was seen that there are significant differences in b-wave amplitude between the treatment groups at the brightest flashes (n = 15 TUDCA; n = 10 vehicle; repeated-measures ANOVA F(6, 138) = 6.364; P < 0.001). No
significant differences were detected between the treatment groups for the small light-adapted a-wave (repeated-measures ANOVA \( F(6, 120) = 2.075; P = 0.061 \)).

Preservation of Photoreceptor Nuclei: Rod and Cone Photoreceptors. To determine whether TUDCA treatments preserved photoreceptors, we examined photoreceptor structure and numbers in rd10 mice. TUDCA treatments significantly preserved photoreceptor numbers and the inner and outer segments in the rd10 mice (Fig. 3C) compared with vehicle-treated controls (Fig. 3D). The number of photoreceptor nuclei in a 0.5-mm field at four locations from inferior to superior in TUDCA-treated rd10 mice at P30 showed significant preservation of photoreceptors (Fig. 3F; ANOVA \( F(3, 12) = 3.013; P < 0.034 \)). TUDCA-treated rd10 retinas have significantly more photoreceptors across all areas sampled than vehicle-treated mice (ANOVA \( F(3, 87) = 3.013; P = 0.034 \)). We conclude that TUDCA treatment is efficacious in preventing or slowing photoreceptor degeneration up to P30 in the rd10 mouse model of RP.

Preservation of Photoreceptor Nuclei: Cone Photoreceptors. To identify the population of cone photoreceptors, we examined cone photoreceptor nuclei and numbers in rd10 mice. TUDCA treatments significantly preserved photoreceptor nuclei and the inner and outer segments in the rd10 mice (Fig. 4C) compared with vehicle-treated controls (Fig. 4D). The number of photoreceptor nuclei in a 0.5-mm field at four locations from inferior to superior in TUDCA-treated rd10 mice at P30 showed significant preservation of photoreceptor nuclei (Fig. 4F; ANOVA \( F(3, 14) = 0.034 \)). TUDCA-treated rd10 retinas contained an average of 353 \( \pm 44 \) photoreceptor nuclei per region compared with only 68 \( \pm 9 \) photoreceptor nuclei per 0.5-mm region in the vehicle-treated rd10 retina (mean \( \pm \) SEM; Figs. 4C, 3D, 3F), a fivefold preservation.

Apoptosis in the rd10 Retina at P30

We have previously shown that rd10 retinas at P18 have numerous TUNEL-positive nuclei, but TUDCA-treated retinas have few apoptotic nuclei. 25,26 Thus, at P30, we expect to see fewer TUNEL-positive nuclei in vehicle-treated retinas because of the prior massive loss of photoreceptors before this stage. In contrast, because of the delay in degeneration produced by TUDCA treatments, we expect to observe some apoptotic nuclei in TUDCA-treated retinas.
Our results show that TUDCA-treated rd10 retinas and vehicle-treated retinas had similar numbers of apoptotic nuclei. The average number of TUNEL-positive nuclei in TUDCA-treated retinas per microscope field was 6.8 ± 1.1 (mean ± SEM; n = 10) compared with 11.2 ± 2.9 (n = 3) in vehicle-treated retinas. These differences were not significant (Student’s t-test, P = 0.29).

**TUDCA Studies in WT Mice**

**TUDCA Treatment Does Not Affect Normal Retinal Function.** To test whether TUDCA has adverse affects on normal retinal function when given early in development, we recorded electroretinograms from TUDCA- and vehicle-treated mice. Figure 6A shows the typical dark-adapted ERG waveform to a series of flash intensities. Waveforms from TUDCA-treated retinas were larger than those from vehicle-treated retinas. The mean dark-adapted ERG amplitudes for the a- and b-waves were nearly identical for the TUDCA- and vehicle-treated mice (Figs. 6B, 6C; repeated-measures ANOVA, F(4, 20) = 2.020, P = 0.130, and F(4, 20) = 3.110, P = 0.138, respectively; n = 5 for TUDCA-treated and n = 2 for vehicle-treated). Light-adapted b-wave amplitudes were also similar between treatment groups (Fig. 6D; repeated-measures ANOVA, F(6, 30) = 0.532, P = 0.779).

**TUDCA Treatment Does Not Affect Normal Retinal Morphology.** Retinal morphology appeared normal in WT mice from all treatment groups (Figs. 3A, 3B, 3E). The average number of photoreceptor nuclei was similar, with 795.4 ± 27.4 and 754.2 ± 46.9 nuclei/region in TUDCA-treated and vehicle-treated retinas, respectively. The number of nuclei

**Figure 4.** Cone opsin labeling in WT and rd10 mice after TUDCA treatments at P30. Each micrograph is presented as a pair, with the DIC image on the right and the cone opsin labeling on the left. (A) Cone opsin immunohistochemistry in a WT retina showing distinct outer segments of cones. (B, C) TUDCA-treated rd10 retinas showing long cone outer segments, similar to WT retina (B) and shorter cone outer segments with more punctuate labeling (C). (D, E) Cone labeling in vehicle-treated rd10 retinas appeared as sparse punctuate labeling. Note that no outer segments are visible in the DIC images of the vehicle-treated mice. These results suggest that TUDCA treatments preserve cone outer segments.

**Figure 5.** Quantitative assessment of cones after TUDCA treatment at P30. Cone nuclei in WT (A) and rd10 (B) mice were counted based on heterochromatin pattern. Heterochromatin that formed two or more clumps was counted as a cone (arrows) according to the criteria established by Carter-Dawson and LaVail. This contrasts with rod nuclear staining, which appears dark and uniformly stained. (C) Cone counts for each 500-μm retinal region did not show any differences between treatment groups for rd10 or WT mice. However, WT mice had two to three times more cone nuclei per retinal area than rd10 mice (Student’s t-test, P < 0.001). Error bars indicate SEM.
across retinal areas was not significant between treatment groups in the WT mice (repeated-measures ANOVA, \( F(3, 12) = 0.397, P = 0.757 \); Fig. 3E).

**TUDCA Treatment Alters Body Weight in rd10 and WT Mice.** Animal weights were also compared across time with TUDCA versus vehicle treatment. TUDCA-treated WT mice were found to have significantly lower body weights than vehicle-injected mice (Fig. 7A; repeated-measures ANOVA, \( F(5, 25) = 4.276, P = 0.006 \)). As treatments progressed, the TUDCA-treated mice gained less weight than did vehicle-treated mice. The largest differences were not reached until P24. TUDCA-treated rd10 mice also showed a significant decrease in body weight over the treatment period (Fig. 7B; repeated-measures ANOVA, \( F(7, 245) = 10.973, P < 0.001 \)). In rd10 mice, the effect of TUDCA on body weight was apparent by P9, much sooner than in the WT mice.

**FIGURE 6.** Studies in WT mice. Retinal function measurements were recorded from WT C57BL/6j mice treated with TUDCA or vehicle from P6 to P30. (A) Representative dark-adapted ERG waveforms for WT TUDCA- and vehicle-treated mice. Dark-adapted a-wave (B), dark-adapted b-wave (C), or light-adapted b-wave (D) amplitudes from WT mice treated with TUDCA (n = 5) or vehicle (n = 2) show no differences in amplitude or timing across different flash intensities (repeated-measures ANOVA, \( F(4, 20) = 2.020, P = 0.130 \); \( F(4, 20) = 3.110, P = 0.158 \); \( F(6, 30) = 0.532, P = 0.779 \); respectively). We concluded that TUDCA treatment from P6 to P30 has no deleterious effect on retinal function, as measured with the ERG.

**FIGURE 7.** Body weights in WT and rd10 mice after TUDCA treatments from P6 to P30. (A) WT mice treated with TUDCA (n = 5) showed a reduction in body weight compared with vehicle (n = 2; repeated-measures ANOVA, \( F(5, 25) = 4.276, P = 0.006 \)). (B) TUDCA-treatment of rd10 mice (n = 24) resulted in significantly lower body weight than vehicle (n = 13) from P15 to P27 (repeated-measures ANOVA, \( F(7, 245) = 10.973, P < 0.001 \)). We conclude that TUDCA treatments suppressed body weight in the WT and rd10 mice. Symbols represent mean ± SD.


DISCUSSION

The present study demonstrates that TUDCA treatment, through the advanced degenerative time point of P30 in the rd10 mouse, is remarkably effective in sustaining photoreceptor cells and their function. We have previously shown successful preservation of rod function and structure through P18 in this model.25 Though slightly delayed compared with that in rd1 mouse, retinal degeneration in rd10 mice is rapid and aggressive. In untreated rd10 mice, the outer nuclear layer (ONL) is reduced to a single nuclear layer by P30.5–7 Functionally, the dark-adapted a-wave is largely undetectable (only 3% of WT response), and the b-wave is greatly diminished (14% of WT response).5–7 At this stage, rods are almost completely absent and cones are degenerating. This study shows that TUDCA treatments starting at P6 sustain retinal function and morphology through this critical stage from 30% (dark-adapted a-wave) to 45% (light-adapted and dark-adapted b-waves and photoreceptor numbers) of WT responses. These results show significant preservation of the retina by a systemic agent at this stage of degeneration.

A pan-retinal preservation of retinal function and photoreceptor nuclei was found in the TUDCA-treated rd10 mice at P30. Rod function and rod photoreceptors were significantly greater in TUDCA-treated rd10 mice (Figs. 1–3). In addition, the number of total photoreceptor nuclei was fivefold greater in TUDCA-treated mice than in vehicle-treated mice (Fig. 5).

Interestingly, TUDCA treatments appeared to preserve cone outer segment morphology compared with vehicle treatment (Fig. 4), which correlates well with the increased amplitudes in the light-adapted TUDCA-treated retinas (Fig. 2). However, no differences were found in the number of cone nuclei between the treatment groups (Fig. 5). These data may suggest that TUDCA causes an overall increase in preservation of the photoreceptors. Given that cone degeneration occurs secondarily in this model, the significant preservation of rods from TUDCA treatments may sustain the cones. A later time point may be needed to exclusively evaluate the effect of TUDCA treatments on cone preservation. However, because humans rely most on cone vision, the finding of greater cone function and healthier cone outer segments after TUDCA treatments may suggest that TUDCA is delayimg the death of photoreceptors in the rd10 model. In our analysis of rd10 retinas at P18, vehicle-treated retinas showed a vastly greater number of TUNEL-positive nuclei compared with TUDCA-treated retinas.25 At P30, as might be expected, the vehicle-treated rd10 retinas do not have many nuclei remaining to undergo apoptosis. Conversely, the TUDCA-treated retinas have many remaining nuclei, some of which are apoptotic. WT mice showed no significant differences between the treatment groups.

Similar to other studies that showed TUDCA to be well tolerated in adult animals,8–12 we found no evidence at the functional or morphologic level that TUDCA itself had any significant adverse effects on the retina in WT mice when given from P6 to P30. However, TUDCA treatments did produce a decrease in body weight in WT and rd10 mice (Fig. 7). The mean body weight of TUDCA-treated WT mice dropped 22%, whereas weights in TUDCA-treated rd10 mice decreased 23%. These values are slightly below the 25% loss of body weight that is considered an end point criterion by our Institutional Animal Care and Use Committee. Other studies have not reported a reduction in body weight after TUDCA treatment, perhaps because other animal models were treated during adulthood8–12 and not early postnatal development, as in this study. Future studies will determine whether a different vehicle would have less effect on body weight. Nevertheless, the lack of toxic effects on the retina complement other studies that showed the safety of UDCA treatment in human patients with liver disease,32,39 of TUDCA treatment in rodent models of Huntington disease,8,9 and of TUDCA treatment in rodent models of stroke.11,12 If TUDCA continues to show effects on body weight in young animals, drug delivery approaches could be pursued to administer TUDCA exclusively to the retina.

Comparing the preservation of the rd10 retina between P18 and P30, TUDCA appears to delay degeneration in rd10 mice by approximately 12 days, or approximately 35%, over the course of the degeneration period. Because it has been reported that different species have surprisingly similar rates of degeneration based on maximal life expectancy90 and that typical RP patients have a linear rate of retinal function loss,41 a prediction of TUDCA ability to preserve vision in RP patients can be made. TUDCA might preserve retinal function for approximately 18 years in a patient whose photoreceptor loss began in his or her 20s and ended in his or her 70s. Coupling this possibility of efficacy with the demonstrated lack of toxicity of hydrophilic bile acids in several animal models8–12 and humans8–2 suggests that hydrophilic bile acids may be relevant to the ophthalmic clinic.

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