Long-term Fluorometholone Topical Use Induces Ganglion Cell Damage in Rats Analyzed With Optical Coherence Tomography


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ABSTRACT

To determine the toxic effects of long-term topical usage of fluorometholone (FLM) on ganglion cells using a direct in vivo retinopathological Brown Norway (BN) rat model. The BN rat retinal model was investigated with a minimum of 3 rats and a maximum of 4 rats per group. Rats received vehicle and 0.02% FLM suspension via topical administration 3 times a day for 28 days. The fundus images and retinal vessels were detected on days 1, 14, and 28 using Micron III retinal imaging microscope and fundus fluorescein angiography (FFA). For retinal structures, spectral-domain optical coherence tomography (SD-OCT) images were taken after FFA on days 1, 14, and 28 using an SD-OCT Imaging System. For retinal function, electrical signal transduction of photoreceptors and bipolar cells was determined by electroretinographic (ERG) recording on days 1 and 28 and IOP detection. At the end of the experiment on day 28, immunohistochemistry and TUNEL assay were performed to investigate apoptosis in ganglion cells. Total retina and nerve fiber layer (NFL) to the inner plexiform layer (IPL) were significantly thinner following 28 days of FLM treatment. Hematoxylin and eosin stain showed that there were NFL and ganglion cell layer deformations in the FLM group. With FLM treatment, TUNEL assay showed approximately a 4.68-fold increase in apoptotic cells. Moreover, FLM decreased ERG b-wave amplitude by about 56%. Using ophthalmofundoscopy devices, after 28 days of topical administration, FLM decreased NFL-IPL and total retina thickness. This suggests that long-term FLM induces adverse effects with respect to ganglion cell apoptosis.

Key words: neurotoxicology, toxicity; chronic; safety evaluation, ocular toxicity; systems toxicology

Glaucoma is the second leading cause of irreversible blindness that affects over 60 million people worldwide, a number that could rise to 80 million in 2020 (Quigley et al., 2006). Progressive degeneration of retinal ganglion cells (RGCs) represents a group of inner retina and axonal ocular neuropathies. RGC condition, which is a vital biomarker in glaucoma, plays an important role...
in visual signal transduction through the optic nerve into the brain (Ju et al., 2005). Primary glaucoma can be classified into 2 categories: primary open- and closed-angle glaucoma (Weinreb et al., 2014). In open-angle glaucoma, the resistance of aqueous outflow pathway through the meshwork increases; conversely, obstruction of the drainage pathway by the iris is related to closed-angle glaucoma. Both types of primary glaucoma can cause intraocular pressure (IOP) abnormalities (Quigley et al., 1981) and lead to mechanical stress on the posterior part of the eye and especially RGC somas (Mac Nair et al., 2014). Hyper IOP may result in lamina cribrosa (LC) compression, remodeling, and deformation of axonal transport to RGCs (Shields, 2008). Meanwhile, neural degeneration, such as RGC impairment, atrophy (Williams et al., 2013), somatic apoptosis (Mac Nair et al., 2014; Wang et al., 2013), and loss of cells in the LC, often accompanies an IOP increase. The symptoms of primary optical neural pathologies can cause secondary neural degeneration of other retinal cells and neurons in the visual pathway by changing their micro-environment and increasing the possibility of damage (Almasieh et al., 2012).

There are 3 layers in the inner retina layer, including the nerve fiber layer (NFL), RGC layer, and inner plexiform layer (IPL). The end of RGC axons is located at synapses that link them to bipolar cells in the IPL (Kim et al., 2012). The morphology of these layers is affected during chronic IOP elevation. Moreover, degeneration of RGCs results in vision loss, but the biological basis of this process is poorly understood.

After ocular surgery, patients experience side-effects, such as ocular surface inflammatory damage and dry-eye diseases, which cause discomfort symptoms, visual interruption, and tear-film disorders. By inhibiting phospholipase A2 and prostaglandin release, glucocorticosteroids (GCSs) have potential immunosuppressive and inflammatory effects. GCSs or nonsteroidal anti-inflammatory drugs (NSAIDs) are ordinarily used systemically or topically to prevent inflammation after ocular surgeries (Jee et al., 2014), such as post-cataract and poststrabismus surgery, chronic diabetic macular edema (Cunha-Vaz et al., 2014), conjunctivitis, optic neuritis, and uveitis (Malik et al., 2010), because of the related postsurgical ocular inflammation. One type of GCS is fluorometholone (21-deoxy-9-fluoro-6-methyl prednisolone), which is commonly used after ocular surgery to prevent oxidative stress; however, it induces toxic effects, like steroid-related glaucoma (Yang et al., 2014), along with an IOP increase. Nevertheless, compared with dexamethasone (DEX), fluorometholone has less of an effect on IOP. Studies have shown that fluorometholone may remarkably induce dose-dependent ocular hypertensive responses in some patients, especially in children with ≥25 mmHg IOP after receiving 4 daily applications of fluorometholone (Yang et al., 2014) and there is concern as to whether it is safe for postsurgical anti-inflammatory treatment. The IOP elevation caused by topical GCS application is sometimes transient and the consequences are often alleviated after GCS withdrawal. However, some patients still suffer from toxic ocular inflammatory effects after surgery (Sihota et al., 2008). Thus, high IOP may induce RGC-related apoptosis in the posterior part of retina and increase the chances of visual disturbance. Today, more evidence into the adverse effects are needed to suggest the adverse effects of topical usage of fluorometholone.

To investigate the adverse effects of fluorometholone eye drops on the posterior eye, we conducted a long-term animal experiment and examined the pathological results. In our study, we demonstrated that endpoint RGC apoptosis is caused by long-term fluorometholone eye drop application and induces ganglion cell apoptosis in a Brown Norway (BN) rat animal model. With advanced in vivo retinal detection devices, we first obtained high quality and excellent resolution rat retinal data to elucidate the toxicity of long-term topical fluorometholone administration.

**MATERIALS AND METHODS**

**Chemicals.** Atropine sulfate 0.125% (KINTROPINE) was purchased from Sympac-Kindom Pharmaceutical Co., Ltd. (Taipei, Taiwan), and 0.02% Fluorometholone (FOXON) was purchased from Winston Medical Supply Co., Ltd. (Taiwan, Taiwan).

**Animal application.** BN rats (250–300 g body weight) were purchased from National Laboratory Animal Center (Taipei, Taiwan) and kept for 7 days in the housing room under controlled conditions consisting of 12:12-h light/dark cycles, 25 ± 1°C temperature, and 39–45% relative humidity; water and food were available ad libitum. All experimental procedures involving the use of animals complied with the Association for Research in Vision and Ophthalmology statements for the use of animals in ophthalmic and vision experimental research, and the animal use protocol listed below was reviewed and approved by the Institutional Animal Care and Use Committee of Taipei Medical University (approval number: LAC-2014-0194). Untreated rats kept in darkness served as controls (n = 4 for each group); the other rats received 28 days of topical fluorometholone administration. The control group received vehicle (normal saline) eye drop treatment 3 times a day for 28 days; the experimental group received fluorometholone eye drop treatment 3 times a day for 28 days.

**Fundus angiography analysis.** We used a Micron III retinal imaging microscope (Phoenix Research Laboratories, Pleasanton, California) to study pathological changes in the fundus. BN rats were anesthetized with an intramuscular injection of ketamine (80 mg kg⁻¹) and xylazine (10 mg kg⁻¹). Before testing, the pupil of the right eye of each rat was dilated with 0.125% atropine sulfate. The vibrissae were trimmed with scissors to avoid them from blurring the photograph. BN rats were held on the microscope platform on their sides. Image focusing was achieved by moving the rats and the platform. Rat position and angle were altered to study different parts of the fundus. The eyes were covered with 2% Methocel gel (OmniVision, SA, Neuhausen, Switzerland) and the fundus images and fluorescein angiography images were captured with a Micron III retinal imaging microscope (Phoenix Research Laboratories). As for fluorescein angiography, 10% sodium fluorescein was injected intravenously and the images were captured immediately after 30s. The injected sodium fluorescein was completely excreted after 24 h. The collected data were quantified using Image-Pro software (Media Cybernetics, Bethesda, Maryland) to determine the length and width of retinal vessels.

**Spectral-domain optical coherence tomography imaging.** We used the 830 nm spectral-domain optical coherence tomography (SD-OCT) Imagine System (Phoenix Research Laboratories), which is customized for retinal imaging of small animals, mice, and rats. This system contains an OCT engine, a scan head, and a computer with software to detect and photograph the retina. The SD-OCT engine has a spectrometer that covers 740–920 nm and is combined with a broadband super-luminescent diode with a 3 dB bandwidth of >150 nm. The spectrometer has the ability to run an A-scan rate of 40 000 lines per second and contains 2048
pixels. The region of the scan mode on the retina is more than 1.8 mm on the X and Y axes in rats. The scan length, rotation, and location can be adjusted within the entire scan region. Following manufacturer’s instructions, we placed the camera appropriately and focused on the retina with an 80-degree angle using A-scan mode. After the retina was positioned in the center of the field, OCT data were collected from inner retina images.

Electroretinographic recording. We connected a MP36 4-channel data acquisition system (BIOPAC Systems, Inc., Pershore, UK) to a Photistimulator, model ps33-plus (GRASS technology, Warwick) to build electroretinographic (ERG) recordings. Before testing, BN rats were kept in the dark overnight and prepared for recording under dim red light using light-emitting diode illumination. Pupils were dilated with 0.125% atropine sulfate for 5 min after anesthesia. Lenses were placed over a cover of 2% Methocel gel before recording via DTL fiber electrodes. ERG signals were amplified (DC to 300 Hz) and digitized at 1 kHz with a resolution of 2 µV.

Immunohistochemistry. All BN rats were sacrificed with a ketamine/xylazine overdose; eyes were removed after 28 days of experimental procedures and the anterior part was discarded. To fix the posterior parts and the lens for paraffin sectioning, we used modified Davidson’s fixative (Latendresse et al., 2002) overnight. After dehydration in ethanol and embedding in paraffin, radial 5-µm sections were collected for hematoxylin and eosin (H&E) staining.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay. We used an in situ cell death detection kit (Roche, Mannhem, Germany) to detect apoptotic programmed cell death in whole-mount eye cup sections according to the manufacturer’s instructions. After eye cup enucleation, the posterior parts of the eye were rehydrated and dewaxed. Using Proteinase K (Roche, Germany) for 30 min at 37°C, antigenicity was recovered by enzyme digestion. Tissue samples were exposed to 50 µL of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture containing enzyme solution at 37°C in the dark. After 1 hour incubation, the tissue samples were treated with the Converted POD followed by DAB (Bethyl Laboratories Inc., Montgomery, Texas). Shown in dark brown color, apoptosis was detected under a light microscope (Nikon, Eclipse Ci, Tokyo, Japan) and images of 3 random fields of view at 200× magnification were taken.

IOP measurement. IOP was obtained with a TonoLab-Tonometer (Icare Finland Oy, Espoo, Finland) in the vehicle group and the experimental group after the animals was enough sedation. We brought the tonometer near to the BN rat’s eye and kept it in a horizontal position. After securing the distance between the probe and the cornea of the eye was 1–4 mm (1/8 inch), we made 6 measurements consecutively. Data of IOP (mmHg) were presented as mean ± SD.

Data and statistical analysis. InSight XL software (Phoenix Research Laboratories) was developed by Voxleron LLC and used for SD-OCT data analysis; it is only used for the analysis of retinal OCT images from animal studies. Due to the fact that the retina is made of different neuronal layers, the core aspect of this analysis is to measure retinal neuron layer thickness. InSight XL has the ability to detect 2–4 layers automatically and edit/add/delete layers manually. In order to investigate fluorometholone induced retinal damage, we first defined 3 layers to elucidate in this study. The first layer was called the upper layer and included the retinal NFL, ganglion cell layer (GCL), and IPL. The second layer was called the middle layers and included the inner nuclear layer, outer plexiform layer, outer nuclear layer, and outer limiting membrane. The third layer was called the lower layer and included the inner segment/outer segment (IS/OS) of photoreceptors and retinal pigment epithelium (RPE). After SD-OCT examination, the OCT data were inputted into the InSight XL software and the thickness of the 3 layers was measured. All data are expressed as the mean ± SD from at least 3 independent experiments (n ≥ 3).

Statistically significant differences between groups were determined using 1-way analysis of variance. A p-value < .05 was considered statistically significant.

RESULTS

Long-term Fluorometholone Eye Drops Exerted No Morphology Deformation on BN Rat Retinas via Fundus and Fluorescein Angiography

To evaluate the toxicity of topical administration of steroids on the eye, we conducted a series of long-term ocular examinations (Fig. 1) using advanced only-for-mouse/rat ophthalmoscopy devices. In this study, with 28 days of 3 times daily fluorometholone drops, we first used fundus and fluorescein angiography to detect the pathological effects on the retina of BN rats. From day 0 to 28, the fundus images showed no retinal vessel morphology deformation or defacement (Fig. 2A). However, significant white turbidity occurred on day 14 and 28 in the fluorometholone-treated group and constrained observation of vessel outlines. Using fluorescein angiography, we took pictures after sodium fluorescein dye injection. No pathological changes in either choroidal or retinal vessels were found, but mild diffusion of fluorescein from the vessels on day 28 was seen in the fluorometholone-treated group (Fig. 2B). In addition, fluorometholone treatment did not affect the vessel length of the retina. In the vehicle-treated group, vessel width increased to 106.50 ± 6.66% on day 14 and 119.48 ± 6.28% on day 28. Meanwhile, vessel width increased to 103.59 ± 8.45% on day 14 and 111.08 ± 10.40% on day 28 in the fluorometholone treated
However, there was no significant difference between both groups with respect to vessel length or width. These data demonstrate that long-term fluorometholone treatment can affect vision articulation and retinal vessel growth.

**Fluorometholone Decreased NFL to IPL Thickness via SD-OCT Examination and Increased the IOP Level**

SD-OCT scan technology allows us to further investigate the structure and pathophysiology of the rat retina in detail beyond the detection of the fundus via fluorescein angiography. In this study, we defined the total retinal as 3 layers. From the inner to the outer retina we defined the following layers: the NFL-IPL layer, IPL-IS/OS layer, and IS/OS-RPE layer (Fig. 3A). Total retina thicknesses were 225.50 ± 1.50 μm on day 0, 223.28 ± 1.88 μm on day 14, and 218.01 ± 5.07 μm on day 28, respectively, in the vehicle treated group; and 206.87 ± 4.45 μm on day 0, 199.94 ± 9.69 μm on day 14, and 196.85 ± 4.86 μm on day 28 in the fluorometholone-treated group. There were significant differences on day 14 and 28 between the vehicle and fluorometholone group (Fig. 3B), but no significant effects in the fluorometholone group from day 0 to 28. NFL-IPL layer thicknesses were 79.35 ± 5.66 μm on day 0, 78.67 ± 3.85 μm on day 14, and 80.88 ± 4.24 μm on day 28 in the vehicle treated group; and 70.11 ± 2.65 μm on day 0, 64.31 ± 1.26 μm on day 14, and 59.58 ± 1.43 μm on day 28 in the fluorometholone-treated group. There were significant differences on day 28 between the vehicle and fluorometholone group. Moreover, compared with day 0, there was a significant difference on day 28 in the fluorometholone group (Fig. 3E). However, there was no significant difference in either the IPL-IS/OS layer or the IS/OS-RPE layer (Fig. 3C and D). Furthermore, we conducted an additional 28-day IOP test on BN rats. We divided these BN rats into 2 groups: the control group and the experimental group. The control
group received vehicle eye drop treatment 3 times a day for 28 days; the experimental group received fluorometholone eye drop treatment 3 times a day for 28 days. On day 0, 5, 10, 14, 21, and 28, we measured the IOP with TonoLab-Tonometer. IOP were 11.92 ± 1.76 mmHg on day 0, 12.00 ± 1.23 mmHg on day 5, 12.39 ± 1.46 mmHg on day 10, 11.50 ± 0.61 mmHg on day 14, 12.60 ± 1.02 mmHg on day 21, and 11.17 ± 0.24 mmHg on day 28, respectively, in the vehicle treated group; and

FIG. 3. SD-OCT examination showed in vivo retina alterations in the NFL to the IPL in BN rats. A. At right is the definition of the 3 layers used in our SD-OCT scan: the first layer (NFL to the IPL), second layer (IPL to the IS/OS), and third layer (IS/OS to the RPE). SD-OCT scan data were taken from the same rats after fundus and fluorescein angiography and show the retina in detail in vehicle and fluorometholone-treated rats on days 0, 14, and 28. Data quantification of both groups was performed on day 0, 14, and 28. The thickness of the total retina and the 3 layers are shown in (B–E). IOP elevation profile in BN rats was shown in (F). Data are expressed as mean ± SE from 3 or 4 independent experiments. *p < .05 and **p < .01 compared with the vehicle treated groups on the same day, #p < .05 compared with the fluorometholone-treated group on day 0.
12.29 ± 0.78 mmHg on day 0, 13.87 ± 0.25 mmHg on day 5, 14.68 ± 0.60 mmHg on day 10, 14.20 ± 0.16 mmHg on day 14, 14.78 ± 0.74 mmHg on day 21, and 12.83 ± 0.62 mmHg on day 28 in the fluorometholone-treated group (Fig. 3F). We found that there were significant differences on days 10, 14, 21, and 28 between the vehicle and fluorometholone group. These data suggested that long-term fluorometholone application exerts toxic IOP elevation effects on the NFL-IPL layer and causes a progressive increase in thickness.

Long-term Fluorometholone Topical Administration Induces GCL Apoptosis

Following 28 days of fluorometholone treatment, there were NFL and GCL morphological alterations in the rat retina seen by H&E stain (Fig. 4A). In the fluorometholone-treated group, fluorometholone diminished NFL structures that caused GCL shrinking comparing with the vehicle group. Moreover, using TUNEL staining, there were large numbers of apoptotic cells in the GCL. TUNEL staining of BN rat retina slices showed 54.67 ± 2.49 and 11.67 ± 2.49 apoptotic cells in the fluorometholone and vehicle groups, respectively (Fig. 4B). Additionally, we further investigated the pathology of lens. Comparing to the vehicle group, we found there was swollen fibers and extensive vacuolization in the lens cortex in fluorometholone group (Fig. 4C). These data suggested that long-term fluorometholone application leads to GCL apoptosis and cataract-like syndromes.

Flurometholone Decreased b-Wave Amplitude on BN Rat ERG

Retinal neuron cell function is correlated with visual signal transduction. Thus, to investigate the influence of long-term fluorometholone application on neuron cells in the rat retina, we conducted an ERG experiment (Fig. 5). After 24 h of dark adaptation, the vehicle-treated group on day 28 showed 24.55 ± 3.44 ms of implicit time and 183.60 ± 65.70 µV of amplitude in a-wave; and 35.00 ± 26.00 ms of implicit time in b-wave. Conversely, the fluorometholone-treated group on day 28 showed 24.55 ± 5.45 ms of implicit time and 183.60 ± 65.70 µV of amplitude in a-wave; 39.15 ± 4.15 ms of implicit time and 219.50 ± 49.50 µV of amplitude in b-wave. There was a significant difference in b-wave amplitude on day 28 between groups.

DISCUSSION

Flurometholone, a GCS, is often used after ocular surgery to attenuate post-operation inflammation and to treat other ophthalmic diseases. Other widespread use of topical NSAIDs and steroid drugs, such as DEX, loteprednol etabonate (LOT), and rimexolone (RIM) are used as anti-inflammatories because of their treatment efficacy. The drugs listed above can impart anti-inflammatory effects and might be a risk factor for GCS-induced ocular hypertension causing a glaucoma-like syndrome. Abnormal IOP is thought to be the greatest risk factor for glaucoma and high IOP can cause progressive and irreversible damage to RGCs and the optic nerve head (Casola et al., 2015). Moreover, glaucoma, which is a progressive and chronic disease, has become the second leading cause of visual impairment and the number of patients suffering from this disease is increasing. However, descriptions and quantification of glaucoma-like syndrome in an animal model have not been widely investigated. Hence, we used advanced only-for-mouse/rat ophthalmofundoscopy devices to investigate the toxicity of long-term fluorometholone eye drop-induced glaucoma-like disease.

Studies have reported that oral GCS usage can cause cataract in 6–39% of patients in a series of randomized and controlled trials (Ponticelli et al., 1997). There are plenty of pathologic mechanisms involved in steroid-induced cataracts, such as the accumulation and agglutination of lens proteins and fibers, glucose in the aqueous and plasma, and blockage of Na+/K+ pump activity (Carnahan et al., 2000). As for eye drops or oral fluorometholone application, there are a few references that indicate the adverse effects of steroid-induced cataract or other conditions, including blurred vision. In this study, we found there was obvious white turbidity in the vitreous from day 14 to 28 in the fluorometholone-treated group, but it remained clear in the vehicle group. After receiving 28 days of fluorometholone eye drops, cataract-like syndromes might occur. Studies showed that 36 days of chronic 0.1% DEX instillation on Sprague-Dawley rat eyes induced IOP elevation by 42.7%. Significant hypotrophy of the thickness of the inner retina and GCL cell density due to the decreased volume of the inner retina and GCL was noted. Moreover, compared which normal rats, rats with SIOH had much thicker TM (Razali et al., 2014). Morphological changes in the TM causing SIOH are thought to be associated with the protein expression of type IV collagen and fibronectin in the extracellular matrix during remodeling and cytoskeleton structure changing (Tektas et al., 2010). Many studies have used different parameters to elucidate pathophysiological GCL morphology, including the number of labeled ganglion cells and the thickness of the inner retina and GCL both in vitro and in vivo (Sun et al., 2011). Our animal model showed that fluorometholone caused total retina and NFL-IPL layer thickness reduction on days 14 and 28, which are similar to the effects of DEX.

Further, we wanted to assess the toxic effects of long-term fluorometholone eye drop use on the retina. It is reported that GCL apoptosis is a consequence of retinal oxygen stress in steroid-treated eyes. Also, such retinal oxygen stress reduces the activity of superoxide dismutase, catalase, and glutathione peroxidase (GSSG/GSH) in RGCs in cultures and mouse models (Liu et al., 2007). The relationship between ROS and apoptosis is well known. Based on the literature, we believe that accumulation of ROS exerts toxic effects on the posterior retina and induces ganglion cell apoptosis. In this study, on immunohistochemistry examination, we found framework deformation in theNFL and GCL. Moreover, histological data revealed that the thickness of the NFL in the fluorometholone group was much thinner than in the vehicle group. In addition, after 28 days of fluorometholone treatment, there were a greater number of apoptotic cell as per the TUNEL assay.

The toxicity of fluorometholone also affected the physiological function of retina. Visual signal transduction is related to the vertical and horizontal pathways in layers that are composed of specialized neurons. After light entering the eye
reaches the retina, photoreceptors sense and convert the light into electric signals. Such neural and electric signals are passed to bipolar cells and then integrate into the optic nerve forming axons of ganglion cells (Ragauskas et al., 2014). To evaluate the physiological functions of the signal transduction abilities of photoreceptor and bipolar cells, conventional ERG is often preferred. Thus, to further examine the toxic effects of long-term fluorometholone application, we assessed visual function with conventional ERG. When compared with the contribution of a-waves to the general physiological functions of photoreceptors in the outer retina, the implicit time and the amplitude of b-waves are more important in bipolar neuron cells in the inner retina, which are correlated with signal transduction (Sieving et al., 1994).

There was a recent report that triamcinolone acetonide (TA) significantly increases IOP after intravitreal injection on days 1,
7, 14, 21, and 28 in New Zealand White Rabbits. The authors also indicated that TA might alter ocular systemic metabolism of proteins, sugars, lipids, or anti-oxidants in a time-dependent manner. Hence, the consequences of a change in metabolites might lead to higher levels of fibronectin and mucopolysaccharide deposition in the TM and thereby cause aqueous outflow occlusion. In ocular hypertensive retinal injury, TA prolonged the implicit time and decreased b-wave amplitude, and damaged retinal and optic nerve function (Song et al., 2011).

In conclusion, we found that fluorometholone induces GCL apoptosis and decreases b-wave amplitude following long-term topical treatment in BN rats. Both OCT and ERG were obtained in real-time from alive animals. When compared with intravitreal injection, we chose the topical administration route to evaluate the ocular trauma and decrease other inflammatory factors to elucidate the toxicity of fluorometholone. Herein, we demonstrate the adverse effects of long-term fluorometholone, and suggest that caution should be used when prescribing fluorometholone as a clinical treatment.

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