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Conjunctival expression of the $P2Y_2$ receptor and the effects of 3% diquafosol ophthalmic solution in dogs

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ABSTRACT

Conjunctival epithelial and goblet cell $P2Y_2$ nucleotide receptors regulate ion transport and secretory function. Diquafosol is a $P2Y_2$ purinergic receptor agonist that stimulates secretion of aqueous tear components from conjunctival epithelial cells and secretion of mucin from conjunctival goblet cells. In humans suffering from keratoconjunctivitis sicca (dry eye), topical administration of diquafosol improves corneal epithelial integrity and stabilises the tear film. The aim of the present study was to investigate $P2Y_2$ receptor expression and to determine the effect of topical administration of diquafosol on mucin and aqueous tear production in dogs.

Canine conjunctival $P2Y_2$ receptor expression was evaluated by Western blotting and immunohistochemical analysis. The effect of diquafosol on mucin secretion was evaluated by examining mucin-5 subtype AC (MUC5AC) concentration in tears. The effect of diquafosol on aqueous secretions was evaluated by performing the Schirmer tear test (STT) and phenol red thread test. Expression of the $P2Y_2$ receptor was confirmed in canine bulbar and palpebral conjunctivae and receptors were identified at the conjunctival epithelial and goblet cell surface. Tear MUC5AC concentration significantly increased after administration of 3% diquafosol ophthalmic solution, although neither STT nor phenol red thread test values showed any significant change after diquafosol instillation. Topical ocular administration of 3% diquafosol might improve corneal epithelial disorders in dogs through stabilisation of the tear film, by virtue of an increase in MUC5AC secretion.

Introduction

On the surface of the eye, the tear film consists of several layers; a mucin layer covers the ocular surface, an aqueous layer lies above the mucin layer, and a lipid layer covers the tear film surface (Holly, 1987; Tiffany, 1994). Mucin helps create a smooth refractive corneal surface, lubricates the corneal and conjunctival surfaces, reduces the shear force between the corneal epithelium and the aqueous layer, inhibits microbe adhesion, and prevents drying (Davidson and Kuonen, 2004).

Mucins can be classified as secretory (MUC2, MUC5AC, MUC5B, and MUC7) or membrane-bound (MUC1, MUC4, and MUC16) (Hicks et al., 1997; Watanabe, 2002). Secretory mucins are present in the aqueous layer and play a complex role in ocular surface health, interacting with both the tear film and the ocular surface epithelium (Dilly, 1994). Interestingly, the main component of the human tear film is not the aqueous component, but mucin (Prydal et al., 1992). This alternative view to the trilaminar model is also applicable to the dog tear film.

Decreased tear production and/or alteration in tear composition occurs in several ocular diseases and can result in dryness of the ocular surface, apoptosis of epithelial cells and decreased goblet cell production of mucin. A lack of mucin reduces tear film stability, which may lead to, or aggravate, ocular pathology (Danjo et al., 1998). Canine tears contain secretory and membrane-bound mucins (Hicks et al., 1997). In canine keratoconjunctivitis sicca (KCS), glycosylation, core protein expression, and/or post-translational modification of ocular surface mucins have been shown (Hicks et al., 1998). In particular, the sialic acid content in ocular mucin has been demonstrated to be depleted in dogs affected with KCS (Corfield et al., 2005).

Adenosine triphosphate (ATP) and uridine triphosphate (UTP) are potent mediators that facilitate chloride ion (Cl^-) and mucin secretion from conjunctival epithelial and goblet cells (Jumblatt and Jumblatt, 1998; Hosoya et al., 1999). Both ATP and UTP act via $P2Y_2$ nucleotide receptors, expressed by conjunctival epithelial and goblet cells, that regulate cellular ion transport and secretory functions.

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The human $P2Y_2$ receptor gene is functionally expressed in the conjunctival epithelium and goblet cells, the corneal epithelium, the sebaceous meibomian glands, and ocular surface ductal cells (Cowlen et al., 2003).

Diquafosol is a $P2Y_2$ purinergic receptor agonist. The activity of diquafosol is equivalent to that of UTP, but it is more stable in aqueous solution than ATP or UTP (Pendergast et al., 2001). In a rabbit model, diquafosol has been shown to act on $P2Y_2$ receptors to stimulate water secretion from conjunctival epithelial cells, mucin secretion from conjunctival goblet cells (Li et al., 2001; Murakami et al., 2004) and has been shown to improve corneal epithelial integrity in experimental dry eye disease (Fujihara et al., 2002). In a rat model of dry eye disease, an increase in aqueous tear volume was observed, with recovery of corneal epithelium barrier function (Fujihara et al., 2001). Furthermore, use of diquafosol eye drops in human KCS patients has led to tear film stabilisation and subsequent improvement in tear break-up time, as well as improved fluorescein and rose Bengal vital staining scores (Kamiya et al., 2012; Matsumoto et al., 2012; Nakamura et al., 2012; Takamura et al., 2012).

Expression of the $P2Y_2$ receptor has not previously been evaluated in the canine conjunctiva. If present, it is feasible that use of diquafosol would increase aqueous and mucin secretion, which might help to stabilise the tear film in canine ocular surface disorders. This would be reflected in an increase in tear break-up time, which could lead to improvements in corneal epithelial disorders, such as superficial punctuate keratopathy. The aim of the present study was to examine canine conjunctival $P2Y_2$ receptor expression and assess the effect of topical diquafosol on ocular aqueous and mucin secretion.

Materials and methods

All experiments complied with the guidelines of the Ethics Committee of Nippon Veterinary and Life Science University, who reviewed and approved this study (Approval number 12-78). Animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement and data reported according to the *Animals in Research: Reporting In Vivo Experiments (ARRIVE)* guidelines.

Western blotting

Samples of bulbar and palpebral conjunctiva from five healthy dogs, euthanased for reasons unrelated to this study, were collected and stored at –80 °C. Tissues were subsequently homogenised in radioimmunoprecipitation assay (RIPA) buffer. Rat conjunctiva, known to express $P2Y_2$ receptors (Fujihara et al., 2001), served as a positive control. Tissue lysates and molecular weight marker (MagicMark, Novex) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins transferred to a polyvinylidene difluoride (PVDF) membrane, and subjected to Western blotting.

Membranes were incubated with 5% blocking reagent (ECL blocking agents; GE Healthcare Life Science) overnight at 4 °C, then probed with rabbit anti-rat $P2Y_2$ polyclonal antibody (1:200; Abcam) for 1 h at room temperature. Membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG polyclonal antibody (1:5000; GE Healthcare Life Science). Immunoreactivity was visualised by chemiluminescence (ECL, GE Healthcare Life Science) and documented with a CCD imager (LAS-4000 film, Fujifilm Corporation).

Immunohistochemical analysis

Conjunctival samples from the same dogs used for Western blotting were fixed in 4% paraformaldehyde (PFA) for 24 h at 4 °C and embedded in paraffin. Tissue sections of 3 μm were mounted onto adhesive slides. After deparaffinisation, slides were incubated with 1% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity, followed by a 2.5% blocking reagent (Block Ace, Dainippon Sumitomo Pharma) in Tris-buffered saline (TBS). Expression of $P2Y_2$ was assessed with a rabbit anti-rat $P2Y_2$ polyclonal antibody (1:100; Abcam), overnight at 4 °C. Immunoreactivity was identified using peroxidase anti-peroxidase complex staining (Histfine, Nichirei Corporation) and visualised using diaminobenzidine as the substrate, with haematoxylin counterstaining.

Measurement of MUC5AC secretion following topical administration of 3% diquafosol

Six healthy laboratory Beagles, aged between 2 and 4 years, were subjected to ophthalmological examination, including vision testing (e.g. menace response, cotton ball test, maze test), Schirmer tear test (STT), intraocular pressure measurement (TonoVet, Icare), slit lamp examination (SL-15, Kowa), and fundus examination (VX-10i, Kowa). Tears were collected from the lacrimal lake of all six dogs by gently placing a microcapillary tube (Microcaps, Drummond Scientific) at the lateral canthus. One drop (approximately 50 μL) of 3% diquafosol ophthalmic solution (Diquas ophthalmic solution 3%, Santen Pharmaceutical) was instilled into the right eye and one drop of 0.1% sodium hyaluronate ophthalmic solution (Hyalein ophthalmic solution, Santen Pharmaceutical) was instilled into the left eye of each dog. Tears were collected from both eyes at various time-points between 10 and 300 min after treatment, using the method described previously. Tear samples were analysed for canine MUC5AC using a specific ELISA kit (TSZ ELISA).

Measurement of aqueous tear secretion following topical administration of 3% diquafosol

A STT was performed in both eyes before drop instillation, using a STT strip (Intervet), placed outside of the third eyelid for 1 min. Immediately following the STT, one drop of 3% diquafosol ophthalmic solution was instilled in the right eye and one drop of 0.1% sodium hyaluronate ophthalmic solution instilled into the left eye. The STT was subsequently performed on each eye at various time-points between 10 and 300 min after treatment. Following a 7-day washout period, the process was repeated, but with diquafosol instilled into the left eye and sodium hyaluronate in the right eye.

Following a further 7-day washout period, the phenol red thread test was performed in both eyes using Zone-Quick (Showa Yakuhin Kako), located outside of the third eyelid for 15 s. After measuring the phenol red thread test value, one drop of 3% diquafosol ophthalmic solution was instilled into the right eye and one drop of 0.1% sodium hyaluronate ophthalmic solution instilled into the left eye. Phenol red thread test measurements were taken for both eyes at various time-points between 10 and 300 min after treatment. Following a 7-day washout period, the process was repeated, but with diquafosol instilled into the left eye and sodium hyaluronate in the right eye.

Statistical analysis

Statistical analyses were performed using the IBM SPSS statistical software package (version 16.0). One way ANOVA followed by a Tukey–Kramer multiple comparison test was used to evaluate the time course of change and a Mann–Whitney *U* test was used to compare groups (diquafosol vs. sodium hyaluronate).

Results

The $P2Y_2$ receptor is expressed on the canine ocular surface

Results of Western blotting demonstrated a single band of approximately 42 kDa present in the conjunctival samples assessed (Fig. 1). Immunohistochemical analysis showed that $P2Y_2$ receptors were expressed by the canine conjunctiva, with both epithelial and goblet cells showing $P2Y_2$ receptor expression (Fig. 2).

Effect of diquafosol on tear MUC5AC concentration

The tear MUC5AC concentration remained fairly constant for 120 min following instillation of diquafosol, after which time the concentration increased and remained elevated until 300 min (Fig. 3). Compared with baseline values, MUC5AC concentrations were significantly higher at 300 min ($P = 0.033$) following diquafosol installation. The tear MUC5AC concentration was consistently low throughout the measurement period after instillation of sodium hyaluronate. Significant differences in tear MUC5AC concentration were observed comparing diquafosol and sodium hyaluronate treatments at 60 min ($P = 0.002$), 180 min ($P = 0.002$), 240 min ($P = 0.002$) and 300 min ($P = 0.002$) after instillation.

Effect of diquafosol on Schirmer tear test and phenol red thread test values

The STT values were lower than baseline at 180 min ($P = 0.047$), 240 min ($P = 0.001$) and 300 min ($P = 0.001$) following instillation of diquafosol (Fig. 4). The STT values in eyes receiving sodium