Ketorolac Administration Attenuates Retinal Ganglion Cell Death After Axonal Injury

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MA-B and RH-V are joint senior authors.
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Purpose. To assess the neuroprotective effects of ketorolac administration, in solution or delivered from biodegradable microspheres, on the survival of axotomized retinal ganglion cells (RGCs).

Methods. Retinas were treated intravitreally with a single injection of tromethamine ketorolac solution and/or with ketorolac-loaded poly(D,L-lactide-co-glycolide) (PLGA) microspheres. Ketonol results were compared to those obtained with ketorolac solution alone or administered in parallel groups. All retinas were dissected as flat mounts; RGCs were immunodetected with brain-specific homeobox/POU domain protein 3A (Brn3a), and their number was automatically quantified.

Results. The percentage of Brn3aþ RGCs was 36% to 41% in all control groups (ONC with or without BSS or nonloaded microparticles). Ketonol solution administered pre-ONC resulted in 63% survival of RGCs, while simultaneous administration promoted a 53% survival. Ketonol-loaded microspheres were not as efficient as ketorolac solution (43% and 42% of RGC survival pre-ONC or simultaneous, respectively). The combination of ketorolac solution and ketorolac-loaded microspheres did not have an additive effect (54% and 55% survival pre-ONC and simultaneous delivery, respectively).

Conclusions. Treatment with the nonsteroidal anti-inflammatory drug ketorolac delays RGC death triggered by a traumatic axonal insult. Pretreatment seems to elicit a better output than simultaneous administration of ketorolac solution. This may be taken into account when performing procedures resulting in RGC axonal injury.

Keywords: NSAIDS, neuroprotection, PLGA, microspheres, ketorolac, Brn3

The rodent retina is often used to study the toxicity of different treatments or pharmacologic formulations on central nervous system (CNS) neurons. The retina is easily reached for experimental manipulations, and injury to it is less detrimental for the animal than injury to other CNS structures. Thus, retinal populations have been well characterized, and a number of injury models have been developed and characterized for the retina, such as ischemia,1,2 ocular hypertension,3–9 or axotomy.10–15 Optic nerve axotomy is a well-established model to study the neuroprotective effects of a given treatment on the survival of injured CNS neurons.16–22 Optic nerve axons arise from retinal ganglion cells (RGCs), the only efferent neurons of the retina. Upon optic nerve axotomy, either crush or transection, the majority of RGCs die within the first 2 weeks13,23 without concomitant death of other retinal neurons. Thus, optic nerve axotomy is an excellent model to carry out in vivo proof-of-concept assays aimed to test the efficacy of a drug, compound, or drug delivery system with potential neuroprotective efficacy.

It is well known that a common physiological response to injury is an inflammatory response. Inflammation is needed to resolve the insult, but in the nervous system this event has a dual role, exacerbating the neuronal loss or impeding regeneration. Corticosteroids present high anti-inflammatory properties and have been tested in animal models of CNS injury24–29; they are often used in patients with ocular diseases such as optic nerve neuropathy or diabetes.30–32 Besides the side effects of corticosteroids,33–34 it has been reported that these therapeutic agents increase neuronal apoptosis and axonal loss.35–37 In ophthalmology, nonsteroidal anti-inflammatory drugs (NSAIDs) have a modulating effect on ocular inflammation and pain through the prevention of prostaglandin synthesis by nonspecific cyclooxygenase enzyme (COX) inhibition.38 This has prompted exploration of the use of NSAIDs in patients and animal models.39–50 Ketorolac belongs to the group of NSAIDs, being a nonselective inhibitor of the COX enzyme. Cyclooxygenase-1 and COX-2 are expressed by RGCs, among other cells, in the...
rodent retina, and they are upregulated in the retina after ischemia and optic nerve injury. The safety, pharmacokinetics, and anti-inflammatory efficacy of intravitreal administration of ketorolac have been studied in rabbits. However, there are no studies related to the neuroprotective effect of ketorolac after optic nerve axotomy.

Intraocular drug delivery systems (IDDS) have been developed to provide sustained concentrations of therapeutic molecules in the target site during the long term. Depending on the size, they are classified as implants (>1 mm), microparticles (1–1000 μm), and nanoparticles (1–100 nm). Among them, implants and microparticles are able to deliver the drug for months, avoiding repeated administrations. Both drug delivery systems have been loaded with different drugs for the treatment of retinal diseases. The inherent advantages over larger devices are that microsize IDDS can be administered as a conventional injection and that different amounts of particles can be administered depending on patient needs. According to their structure, microparticles are referred to as microcapsules (reservoir) or microspheres (matrix). Biodegradable microspheres release the drug and then undergo degradation at the site of administration. The most frequently used polymers to prepare biodegradable drug delivery devices belong to the group of polyesters, mainly poly(lactic) and poly(glycolic) acid and their derivatives. These biomaterials degrade to metabolic products and are easily eliminated from the body. The use of biodegradable microspheres for intraocular delivery has become more popular over the last few decades. Biodegradable microspheres have been loaded with different active substances and intended for posterior segment diseases such as diabetic retinopathy, uveitis, glaucoma, macular edema, or age-related macular degeneration. Once in contact with aqueous media, an initial release due to the penetration of water on the matrix pores is typically observed. After that, the controlled release is governed by a combination of polymer diffusion and erosion due to hydrolysis of ester bonds. These long-acting injectable drug formulations represent an effective alternative to repeated intraocular injections.

The question arises as to the potential advantage of using ketorolac in solution as a single injection or delivered in low amounts from a biodegradable drug delivery system (microspheres) in an acute injury model of axonal trauma. In the present work we have tested the effect of an intravitreal solution of ketorolac and/or delivered from poly(D,L-lactide-co-glycolide) (PLGA) microspheres on RGC survival after optic nerve crush in rats. The two treatments have been evaluated after administration simultaneous with the optic injury or before the injury.

**Materials and Methods**

**Materials**

Ketorolac was supplied by Chemos GmbH (Regentstauf, Germany). Poly(D,L-lactide-co-glycolide) (PLGA) 85:15 (MWt 87,000 g/mol, inherent viscosity = 0.62 dL/g in CHCl₃) was purchased from Lactel (Durect Corporation, Burlington Division, Pelham, UK). Polyvinyl alcohol 67,000 g/mol (PVA) was supplied by Chemos GmbH (Regentstauf, Germany). Tetrahydrofuran, HPLC grade (THF; Romil, Teknokroma, Barcelona, Spain), and ammonium dihydrogen-phosphate crystaline extrapur (NH₄H₂PO₄; Merck KGaA) were used to prepare the mobile phase in HPLC determinations.

**Microsphere Preparation**

Microspheres (MP) were prepared according to the O/W emulsion solvent extraction-evaporation technique. Briefly, 400 mg PLGA were dissolved in 1400 μL of a mixture of methylene and acetonitrile (3:1). After dissolution, 60 mg ketorolac were suspended in the organic solution by ultrasonication in a water/ice bath for 5 minutes (Transsonic 460; Elm, Singen, Germany). Then the suspension was gently sonicated at low temperature (Sonicator XL; Heat Systems, Inc., Farmingdale, NY, USA) for 1 minute. This prepared organic phase was emulsified with 5 mL PVA MilliQ water solution (1% wt/vol). Emulsification was performed in a homogenizer (Polytron PT 3000, RECO; Kinematica GmbH, Lucerna, Switzerland) at 9500 rpm for 2 minutes. Subsequently, 10 mL PVA solution (0.1%) was added to the emulsion and kept at the same homogenization conditions for an extra minute. The formed emulsion was finally poured onto 90 mL aqueous PVA solution (0.1%) and maintained under agitation for 3 hours to allow the evaporation of the organic solvents. Once formed, MP were washed with MilliQ water to eliminate PVA and sieved according to their particle size (40, 10, and 1 μm). The mature MP were then frozen and freeze-dried. Lyophilized MP were kept at −20°C under dry conditions until use.

**Microsphere Characterization**

The production yield percentage (PY%) of each batch was calculated from the following equation:

\[
PY(\%) = \frac{M_r(\text{microspheres})}{M_r(\text{PLGA}) + M_r(K)} \times 100
\]

where \( M_r \) is the mass of each component in the formulation.

Morphologic evaluation of MP was performed by scanning electron microscopy (SEM) (JSM-6335F; JEOL, Tokyo, Japan).

**Encapsulation Efficiency**

The content of the encapsulated anti-inflammatory agent in the MP was determined as follows: Briefly, an amount (10 mg) of ketorolac-loaded MP was placed in methylene chloride (5 mL). After dissolution of the PLGA, the drug was extracted with 12 mL ethanol that promoted polymer precipitation. After vortex mixing, the samples were centrifuged at 5000 rpm for 5 minutes and the ethanolic supernatant was recovered and filtered (0.45 μm). The ketorolac content in ethanol was quantified by HPLC as described below.

**In Vitro Release Studies**

Release experiments were performed by suspending an amount (10 mg) of MP in 1.5 mL phosphate buffer saline (PBS). Then the samples were placed in a water shaker bath with a constant agitation of 100 rpm at 37°C (Clifton Shaking Bath NE5; Nickel Electro Ltd., Avon, UK). At defined time intervals (1 hour, 24 hours, 3 days, and once a week until the end of the assay, 42 days), MP suspensions were centrifuged at 5000 rpm for 5 minutes, and the supernatants were removed and replaced by the same volume of fresh medium to continue the release study. After filtration (0.45 μm), the concentration of the released ketorolac from the MP was measured by HPLC as described below.

**Ketorolac Quantification by High-Performance Liquid Chromatography (HPLC)**

Ketorolac HPLC quantification was performed according to the 1755 monograph of the European Pharmacopoeia 8.5 (2015). The
Table 1. Experimental Design

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Lesion to Optic Nerve: Toxicity Assay</th>
<th>ONC and Treatment</th>
<th>Treatment → 7 d Later ONC</th>
<th>ONC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS, vehicle</td>
<td>Analysis at 14 d</td>
<td>7 d therapy and lesion</td>
<td>7 d lesion</td>
<td>7 d lesion</td>
</tr>
<tr>
<td>Ketorolac solution, KET-SOL</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Nonloaded microspheres, NL-MP</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Ketorolac microspheres, KET-MP</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Ketorolac microspheres + ketorolac solution, KET-MP + KET-SOL</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6* (naive)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number of retinas analyzed per group.

Table 2. Ketorolac Dose in the Different Formulations Tested in This Work

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Total Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>KET-SOL</td>
<td>84.35 μg ketorolac tromethamine: equivalent to 57.15 μg ketorolac</td>
</tr>
<tr>
<td>KET-MP</td>
<td>57.15 μg ketorolac</td>
</tr>
<tr>
<td>KET-SOL + KET-MP</td>
<td>114.3 μg ketorolac: 84.35 μg ketorolac tromethamine + 57.15 μg ketorolac in MP</td>
</tr>
</tbody>
</table>

Analysis was carried out on a Waters 717 Plus system composed of 600E pump, an autosampler Waters 717 plus (Waters, Barcelona, Spain). The reverse-phase liquid chromatography analyses were performed with a Mediterranean Sea 18 column (15 cm × 0.4 mm, 5-μm particle size; Teknokroma) preceded by a guard cartridge SEA 18 (100 × 4.0 mm; Teknokroma). The column was maintained at 40°C using a heating column system (Waters TCM/MM). The mobile phase was a mixture of an aqueous solution of ammonium dihydrogen-phosphate 0.05 M (pH 3): tetrahydrofurane (70:30, vol/vol). The flow rate was set at 1 mL/min, and the absorbance was monitored at 313 nm (Waters 486 UV/Vis detector). The column was eluted over minutes and peak areas were determined with the Empower Logix software (Waters, Milford, MA, USA). The concentration of ketorolac was calculated from a calibration curve.

Animal Handling, Anesthesia, and Analgesia

Two-month-old female albino Sprague-Dawley (180–220 g body weight) rats were obtained from the University of Murcia breeding colony. All experimental procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Union guidelines for the use of animals in research and were approved by the Ethical and Animal Studies Committee of the University of Murcia (Spain).

For anesthesia, a mixture of xylazine (10 mg/kg body weight; Rompun; Bayer, Kiel, Germany) and ketamine (60 mg/kg body weight; Ketolar; Pfizer, Alcobendas, Madrid, Spain) was used intraperitoneally (IP). After surgery, an ointment containing tobramycin (Tobrex; Alcon S.A., Barcelona, Spain) was applied on the cornea to prevent its desiccation. Rats were given oral analgesia (Buprex; Iupurephine 0.3 mg/mL; Schering-Plough, Madrid, Spain) at 0.5 mg/kg (prepared in strawberry-flavored gelatin) the day of surgery and during the next 3 days.

All animals were euthanized with an IP injection of an overdose of pentobarbital (Dolethal, Vetoquinol; Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain).

Experimental Design

Animals were divided into several groups (Table 1). To assess whether ketorolac protected RGCs from axotomy-induced death, retinas were intravitreally treated with a single injection of ketorolac-tromethamine solution and/or with ketorolac-loaded MP. Two experimental designs were carried out: intravitreal administration 1 week before the ON lesion and intravitreal administration right after the ON lesion. In either case, all animals were euthanized 7 days after the optic nerve (ON) injury. Nonloaded MP or ketorolac vehicle (balanced salt solution, BSS) was administered to animals subjected to the same procedures as the animals treated with ketorolac. As negative control of the RGC population, naive animals were used, while the injury control (positive) was a group of animals undergoing optic nerve crush (ONC) without treatment.

To investigate the possible toxic effect of ketorolac and MP, three groups of animals were used: intravitreal injection of ketorolac-tromethamine solution, intravitreal injection of non-loaded MP, and intravitreal injection of ketorolac-loaded MP. Rats were euthanized 1–4 days later to match the longest time of treatment in the injured groups.

Surgery

For ONC, the left ON was intraorbitally crushed at 0.5 mm from the optic disc following standard procedures in our laboratory.12,14,20,62 Right after the injury, the eye fundus was checked to verify that the retinal blood supply was intact. Animals were euthanized 7 days later.

Animals received an intravitreal injection (5 μL) of either vehicle (BSS), ketorolac-tromethamine solution (16.87 mg/mL corresponding to 57.15 μg ketorolac base), nonloaded MP, ketorolac-loaded MP (10% wt/vol), or simultaneous ketorolac-loaded MP and ketorolac solution. For the treatments, an initial suspension of MP in BSS (10% wt/vol) was prepared and briefly vortexed immediately before each injection to ensure a uniform dispersion of MP in the injected fluid. Five microliters of each sample was then injected into the vitreous using a Hamilton Syringe (Sigma Aldrich, Madrid, Spain) with a 30-gauge needle. Rats received 0.5 mg MP containing 57.15 μg ketorolac and the same dose in solution (Table 2).

Retinal Dissection and RGC Immunodetection

Animals were perfused transcardially with 4% paraformaldehyde in phosphate buffer after a saline rinse. Eyes were enucleated and all retinas dissected as flat mounts as previously described.12,14,15,63,64 To identify RGCs, brain-specific homeobox/POU domain protein 3A (Brn3a) was immunodetected in all retinas as
previously reported\textsuperscript{12,14,15,63,64} using goat-anti Brn3a (C-20) (1:750; Santa Cruz Biotechnologies, Heidelberg, Germany) and as secondary detection donkey anti-goat IgG(H+L)-Alexa 594 (1:500; Molecular Probes, Life Technologies, Madrid, Spain).

**Image Acquisition**

All retinas were photographed with an epifluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany) equipped with a computer-driven motorized stage (ProScan H128 Series; Prior Scientific Instruments, Cambridge, UK), controlled by the Image Pro Plus software (IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA) as previously described.\textsuperscript{12,14,15} To make reconstructions of retinal whole mounts, retinal multiframe acquisitions were taken in a raster scan pattern. The 154 frames/retina were combined automatically into a single tiled high-resolution photomontage using IPP for Windows.

**Automated Quantification of RGCs and Isodensity Maps**

Brn3a\textsuperscript{+}RGCs were automatically quantified and their topographical distribution assessed by isodensity maps as reported before.\textsuperscript{12,14,15,63,64}

**Statistical Analysis**

Data were analyzed with SigmaStat for Windows version 3.11 (Systat Software, Inc., Richmond, CA, USA). Differences were considered significant when $P \leq 0.05$, and tests are detailed in Results.

**RESULTS**

**Microsphere Characterization**

The MP prepared by the O/W solvent evaporation technique were spherical and exhibited smooth surfaces with the
absence of drug crystals on the MP surface (Figs. 1A, 1B). The encapsulation method led to a production yield of 47.6%. The 10- to 40-μm size fraction, with a mean particle size of 26.99 ± 3.46 μm, was selected for intravitreal injection in animals (Fig. 1C). The ketorolac loading in the MP resulted in 114.3 μg ketorolac/mg MP (encapsulation efficiency of 87.69% ± 1.92%).

According to the in vitro release profile, MP were able to release the active molecule for 30 days in a controlled fashion (Fig. 1D). During the first 24 hours, a burst release effect was observed (47.25 ± 0.45 μg ketorolac/mg MP) that resulted in 44% ± 0.39% of the encapsulated drug. After that, ketorolac was delivered for 30 days according to a zero order release kinetic (release rate: 2.338 μg ketorolac/mg MP/day). At the end of the release study (42 days), MP had delivered almost all the encapsulated drug.

Toxicity Assay

First we studied the fate of MP after intravitreal administration. Microspheres remained in the vitreous during the time of the study (7 or 14 days) (Fig. 2). They were brushed away when cleaning the vitreous; thus, the retina in Figure 2 was photographed before vitreous cleansing. Fourteen days after administration, many MP were still observed across the retina. Most of them were found on the ventral retina, where they sunk after being injected through the dorsotemporal quadrant (red circle in Fig. 2).

In a second round of experiments, the toxicity on RGCs of all formulations was tested. Thus, ketorolac solution (KET-SOL), nonloaded microspheres (NL-MP), or ketorolac-loaded microspheres (KET-MP) were administered to uninjured retinas (Table 3) and analyzed 14 days later to match the longest treatment time (see Table 1).

In all groups, the mean number of RGCs did not differ from the number of RGCs quantified in naive animals (Table 3). This is also observed in the isodensity maps shown in Figures 3A through 3E.

Effect of Ketorolac Treatment on RGC Survival After ONC

As previously described, in order to evaluate the neuroprotective effect of ketorolac, the molecule was delivered in two formulations: in solution, dissolved in BSS, or in MP for a slow and continuous release (see above). In a series of experiments, each formulation and the respective controls were administered 1 week before the injury. In a second series, the treatment was administered simultaneously with the lesion. Quantitative results are shown in Table 4 and Figure 4.

As control of the RGC population, naive intact retinas were used because it has been described as a contralateral response to unilateral injury.16 However, in this work the number of retinal ganglion cell survival by ketorolac.
RGCs in the right eyes, contralateral to the lesion and treatment, was within normality. There was no difference between ONC alone, ONC and vehicle, and NL-MP treatment (prelesion or simultaneous). In these groups the percentage of surviving RGCs was 36% to 41% (Fig. 4).

In all groups in which ketorolac was administered in solution, there was a significantly higher number of RGCs compared to the values in all control groups. The best neuroprotection was observed after intravitreal injection of KET-SOL 1 week before the injury, where 63% of the RGCs were still present at 7 days after the injury. Retinal ganglion cell survival was similar between KET-SOL injected at the time of the injury and KET-MP either simultaneous or preinjury (53% or 55% of survival, respectively). Ketorolac delivered by MP alone (KET-MP) was not as efficient as in solution (42% simultaneous with ONC, 43% pre-ONC); and although the number of surviving RGCs was higher than after ONC alone, it did not reach statistical significance. However, compared to NL-MP, KET-MP delayed RGC death in significant numbers.

In Figure 3 are shown representative isodensity maps from each group. These images support the quantitative data and document that ketorolac protection occurs across the retina, in spite of the fact that ketorolac solution was injected in the dorso temporal quadrant.

**DISCUSSION**

Here we show for the first time that a single administration of ketorolac, a NSAID, delays neuronal death in an acute model of axonal injury. The elicited neuroprotection is better when the drug is administered before the injury and when it is delivered...
in aqueous solution. Furthermore, as has been reported in rabbits, intravitreal administration of ketorolac did not show toxic effects on RGC survival for the period of study of 1 week.

Corticoids are widely used to treat injuries of the CNS, and also as therapy in patients with ocular diseases. It has been reported that cortisol administered intravitreally in rats neuroprotects RGCs after axotomy. The authors showed that 50% of RGCs survive at 15 days post axotomy when treated, while without treatment only 17% of the original RGC population is.

The use of NSAIDs as neuroprotectors has gained a lot of interest during the last decade. The neuroprotective effect of several NSAIDs has been demonstrated in different animal models. Their effect is mainly based on the suppression of microglial activation occurring by different pathways. The two isoforms of cyclooxygenase (COX), the constitutive (COX-1) and inducible (COX-2) forms responsible for tissue prostaglandin synthesis, are clearly involved in the inflammatory process. Subsequently, their inhibition has been reported to suppress toxic actions of microglia.

In this work, neither the initial drug release from MP (47.25 µg in the first 24 hours) nor the continuous and slow release of ketorolac from the MP (1.169 µg ketorolac/day released from 0.5 mg of MP) observed in vitro promoted a moderate rescue of RGCs; it did not show an additive effect with ketorolac in solution, indicating that low ketorolac doses are not enough to protect RGCs in a chronic injury. In this study, a bolus ketorolac concentration of 57.15 µg was needed to observe a neuroprotective effect in RGC cells in this animal model.

All these findings suggest that the injury produced by axotomy probably requires higher doses of the active

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**TABLE 4. Ketorolac Administration Attenuates RGC Death After ONC**

<table>
<thead>
<tr>
<th></th>
<th>No. ± SD of RGCs</th>
<th>7 d After ONC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>Right retinas: contralateral to injured ones</td>
<td>80,193 ± 1,066</td>
<td>Injured</td>
<td>30,543 ± 6,742</td>
</tr>
<tr>
<td></td>
<td>Vehicle: BSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Treatment—ONC: 14 d therapy</td>
<td>32,333 ± 6,130</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONC−treatment</td>
<td>Treatment—ONC: 7 d therapy</td>
<td>29,022 ± 4,808</td>
<td></td>
<td>29,772 ± 3,686</td>
</tr>
<tr>
<td>NL-MP</td>
<td>Treatment—ONC: 14 d therapy</td>
<td>50,173 ± 3,260</td>
<td></td>
<td>43,249 ± 4,460</td>
</tr>
<tr>
<td>KET-SOL</td>
<td>Treatment—ONC: 14 d therapy</td>
<td>34,142 ± 2,711</td>
<td></td>
<td>33,568 ± 1,646</td>
</tr>
<tr>
<td>KETMP−KET-SOL</td>
<td>Treatment—ONC: 14 d therapy</td>
<td>43,915 ± 5,140</td>
<td></td>
<td>42,412 ± 5,028</td>
</tr>
</tbody>
</table>

**FIGURE 4.** Ketorolac delays RGC death after optic nerve crush. Bar graph showing the percentage of surviving RGCs in all the analyzed groups compared to naive retinas (100%). n.s., not significant. Statistical analysis: one-way ANOVA all pairwise multiple comparison procedures.
Retinal Ganglion Cell Survival by Ketorolac

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