Preventing Corneal Calcification Associated With Xylazine for Longitudinal Optical Coherence Tomography in Young Rodents

Tianwei Ellen Zhou,1,2 Diane N. Sayah,2,3 Baraa Noueihed,1,2 Javier Mazaferri,2,4 Santiago Costantino,2,4 Isabelle Brunette,2,4 and Sylvain Chemtob1,2,4–6

1Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada
2Hôpital Maisonneuve-Rosemont Research Center, Montréal, Québec, Canada
3École d’Optométrie, Université de Montréal, Montréal, Québec, Canada
4Department of Ophthalmology, Université de Montréal, Montréal, Québec, Canada
5Department of Pharmacology, Centre Hospitalier Universitaire Sainte-Justine Hospital, University of Montréal, Montréal, Québec, Canada
6Department of Pediatrics, Centre Hospitalier Universitaire Sainte-Justine Research Center, Université de Montréal, Montréal, Québec, Canada

Correspondence: Tianwei Ellen Zhou, Zhou, Centre de Recherche, Hôpital Maisonneuve-Rosemont, 5345 Boulevard de l’Assomption, Montréal, QC H1T 4B3, Canada; ellen.zhou@mail.mcgill.ca.
Sylvain Chemtob, Centre de Recherche, Hôpital Maisonneuve-Rosemont, 5345 Boulevard de l’Assomption, Montréal, QC H1T 4B3, Canada; sylvain.chemtob@umontreal.ca.
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Purpose. Spectral-domain optical coherence tomography (SD-OCT) is widely used in clinical ophthalmology and recently gained popularity in laboratory research involving small rodents. Its noninvasive nature allows repeated measurements, thereby decreasing the number of animals required. However, when used at a conventional dosage, xylazine (an α2-adrenoceptor) can cause irreversible corneal calcification, especially among young rodents. In the present study, we test whether corneal calcification associated with xylazine is mediated by the α2-adrenoceptor.

Methods. Our study tested Sprague-Dawley rats, Long-Evans rats, and CD-1 mice (postnatal day [P]14). Retinal images were captured by SD-OCT. Quantitative PCR (qPCR) was used to study gene expression, whereas receptor localization was examined by immunofluorescent staining followed by confocal microscopy. Calcium deposits were detected via von Kossa staining.

Results. When used at dosages appropriate for adult animals, ketamine-xylazine anesthetics led to a high rate of respiratory failure, increased apoptotic activity in the corneal epithelium, and irreversible corneal calcification in P14 rat pups. Meanwhile, OCT image quality decreased drastically as a result of corneal calcification among animals recovering from anesthesia. α2-Adrenoceptor subtypes were highly expressed on P14, in line with rodents’ age-specific sensitivity to xylazine. Clonidine, a potent α2-adrenoceptor agonist, dose-dependently induced corneal calcification, which could be prevented by an α2-adrenoceptor antagonist.

Conclusions. These data suggest that α2-adrenoceptors contribute to corneal calcification in young rodents. Therefore, we developed a suitable OCT imaging protocol for this cohort, including a carefully tailored ketamine-xylazine dosage (60 mg/kg and 2.5 kg/mg, respectively).

Keywords: corneal calcification, optical coherence tomography, α2-adrenoceptor, xylazine

Spectral-domain optical coherence tomography (SD-OCT), a technology widely used to examine retinal pathologies in humans, has revolutionized the practice of ophthalmology and has become part of the standard care.1,2 Optical coherence tomography also gained increasing popularity in laboratory research involving small rodents. Based on low-coherence interferometry, it provides high-resolution cross-sectional imaging of ocular structures, allowing rapid and reliable repeated measures of delicate retinal layers in rodents. Contrary to the traditional histology techniques that require euthanizing animals and extensive sample process time, the noninvasive nature of OCT allows longitudinal studies of the same cohort, thereby decreasing the number of animals and minimizing intersubject variability.

Ketamine hydrochloride is a noncompetitive, centrally acting, dissociative general anesthetic that provides amnesia, analgesia, and immobility.3 Ketamine offers a wide margin of safety in most species, as well as a residual analgesia following anesthetic recovery. Meanwhile, xylazine provides additional analgesia and muscle relaxation. As an agonist for α2 adrenoceptor (a G protein-coupled receptor), xylazine activates the inhibitory Gi/o heterotrimeric G protein. Thus far, three distinct subtypes of α2-adrenergic receptors, α2A, α2B, and α2C, have been identified.4 The ketamine-xylazine (K-X) combination is the most commonly used drug combination for injectable anesthesia in small rodents.3

However, rodents, especially young pups (postnatal day [P]14), receiving K-X at the conventional dose (85 and 5.0 mg/
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Materials and Methods

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Anesthesia and Follow-Up Schedule

Ninety-six Sprague-Dawley rats, 27 Long-Evans (LE) rats, and 20 CD-1 mice were used in our study. All animals were P14. At days 1 and 3 of the study, animals were anesthetized by intramuscular (i.m.) injection of a combination of ketamine (K) (Vetalar, DIN 01989529; Bioniche Animal Health, Belleville, Ontario, Canada) at 50, 70, or 85 mg/kg and xylazine (X) (Rompun, DIN 02169592; Bayer HealthCare, Mississauga, Ontario, Canada) at 2.5 or 5.0 mg/kg. Optical coherence tomography imaging was performed under anesthesia at three time points: 5 minutes, 30 minutes (day 1), and 3 days later, and slit-lamp corneal examination was performed on day 3. Following recovery of spontaneous locomotion, all animals were returned to their home cages. The Sprague-Dawley rats were euthanized immediately after day 3 OCT scans and histology studies were performed.

Optical Coherence Tomography

Time course SD-OCT (Spectralis OCT Plus; Heidelberg Engineering GmbH, Heidelberg, Germany) with enhanced depth imaging was carried out on all rats. The anesthetized rats were placed on a horizontal platform in front of the OCT device. Tropicamide 1% (Mydriacyl; Alcon Laboratories, Inc., Fort Worth, TX, USA) dilating drops were instilled in the studied eye. Volume scans of 15° × 5° (seven B-scans 240 μm apart, ART 100 frames including 768 A-scans) were carried out in the rats’ right eyes, by convention. If the imaging was rendered difficult due to rapid breathing or movement that disrupted the eye tracker, the experimenter gently stabilized the rat’s head, with light pressure. The eye tracker was directed at the center of the eye, and all OCT scans were obtained at the temporal side of the optic nerve (equivalent to the human macula). To circumvent the central corneal plaque when present (Fig. 1A), images were acquired through the peripheral cornea.

Quantification of Image Quality

Signal-to-noise ratio was calculated for all images obtained by OCT using MATLAB (The MathWorks, Inc., Natick, MA, USA). The signal was defined as the 90th percentile of the intensities displayed in the retinal scan. The noise was defined as the SD of intensities and was computed from a square of constant size cropped from the vitreous region of each image. The quality indices were then analyzed for each study condition by 1-way ANOVA using the optimal condition (i.e., 60 mg/kg K + 2.5 mg/kg X at 5 minutes) as a control.

Slit-Lamp Biomicroscopy

Corneal opacities were observed using a slit-lamp (Coherent LDS10A; Coherent Medical Group, Palo Alto, CA, USA). The rats were placed on a horizontal platform in front of the biomicroscope. The cornea and anterior segment were examined and photographed through the slit-lamp ocular.

Corneal Tissue Preparation

All eyes were enucleated, fixed in 4% paraformaldehyde for 1 hour at room temperature, and dehydrated in 30% sucrose overnight at 4°C. Corneas were then dissected and frozen in Optimal Cutting Temperature medium (Tissue-Tek; Sakura Finetek, Inc., Torrance, CA, USA). Samples were cut into 10-μm-thick sagittal sections (Microm HM5000; Microm Laborg-
Ketamine analyses were performed. Eyes were rapidly enucleated and Slc4a4 NBC-1 (gene symbol: Adrar, Adrar1 gene symbols: acidosis.12 To evaluate whether the increased sensitivity to while, sodium-bicarbonate cotransporter-1 (NBC-1) was linked for 2 minutes, and rinsed again in three changes of distilled water. Thiosulfate (cat. no. 217247; Sigma-Aldrich Corp.) solution for 3% silver nitrate solution for Cryo-section samples were placed in 3% silver nitrate solution (cat. no. 209139; Sigma-Aldrich Corp., St. Louis, MO, USA) and exposed to sunlight or UV light for 30–60 minutes as previously described.11 They were subsequently rinsed in three changes of distilled water, placed in 5% sodium thiosulfate (cat. no. 217247; Sigma-Aldrich Corp., Dallas, TX, USA) and processed for immunohistochemistry.

**Immunohistochemistry**

Cryo-section samples were stained with primary antibodies against cleaved caspase-3 antibodies (Cell Signaling Technology, Danvers, MA, USA; sc-1478 at 1:200 dilution). The slides were then counterstained with 4,6-diamidino-2-phenylindole, dihydrochloride, Danvers, MA, USA; at 1:500 dilution), neuron-specific tubulin (R&D Systems, Minneapolis, MN, USA; at 1:400 dilution), and/or a-2-adrenoceptors (Cell Signaling Technology, Danvers, MA, USA; at 1:200 dilution). The slides were then counterstained with 4,6-diamidino-2-phenylindole, dihydrochloride (DAP; 0.1 µg/mL; Molecular Probes, Eugene, OR, USA). Stained slides were examined under a confocal microscope (Zeiss, Toronto, Ontario, Canada).

**RNA Isolation and Quantitative Real-Time PCR**

Xylazine is considered to be responsible for corneal calcification in young rats by activating a2-adrenoceptors.5,6,8 Meanwhile, sodium-bicarbonate cotransporter-1 (NBC-1) was linked to corneal calcification associated with proximal renal tubular acidosis.12 To evaluate whether the increased sensitivity to xylazine in rat pups (P14) would be due to developmental gene expression differences for a2-adrenoceptors (a2A, a2B, a2C; gene symbols: Adrar, Adrar1, and Adrar2, respectively) and/or NBC-1 (gene symbol: Slc4a4), quantitative PCR (qPCR) analyses were performed. Eyes were rapidly enucleated and placed into a sterile petri dish resting on ice. The cornea was dissected and processed for RNA extraction using TRIzol (Invitrogen, Thermofisher Scientific Corporation, Carlsbad, CA, USA), followed by treatment with DNase I (Qiagen, Hilden, Germany) to remove any contaminating genomic DNA. The DNase-treated RNA was then converted into cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). Polymerase chain reaction primers targeting rats were designed using National Center for Biotechnology Information (NCBI) Primer Blast (Table 1). Quantitative analysis of gene expression was generated using an ABI Prism 7700 sequence detection system and the SYBR Green Master Mix Kit (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression was calculated relative to 18s universal primer pair (Ambion, Thermofisher Scientific, Inc., Waltham, MA, USA) expression using the ΔΔct method.

**Effects of Clonidine Hydrochloride, Yohimbine Hydrochloride, and Nifedipine**

Clonidine hydrochloride (cat. no. C7897; Sigma-Aldrich Corp.) was injected intramuscularly into P14 pups (N=12; n=3 pups per group) with a dose varying from 0.075 to 0.600 mg/kg. Three days later, pups were euthanized, and their corneas were processed for cryo-section and histologic studies.

Nine P14 Sprague-Dawley rat pups received i.m. injection of either xylazine (5.0 mg/kg), yohimbine hydrochloride (2.0 mg/kg; cat. no. Y3125; Sigma-Aldrich Corp.) or xylazine + yohimbine (n=3 pups per group). For the last group, yohimbine was administered 30 minutes after initial xylazine injection. All pups were euthanized 24 hours after, and their corneas were processed for qPCR or histologic studies.

Six P14 Sprague-Dawley/LE rats and CD-1 mice received topical nifedipine (an L-type calcium channel blocker; cat. No. N7634; Sigma-Aldrich Corp.) 30 minutes after xylazine (5.0 mg/kg) or xylazine (5.0 mg/kg)–ketamine (60 mg/kg) injections. Nifedipine (20 µM) was applied topically on the cornea to avoid severe adverse hemodynamic effects in young rodents. The time at which corneal calcification was observed was recorded.

**Table 2. Initial and Tailored Doses of Anesthetics (SD Rats)**

<table>
<thead>
<tr>
<th>Anesthetic Combination</th>
<th>Ketamine (mg/kg)</th>
<th>Xyazine (mg/kg)</th>
<th>Number of Rats</th>
<th>Number of Deaths</th>
<th>Number of Eyes With Corneal Opacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>5.0</td>
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<td>32/34</td>
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<td>60</td>
<td>2.5</td>
<td>24</td>
<td>3/24</td>
<td>4 (from 3 rats)/48</td>
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</table>

**Table 3. Different Ketamine-Xyazine Doses and Their Effects (SD Rats)**

<table>
<thead>
<tr>
<th>Anesthetic Combination</th>
<th>Ketamine (mg/kg)</th>
<th>Xyazine (mg/kg)</th>
<th>Number of Rats</th>
<th>Number of Deaths</th>
<th>Number of Eyes With Corneal Opacity</th>
</tr>
</thead>
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<tr>
<td>60</td>
<td>2.5</td>
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<td>0/2</td>
<td>0/4</td>
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<tr>
<td>60</td>
<td>5.0</td>
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<td>0/2</td>
<td>4/4</td>
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<td>1/4</td>
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<td>85</td>
<td>5.0</td>
<td>2</td>
<td>1/2</td>
<td>3/4</td>
<td></td>
</tr>
</tbody>
</table>
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RESULTS

Xylazine 5.0 mg/kg in P14 Young Rats Is Strongly Associated With Irreversible Corneal Opacification

Twenty-four P14 Sprague-Dawley pups (initially destined to a different experiment) were anesthetized using ketamine (85 mg/kg) and xylazine (5.0 mg/kg). Clear OCT fundus images were obtained; however, seven young rats never recovered from anesthetics and died (Table 2). On P21, all remaining 17 rats (10 males and 7 females) had developed a white, opaque, chalky plaque in the central cornea of both eyes. In addition, significant neovascularization extended from the limbal vascular plexus to the central cornea (Fig. 1A). Because OCT scans could not proceed to completion due to low image quality (Spectralis quality score < 10), sample images were obtained via print screen (Fig. 1B). Similar corneal opacification induced by xylazine (5.0 mg/kg) used alone or in combination with ketamine was observed in CD-1 mice and LE rats (Tables 4, 5).

Statistical Analysis

Results are presented as means ± SEM. 1-way or 2-way ANOVA with significance (α = 0.05) was used for processing data. Bonferroni post hoc analysis was used for calculating significance between groups. Two-tailed Student’s t-tests were used to test for significance between two means.

Identifying an Appropriate K-X Dose Combination for Young Rats

A series of anesthetic combinations was then tested using dosages within the ranges recommended by the National Institutes of Health Office of Laboratory Animal Welfare13 (i.e., ketamine: 60–85 mg/kg; xylazine: 2.5–5.0 mg/kg; Table 3). All tested combinations resulted in rapid onset of anesthesia within 5–10 minutes, with noticeable muscle relaxation.

Three of four Sprague-Dawley rats that received 85 mg/kg ketamine had irreversible respiratory failure (Table 3). The remaining rat slowly regained consciousness after 4 hours but appeared lethargic for another day. Similar observations held true for pups that received ketamine at 70 mg/kg, except for a slightly shorter duration before regaining consciousness and full activity. All Sprague-Dawley/LE rats and CD-1 mice survived with 60 mg/kg ketamine (Tables 3–7). Among SD rats, xylazine at 5.0 mg/kg was strongly associated with the development of corneal opacity (Table 3). Likewise, the majority of CD-1 mice and LE rats receiving xylazine (5.0 mg/kg; alone or with ketamine) showed corneal lesion (Tables 4, 5).

The K-X combination at 60 and 2.5 mg/kg was later used in a separate experiment with 24 Sprague-Dawley rats (P14). Three pups went into respiratory failure, and only four eyes from three rats exhibited corneal opacification (Table 2). The same regimen was also found to be relatively safe in CD-1 mice and LE rats (Tables 4, 5).

Longitudinal OCT Imaging Using Ketamine 60 mg/kg and Xylazine 2.5 mg/kg Yielded High-Quality Images

Because corneal opacity appeared to be linked to xylazine dosage in young rats (P14), we compared the OCT image quality for two different xylazine dosages (group 1, 2.5 mg/kg versus group 2, 5.0 mg/kg) using a constant dose of ketamine (60 mg/kg) at different time points after injection (Fig. 2).

Five and 30 minutes after anesthetics injection, both groups yielded high-quality OCT images, with clearly identifiable inner nuclear layer (INL), outer nuclear layer (ONL), and choroid (Ch) in all eyes (Figs. 2A–D).

On day 3, visual inspection and slit lamp confirmed white, chalky corneal opacification in group 2 rats only (Fig. 2H). Day 3 OCT scans from group 1 rats (2.5 mg/kg xylazine; Fig. 2E) were crisp compared with those from group 2 (5 mg/kg xylazine; Fig. 2F), as confirmed by quality analyses (Fig. 2I). In addition, OCT image acquisition for group 2 rats was much more laborious as direct scanning was prevented by the corneal plaque; we obtained only two scans by deviating the laser beam through the peripheral cornea devoid of calcification.

Histology Revealed Calcific Deposits and Heightened Caspase-3 Activity in Affected Corneas

von Kossa staining of corneal sections of rats exposed to higher-dose xylazine (group 2) revealed a thick dark band in the anterior stroma and Bowman’s membrane (Fig. 3A) in all of the animals, in line with the calcific deposit shown by the slit lamp (Fig. 2H). In comparison, animals exposed to lower-dose xylazine (group 1) showed normal cornea histology (Fig. 3B) and slit-lamp appearance (Fig. 2G).

Cleaved caspase-3 signal in the surface squamous epithelium of the cornea was observed in both groups (Figs. 3C, 3D). However, only group 2 rats showed cleaved caspase-3–positive columnar basal cells (Fig. 3D), which are the only corneal epithelial cells capable of mitosis.14 Cleaved caspase-3 signal was also sparsely observed in the anterior and mid-corneal stroma of group 2 rats.

Table 6. Effects of Xylazine and/or Yohimbine on the Cornea (SD Rats, P14)

<table>
<thead>
<tr>
<th>Medications</th>
<th>Number of Rats</th>
<th>Number of Deaths</th>
<th>Number of Eyes With Corneal Opacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylazine (mg/kg)</td>
<td>Yohimbine (mg/kg)</td>
<td>Number of Rats</td>
<td>Number of Deaths</td>
</tr>
<tr>
<td>0 2.0</td>
<td>3</td>
<td>0/3</td>
<td>0/6</td>
</tr>
<tr>
<td>5.0 0</td>
<td>3</td>
<td>0/3</td>
<td>6/6</td>
</tr>
<tr>
<td>5.0 2.0 (30 minutes later)</td>
<td>3</td>
<td>0/3</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Table 7. Effects of Different Regimens on the Cornea of SD and LE Rats (P21)

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Number of Rats</th>
<th>Number of Deaths</th>
<th>Number of Eyes With Corneal Opacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketamine (60 mg/kg) + xylazine (5.0 mg/kg)</td>
<td>SD LE</td>
<td>6 (3F 3M)</td>
<td>4 (2F 2M)</td>
</tr>
<tr>
<td>Ketamine (60 mg/kg) + xylazine (2.5 mg/kg)</td>
<td>SD LE</td>
<td>6 (3F 3M)</td>
<td>4 (2F 2M)</td>
</tr>
<tr>
<td>Ketamine (60 mg/kg)</td>
<td>SD LE</td>
<td>4 (1E 3M)</td>
<td>4 (1E 3M)</td>
</tr>
<tr>
<td>Xylazine (5.0 mg/kg)</td>
<td>SD LE</td>
<td>6 (3F 3M)</td>
<td>4 (2F 2M)</td>
</tr>
<tr>
<td>Xylazine (2.5 mg/kg)</td>
<td>SD LE</td>
<td>5 (2F 3M)</td>
<td>4 (1E 3M)</td>
</tr>
</tbody>
</table>
adrenoceptor was also highly expressed at P14 (Fig. 4C). The expression level of NBC-1, however, did not change significantly from P6 to P21 (Fig. 4D). In line with qPCR results, confocal microscopy confirmed the colocalization of corneal nerve fibers (labeled with βIII tubulin, red) and the α2A-adrenoceptor (Figs. 4E, 4F) on corneal flatmounts.

**Corneal Calcification in P14 SD Rats Is Dose-Dependently Induced by Clonidine and Inhibited by Yohimbine**

To confirm the relation between α2-adrenoceptor and corneal calcification, clonidine, an α2-adrenoceptor agonist more potent than xylazine, was injected intramuscularly into P14 rats at increasing doses (0.075, 0.150, 0.300, or 0.600 mg/kg). At 0.075 mg/kg, clonidine did not cause calcium deposit in cornea, as indicated by the lack of von Kossa staining (Fig. 5A). With a rising dose of xylazine, increasing amounts of calcium (brown streaks) were detected in Bowman’s layer and anterior/midstroma (Figs. 5B–D). Accordingly, corneal calcification secondary to xylazine (5.0 mg/kg) was prevented by the α2-adrenoceptor agonist yohimbine (2.0 mg/kg, intraperitoneally) 30 minutes after xylazine administration (Table 6; Figs. 5E–G).

**Effect of Topical Nifedipine (20 μM) on Corneal Calcification**

Because activation of axonal α2-adrenoceptors decreases neuronal excitation by blocking calcium influx,15 our original hypothesis was that xylazine closes calcium channels, causing buildup of extracellular calcium. Therefore, we used nifedipine (topical drops, 20 μM), a potent L-type calcium channel blocker, to see whether it is sufficient to induce corneal calcification. Contrary to our expectation, nifedipine alone does not induce corneal calcification in Sprague-Dawley/LE rats or CD-1 mice. However, the drug seems to accelerate toxicity (Figs. 4A–C). This is corroborated by confocal microscopy showing colocalization of corneal nerve fibers (labeled with βIII tubulin, red) and the α2A-adrenoceptor (Figs. 4E, 4F) on corneal flatmounts.

**Expression of α2-Adrenoceptors, but Not NBC-1, Are Elevated in Young SD Pups**

Quantitative PCR analyses of α2-adrenoceptors (Adrar, Adrar1, and Adrar2 genes) and NBC-1 (Slc4a4 gene) were performed. They revealed that the expression of α2A and α2B-adrenoceptors was significantly higher in the cornea of P14 rats (2.18- and 3.20-fold, respectively) than at P21 (Figs. 4A, 4B); the α2C-adrenoceptor was also highly expressed at P14 (Fig. 4C). The expression level of NBC-1, however, did not change significantly from P6 to P21 (Fig. 4D). In line with qPCR results, confocal microscopy confirmed the colocalization of corneal nerve fibers (labeled with βIII tubulin, red) and the α2A-adrenoceptor (Figs. 4E, 4F) on corneal flatmounts.

**DISCUSSION**

The K-X combination is widely used to anesthetize rodents. Corneal calcification secondary to K-X anesthetics was first reported in the 1980s. Calderone et al. reported “lens opacification”5 among rodents that received the K-X mix or xylazine (13 mg/kg), but not ketamine alone. Two years later, Guillet et al. demonstrated the problem was in fact calcification of the cornea and suggested that anesthetic sensitivity is age dependent.6 Using clonidine and yohimbine, Tita et al. and Koehn et al. studied the relation between α2-adrenoceptors and histologic alterations in rodent cornea.16,17 However, the former did not study the composition of the corneal deposits. In addition, neither paper explained why corneal calcification is age dependent or explored the mechanism. A transparent cornea is key to acquiring high-quality OCT imaging and performing fundoscopy. Herein, we provide strong evidence to relate rodents’ (in both rats and mice) age-specific sensitivity to xylazine with corneal calcification. In this process, we also optimized the protocol for repeated OCT measures.

A feature of this study is the expression profile of α2-adrenoceptors: the mRNA levels of three subtypes simultaneouly peaked at P14, coinciding with the timing of xylazine toxicity (Figs. 4A–C). This is corroborated by confocal microscopy showing colocalization of corneal nerve fibers and α2-adrenoceptor (Figs. 4E, 4F). Along with previous reports, corneal calcification is likely mediated through α2-adrenoceptors based on the following observations: (1) elevated α2-adrenoceptors expression in cornea of young pups.
Corneal opacification is linked to xylazine (5.0 mg/kg) in young rats. (A) Corneal opacification was observed 1–3 hours after i.m. xylazine at 5.0 mg/kg in P14 rats. von Kossa staining revealed dense calcium deposit (brown-colored) at Bowman’s membrane and anterior stroma. (B) In contrast, corneal calcification was avoided by lowering xylazine dosage to 2.5 mg/kg. Scale bar denotes 100 μm. (C, D) Immunohistochemistry staining showed baseline, physiologic cleaved caspase-3 signals at the squamous cell layer of the epithelium. Pups receiving xylazine at 5.0 mg/kg exhibited cleaved caspase-3 signals at both squamous and basal cell layers (yellow arrows). Scale bar denotes 20 μm.

**Figure 3.**

(P10 and P14) corresponds to the age for heightened xylazine sensitivity (Fig. 1A; Table 2); (2) despite the use of a heating pad and proper hydration, corneal calcification at P14 in rats occurred within 1–2 hours after xylazine injection (i.m., 5.0 mg/kg)17 (the rapid onset of symptoms pointed toward a receptor-mediated process); and (3) clonidine, a potent α2-adrenoceptor agonist, dose-dependently induced corneal calcification (Figs. 5A–D); whereas (4) yohimbine (an α2-adrenoceptor antagonist) was able to prevent corneal calcification in xylazine-treated pups (Figs. 5E–G).

The mechanism for α2-adrenoceptor-dependent calcification remains unclear. Nifedipine accelerates corneal calcification; yet the drug does not induce the problem (Fig. 6), suggesting that the lack of calcium re-entry alone cannot account for calcification. Other mechanisms, such as active secretion of Ca²⁺ may be involved.

In rodents, the density of corneal nerve fibers markedly increases postnataally.18 During this period, nerve fibers gradually form a swirl pattern near the apex of the cornea.19 As a result, the central cornea has the highest density of axon terminals.20 This anatomical feature supports the observation that corneal calcification is centrally located (Figs. 1A, 2H).

Thus far, mechanistic explanation for corneal calcification remains scant. Usui et al. suggested the involvement of NBC-1, a family of ion transporters linked to proximal renal tubule acidosis12,21; NBC-1 is expressed in human cornea,22 and mutations on this gene are postulated to cause ion imbalance and, subsequently, corneal damages.12 However, we did not find differential NBC-1 expression among young and adolescent rodents (Fig. 4D). Hence, NBC-1 alone may not be sufficient to account for rodents’ age-dependent sensitivity to xylazine.6

Another notable observation in this study is the detection of excessive apoptosis in corneal columnar basal cells in pups that received the higher dose of xylazine (5.0 mg/kg) (Fig. 3D). The upper squamous epithelium is known for orderly apoptosis and desquamation as part of physiologic turnover.14 The columnar basal cells, however, are the only corneal epithelial cells capable of mitosis23 (to replace apoptotic cells). Increased cleaved caspase-3 activity in columnar basal cells suggests a depletion of stem cell population in corneal epithelium. This may explain the lack of sufficient corneal repair following calcific injury.

Other anesthetic alternatives for OCT experiments have been suggested. The most common alternative is isoflurane inhalation using a rodent facemask.24,25 Although facemasks have been redesigned over time, the frequent occurrence of gas leakage still poses a hazard to personnel.24 In addition, the bulkiness of the facemask and tubing is cumbersome, it hinders proper alignment of the rodent eye with the OCT.
optical axis, and care must be taken to avoid contact with the OCT’s objective lens, making it difficult to scan the desired location. Last, KX anesthetics offers better control on eye movement (excessive movement decreases OCT quality) than isoflurane. Thus, KX remains a better option than inhalation isoflurane in OCT acquisition.

Several external factors have been reported to be linked to corneal calcification in the interpalpebral zone. Ocular surface dryness resulting from tear evaporation leads to increased concentration of ions such as calcium and phosphate. Carbon dioxide release in this same zone also leads to pH elevation. Alkalization facilitates the precipitation of calcium phosphate, which already has low solubility and becomes supersaturated in the fluids of the eye. The loss of body heat is also considered a contributing factor to crystalline lens opacification and calcium deposition in the cornea. However, in our study and that of Guillet et al., lubricating gel and warming pads failed to prevent corneal calcification (Fig. 3A). Of note, because KX combination reduces body temperature and P14 pups still lack sufficient fur, all anesthetized pups should be placed on warming pads until they regained consciousness.

One caveat in this study is that only two strains of rats (Sprague-Dawley and LE) and one stock of mice (CD-1) were tested. However, Sprague-Dawley (albino)/LE (pigmented) rats and CD-1 (albino) mice are outbred stocks (i.e., different genetic makeup), yet their responses to xylazine (5.0 mg/kg) were similar. Notably, C57BL/6J (inbred, pigmented), C57BLKS/J (inbred, pigmented), and SJL/J (inbred, albino) mice also suffer from corneal calcification following KX.
Meanwhile, Wistar (inbred, albino), LE (outbred, pigmented), and Fischer 344 (inbred, albino) rats are found to be more susceptible to xylazine-induced corneal calcification than Sprague-Dawley (outbred, albino) and Lewis (inbred, albino) rats. This is in agreement with our data for P21 Sprague-Dawley and LE rats (Table 7), although the two strains of rats showed similar sensitivity at P14 (Tables 2, 5). Collectively, our data suggest that sensitivity to xylazine is a general feature of laboratory rodents: some stocks/strains may be more susceptible; of note, all rodents tested by us and studies mentioned above were wild-type animals. In this context, pigmentation does not seem to alter susceptibility. Last, there does not seem to be any sex predilection for xylazine-induced corneal lesions.

In conclusion, we hereby provide solid evidence that corneal calcification in young rodents is likely mediated by α2-adrenoceptors. Our study is relevant for longitudinal evaluations of intraocular structures by OCT and other means as it highlights the detrimental effects of corneal calcification; we propose a new protocol to enable such longitudinal evaluations including by OCT (Table 8).

**Table 8. Maisonneuve-Rosemont Hospital Protocol for High Caliber OCT Retinal Images in Young (P14) Rats**

1. Anesthesia: Ketamine 60 mg/kg and xylazine 2.5 mg/kg to avoid animal demise and corneal calcification.
2. Pupil dilation: Tropicamide 1% (Mydriacyl; Alcon Laboratories, Inc., Fort Worth, TX USA), one drop in the study eye.
3. Temperature: Keep anesthetized rat pups on warming pads (35–37°C) throughout the experiment and until they regain full consciousness.
4. Corneal hydration: Hydrate the cornea of the tested eye every 12–15 seconds with PBS to allow clear fundus images and crisp OCT scans. A normal rat blinks about five times per minute. Keep the unexamined eye constantly covered with Tear gel (Alcon).
5. Restrained the head gently if rapid breathing interferes with OCT acquisition.
6. Post-OCT precautions: both eyes should be covered with tear gel until rats regain consciousness. Be mindful not to have wood chips (from bedding) attached to their cornea.
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References