CASE REPORT

Clinical management of *Candida albicans* keratomycosis in a bottlenose dolphin (*Tursiops truncatus*)

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Abstract

Objective Corneal ulceration secondary to trauma commonly affects marine mammals, often with opportunistic secondary bacterial or fungal infections. This report characterizes the combined use of auriculopalpebral and ophthalmic nerve blocks, adipose-derived stem cells, and subconjunctival injections for successful treatment of corneal trauma and infection in dolphins.

Animal studied An 11-year-old, female bottlenose dolphin (*Tursiops truncatus*) presented with bilateral diffuse corneal opacities, which progressed to keratomycosis caused by *Candida albicans*.

Procedure Aggressive medical management was employed, including the use of subconjunctival injections of adipose-derived stem cells, plasma, topical and oral antifungals and antibiotics, and anti-inflammatory and pain medications. Anesthetic block of the auriculopalpebral and ophthalmic nerves was employed to evaluate the corneas.

Conclusion Subconjunctival injections were employed over 52 days, followed by topical drops for 5 months. At last evaluation, there was no evidence of blepharospasm bilaterally. Only a faint superficial gray corneal opacity remained OS. A temporal paraxial corneal opacity was present OD, with receding inactive vascularization and a small amount of melanosis temporally.

Key Words: bottlenose dolphin, *Candida albicans*, keratomycosis, nerve block, stem cells, *Tursiops truncatus*

CASE REPORT

This report characterizes the case progression for an 11-year-old, female bottlenose dolphin (*Tursiops truncatus*) that presented with bilateral progressive keratomycosis caused by *Candida albicans*.

On October 27, 2012 (day 0), an axial diffuse corneal opacity was noted OD, with no blepharospasm present. Two days later, multifocal white opacities were noted scattered throughout the cornea, ranging from 1 to 3 mm in diameter (Fig. 1a). On day 4, complete blepharospasm was noted OD, which progressed to complete bilateral blepharospasm the next day. Tramadol (150 mg P.O. q 24 h) was started. On day 9, complete blepharospasm, and marked periocular swelling and bruising were present OD, and two focal white lesions were noted on the ventral cornea OS (Fig. 1a). Fluorescein staining showed positive uptake in the center of the lesions. Doxycycline administration (400 mg P.O. B.I.D.) was initiated, and tramadol frequency was increased to B.I.D.

On day 12, the animal was brought to the hospital to perform a thorough ophthalmic evaluation and to implement treatments. For all procedures, the dolphin was removed from the water immediately prior to the procedure and returned to the water immediately following completion. Local nerve block technique was developed with a combination of published equine nerve blocks and extrapolation to known dolphin skull anatomy (Fig. 2).[1,2]

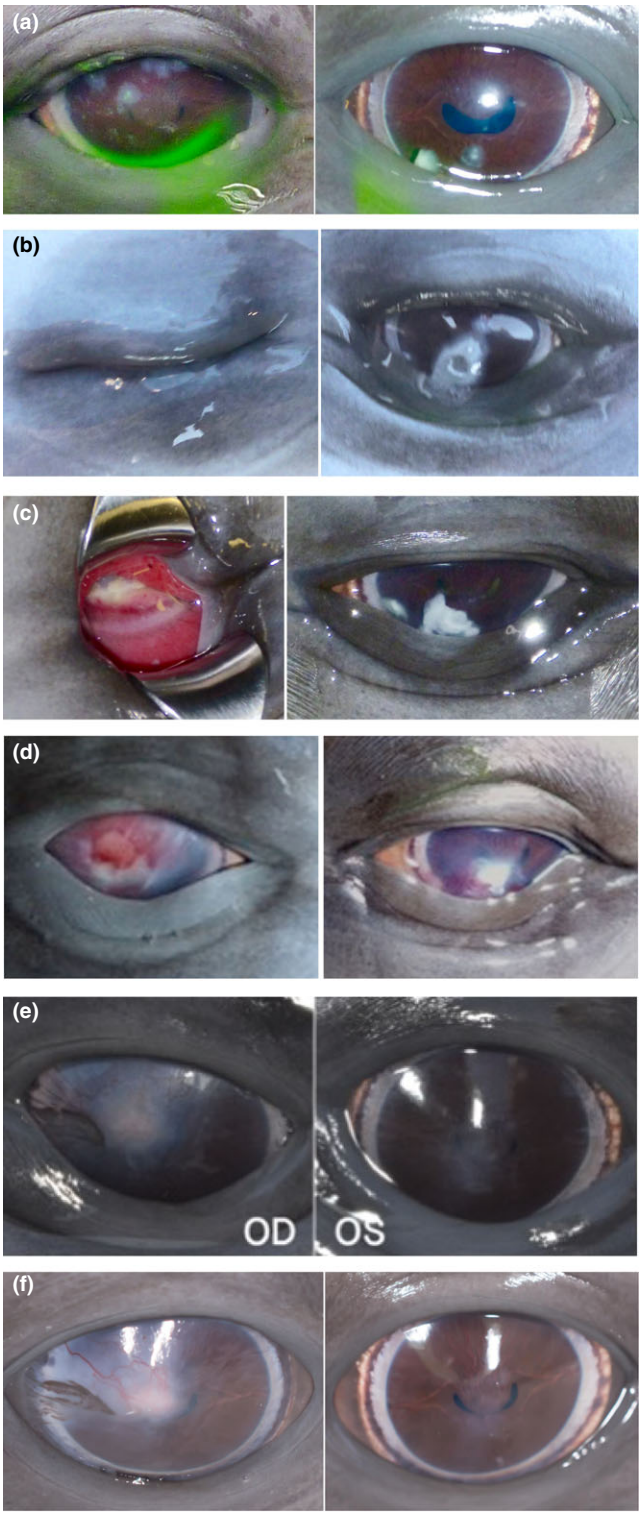

Figure 1. (a) Day 2 (OD). Note white focal corneal lesions present bilaterally. Fluorescein stain uptake noted at center of lesions. Day 9 (OS). By day 9 complete blepharospasm was noted OD. (b) Day 19. Note marked blepharospasm and blepharitis OU. White raised fungal lesion encompasses one-third cornea OS. (c) Day 25. OD: Note moderate blepharospasm, with marked conjunctival hyperemia and chemosis, corneal cellular infiltrates, and vascularization affecting the entire visible cornea. OS: Yellow-white corneal opacity no longer appears raised. (d) Day 52. Intermittent blepharospasm reported OU. OD: Note edema, yellow-white opacity within the vascularization consistent with cellular infiltrates noted encompassing the majority of the cornea. OS: Yellow-white opacity has mild diffuse edema surrounding the lesion with more diffuse margins and 4–5 mm of vascularization arising from the ventral limbus from 5 to 8 o'clock. (e) 5 months following termination of subconjunctival injections. No blepharospasm present. OD: Note temporal paraxial gray-white corneal opacity consistent with fibrosis, inactive vascularization, and pigment streaming in from temporal aspect and just abutting the temporal aspect of the opacity, OS: Note faint axial superficial gray corneal opacity consistent with fibrosis. (f) 2.5 years following initial presentation. OD: Note the dense gray-pink axial opacity with active vascularization, diffuse gray-white opacity consistent with fibrosis temporal and dorsal to the denser opacity, and pigment streaming in from temporal aspect, OS: Note faint, superficial gray corneal opacity, consistent with fibrosis.

MRI images of the dolphin head were studied, but the small size of the nerves prevented visualization via this method. The auriculopalpebral branch of the facial nerve was targeted to cause motor paralysis to the upper eyelid, and branches of the ophthalmic nerve of the trigeminal nerve were targeted to provide analgesia to the eyelids. A 25-g, 1-inch needle was inserted up to the needle hub at an angle perpendicular to the surface of the skin (Fig. 3). While total relaxation of the eyelids was not achieved throughout the course of treatment, the upper lid was sufficiently blocked to provide access to the corneal and conjunctival surfaces. The eyelids were retracted using Desmarres lid retractors, and the tear film was wiped with sterile gauze. Topical proparacaine was applied to the corneal surface. The protocol for the nerve blocks (for either one or both eyes), topical proparacaine, and eyelid retraction was repeated in all subsequent procedures. During the first procedure, profound bradycardia of 35–40 beats per minute with no evidence of a normal sinus arrhythmia was noted with manipulation of the eyes (normal resting heart rate for *T. truncatus* range 100–110 beats/min).[3] The heart rate returned to normal when the retractors were removed and the animal was placed in sternal recumbency. Bradycardia was not noted at any subsequent procedure.

Allogeneic, adipose-derived stem cells were obtained from VetStem Pharma (Poway, CA). The cells had been previously harvested from the sire of the patient and were collected, cultured, identified as ASCs via cell markers, and banked as previously described.[4] The stem cells were thawed the night before the procedure and resuspended with the patient's own serum for an estimated cell concentration of (minimum) 9 000 000 cells/mL. The stem cell solution (0.6 cc) was gently mixed with plasma (1.4 cc). This preparation protocol was used in all subsequent procedures when stem cells were used. During the first procedure, half of the mixture (1.0 cc) was administered topically to the corneal surface OU. The topical

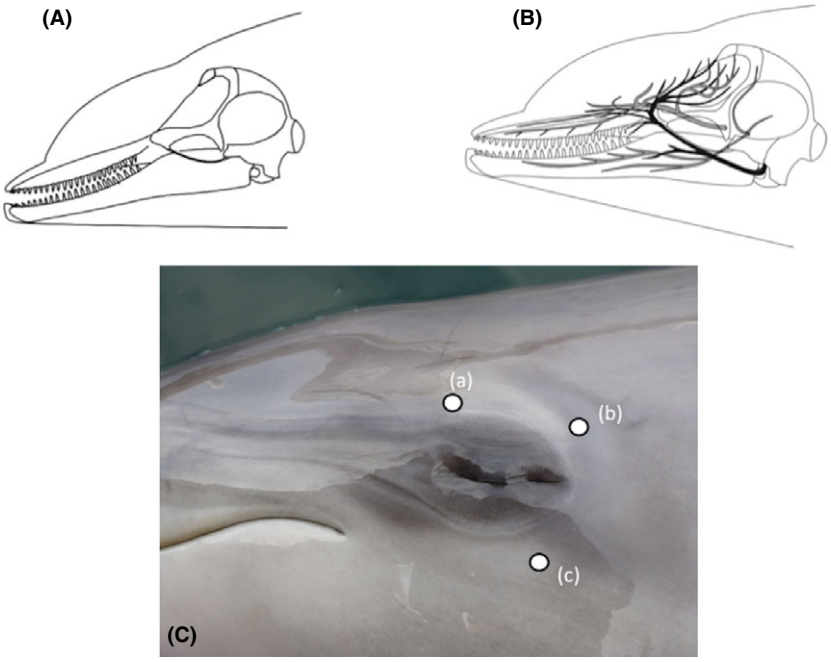

Figure 2. Location of auriculopalpebral and ophthalmic nerve block injection sites in *Tursiops truncatus*. (A) Diagram of skull and soft tissue structures, (B) pathways of trigeminal nerve (gray) and facial nerve (black) superimposed over skull, (C) photograph of lateral view of head, with injection sites labeled by white circles. Motor innervation to the upper eyelid was targeted by (a) infusion around the auriculopalpebral nerve, and sensory innervation to the eyelids was targeted by (b) infusion around the ophthalmic nerve. Additional targeting of proximal nerve branches of the facial and trigeminal nerves was targeted with (c). (A) and (B) copyright S. A. Rommel, borrowed with permission from Rommel *et al.* 2009.

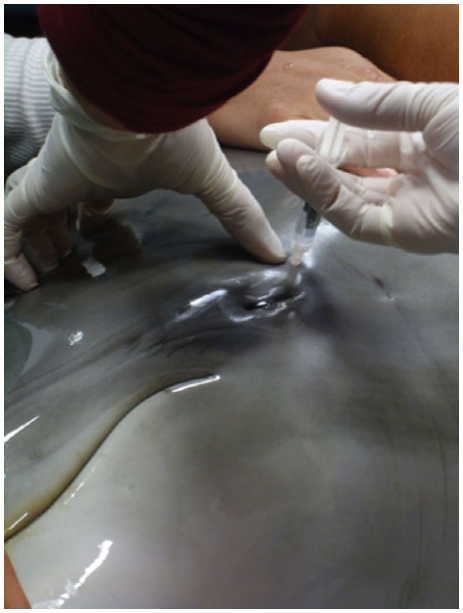

Figure 3. Ophthalmic nerve block placement in *Tursiops truncatus*. Note palpation of frontal bone as reference point, and 25 g, 1 inch needle is inserted to the needle hub.

administration was repeated with the other half of the mixture 15 min later. Neomycin-polymyxin-gramicidin ophthalmic drops (Bausch & Lomb Incorporated, Tampa, FL) were started at that time (three drops topically OU B.I.D.).

Blood was collected from the periarterial venous rete at the distal ventral peduncle and processed to collect platelet-rich plasma (PrP) using a veterinary benchtop kit primarily utilized in equine patients, which included spinning whole blood at 720 g for 15 min (Genesis CS-2, Vet Stem Pharma, Poway, CA). Recent studies have shown that dolphin platelets have a significantly larger area compared with equine and human platelets, and an ideal centrifugation protocol for dolphin platelet concentration is 106 g (900 rpm) for 3 min, a much lower speed and shorter duration than was recommended with the benchtop kit.[5] Because of the size difference between equine and cetacean platelets, this protocol was not successful in collecting PrP, and fresh plasma was used in all procedures.

Sterile cotton swabs were used to gently debride the corneal lesions during this procedure, and the swabs were submitted for bacterial and fungal culture (Cornell Animal Health Diagnostic Center, Ithaca, NY), universal fungal PCR (University of Illinois Veterinary Diagnostic Laboratory, Urbana, IL), and virus detection (Athens Veterinary Diagnostic Laboratory, Athens, GA).

Bacterial culture revealed *Staphylococcus aureus* and *Staphylococcus warnerii* OD, and few *Vibrio spp.* OS. Fungal culture revealed *Candida albicans* OU, on both original cultures, as well as follow-up culture 1 week later. Universal fungal PCR produced a 200-bp fragment of DNA, 100% compatible with *Candida albicans* OU. Virus detection via negative-stain electron microscopy and herpesviral PCR were negative OU. The *Candida* organisms were sensitive to fluconazole and voriconazole, and *Staphylococcus* spp were sensitive to doxycycline, among a variety of other antibiotics. Following culture results, oral fluconazole (800 mg P.O. q 24 h) was initiated.

On day 19, marked blepharospasm and blepharedema continued OU, and retraction of the eyelids revealed marked chemosis and conjunctival hyperemia OD, which made visualization of the cornea impossible. The white corneal opacities previously noted OS had coalesced into a lesion that encompassed one-third of the cornea, with an

irregular, raised surface in the center (Fig. 1b). Platelet analysis of PrP showed 275 × 10(9)/L (in-house established reference range for whole blood: 150–450 × 10(9)/L). The stem cell/plasma mixture was administered as a subconjunctival injection (0.3 cc OU) in the temporal bulbar conjunctiva, which was followed by topical administration (1 cc OU) 15 min later. The location of the injection varied, but the temporal bulbar conjunctiva was used to standardize observations for adverse effects of the injection. Topical antibiotic drops were discontinued at this time, due to the difficulty of administration in an animal with complete blepharospasm, as well as a concern for potential cytotoxicity of the stem cells from topical medications, which have been described as reducing stem cell viability in a variety of settings.[6,7]

On day 25, the lesion OS showed improvement, with a reduction in size and no associated corneal edema, while OD showed moderate blepharospasm, with marked conjunctival hyperemia and chemosis, corneal cellular infiltrates, and vascularization affecting the majority of the cornea (Fig. 1c). As the optimal concentration of stem cells was unknown, administration of stem cells without additional plasma was performed to concentrate the maximum number of cells at the site. An injection of 0.5 cc stem cells was placed subconjunctivally OD, followed by 0.5 cc topically OU (Fig. 4).

On day 33, the lesion OS continued to improve, while marked blepharospasm and dorsal symblepharon were noted OD. A sterile cotton swab and saline were used to gently detach the adhered conjunctiva following application of topical proparacaine. The stem cell/plasma mixture was administered as a subconjunctival injection (0.5 cc OU), and topically (1 cc OU) 15 min later. The symblepharon recurred to a lesser degree after detachment, and manual detachment was repeated during two subsequent procedures.

There was concern over the lack of response to therapy OD. While OS showed improvement at each procedure, OD continued to have marked chemosis and blepharospasm, with no appreciable change in the corneal lesion. To provide more aggressive antifungal therapy, oral fluconazole was increased from q 24 h to B.I.D., and voriconazole 1% ophthalmic drops were started OU B.I.D. The drops were prepared as previously described,[8] as there are currently no commercially available topical ophthalmic voriconazole preparations. Hepatic function was monitored throughout fluconazole treatment, and there was no evidence of dysfunction.

On day 38, the lesion OS continued to reduce in size, and OD only had moderate blepharospasm, with a small portion of cornea visible for the first time. A white opacity encompassed the entire visible cornea. Plasma and voriconazole were administered as separate subconjunctival injections (1 cc each OU). Oral diazepam (20 mg) was administered 45 min prior to subsequent procedures for light sedation.

Procedures on day 40, 45, and 47 noted static lesions OU. Subconjunctival injections, using separate subconjunctival injections to avoid any medication interaction, of stem cells and voriconazole (d40), plasma and voriconazole (d45), or stem cells alone (d47) were administered OD due to the severity of the lesion (Fig. 4). The lesion OS was responding well to therapy, so the stem cells/plasma/voriconazole treatment that was employed with subconjunctival injections OD were administered topically OS.

On day 52, the lesion OS appeared static, with vascularization noted ventrally, and only intermittent blepharospasm (Fig. 1d). The right eye was comfortable, without photophobia or blepharospasm, although it still had yellow-white opacity within the vascularization consistent with cellular infiltrates encompassing the majority of the cornea, and vascularization noted across the majority of the cornea (Fig. 1d). Plasma was administered (0.5 cc subconjunctival OD; 0.5 cc topical OS). While there was no evidence of inflammation at the previous injection sites, it was suspected that one of the therapies may have been irritating, as intermittent blepharospasm had continued to be reported. Topical voriconazole and all subconjunctival injections were discontinued at that time. Topical medications, including nepafenac (three drops OU B.I.D.), 5% sodium chloride (three drops OU B.I.D.), and triple antibiotic drops (three drops OU B.I.D.), were administered for the next 5 months.

In June 2013, 9 months after initial presentation and treatment, there was no evidence of blepharospasm in either eye. The lesion OS had regressed so that only a faint superficial gray corneal opacity was present (Fig. 1e). The lesion OD had regressed so that a temporal paraxial corneal opacity remained, with receding inactive

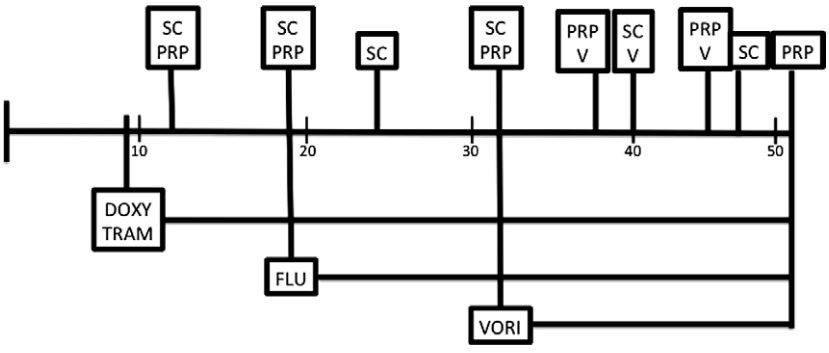

Figure 4. Timeline of treatment administration from day 0 to day 52. SC = stem cells subconjunctival and topical; PRP = platelet-rich plasma subconjunctival and topical; V = voriconazole subconjunctival; DOXY = doxycycline PO; TRAM = tramadol PO; FLU = fluconazole PO; VORI = voriconazole ophthalmic drops.

vascularization and a small amount of melanosis temporally (Fig. 1e).

In April 2015, two and a half years following the initial insult, the animal was visual in both eyes, with no reported blepharospasm. Only a faint, superficial corneal opacity was noted OS consistent with very mild fibrosis (Fig. 1f). The cornea OD had a dense gray-pink axial opacity with moderately active vascularization, diffuse gray-white opacity consistent with fibrosis temporal and dorsal to the gray-pink opacity, and pigment streaming in from the temporal aspect (Fig. 1f).

In total, the anesthetic blocks were performed ten times (twice OU and six OD, when subconjunctival injections were employed) with a similar level of success each time. Over 52 days, the patient received three treatments of stem cells/plasma, two treatments of stem cells alone, two treatments of plasma/voriconazole, and one treatment each of stem cells/voriconazole or plasma alone (Fig. 4).

DISCUSSION

Corneal ulceration secondary to trauma commonly affects cetaceans, often with opportunistic secondary bacterial or fungal infections.[9–11] Scarring is a common sequela that can affect vision if sufficiently severe.[12] Strong periocular muscles cause a marked blepharospastic response in this species and can make frequent topical medication administration difficult.[13] Novel therapies are desired to decrease healing time and reduce residual corneal damage.

Keratomycosis is commonly seen in equine patients and results from disruption of the corneal epithelial layer, often secondary to traumatic insult, and opportunistic yeast and fungi in their environments and periocular surfaces.[14] It is a potentially sight-threatening disease that requires early diagnosis, and appropriate aggressive medical or surgical therapy. There is a single case of keratomycosis described in a cetacean, which presented in a similar manner to the case above, with multiple punctiform lesions that coalesced into a white, raised lesion occupying 70% of the cornea.[15] The animal was treated with oral fluconazole for only 35 days due to medication interactions, and 15 months after the start of clinical signs, the majority of the cornea was clear, save a 4 mm axial corneal scar. This could suggest that the fungal infection in these cases may have been cleared early, with the prolonged healing of the lesion due to remodeling of corneal scar tissue.

In this case, the exact etiology of the initial lesion could not be determined. Voriconazole was elected due to the sensitivity profile of the cultured *Candida*. Voriconazole appears to be the most effective antifungal drug for initial treatment of equine keratomycosis, because it penetrates the cornea well, and a variety of common fungal pathogens have *in vitro* susceptibility to the drug.[16–18] Because voriconazole has not been used frequently via a topical or subconjunctival route in cetaceans, its efficacy was unknown. Therefore, oral fluconazole was administered as well. Doxycycline was used to treat the bacterial infection because of its immunomodulatory effects and inhibition of MMP-9, which is responsible for corneal malacia and stromal loss.[19,20] Doxycycline is also secreted in the tear film, helping to ensure adequate tissue concentrations.[21–23] Focal application of voriconazole likely contributed to the clinical improvement noted as the infection was cleared. Applying voriconazole directly to the lesion via topical administration, as well as providing prolonged concentrations with subconjunctival injections, avoids systemic hepatic effects and is less expensive than oral therapy.[24] Additionally, topical voriconazole is often much more effective for fungal keratitis than oral therapy unless there is dense vascularization of the lesion.

At each procedure, the conjunctiva OU were examined for gross evidence of a tissue reaction to the subconjunctival injections. A variety of volumes (0.3–1.0 cc) were used, and there was no visual evidence of local inflammation, discoloration, granuloma formation, or necrosis at the sites. While the 1.0 cc was not associated with visible adverse effects, this is a large volume to inject in a single site, and the authors recommend restricting volumes to 0.5 cc at a single site. The only adverse effect noted was blepharospasm temporally associated with the subconjunctival voriconazole injections, suggesting they may have been irritating to the eye. Solutions injected into the subconjunctival space form a localized depot, which may allow for long-term sustained delivery.[25] Subconjunctival injections show promise for treating ocular infections in dolphins. Subconjunctival injections have the potential for side effects such as conjunctival necrosis, when used with steroids.[26] As cetaceans have extremely strong orbicularis oculi muscles, complete blepharospasm from any cause can persist for weeks to months. This makes topical medication administration difficult to impossible, and uveitis, corneal ulceration, and perforation of the globe are a concern when the eye cannot be re-evaluated or adequately treated.

Adipose-derived stem cells (ASC) are a type of mesenchymal cell, which can assist in the regeneration of tissues and wound healing, and can be derived from a variety of sources.[27,28] The most impactful effect of ASC pluripotent cells is probably their immunomodulatory effect on both the innate and adaptive immune response.[29,30] They are often used as a source due to their availability, ease of collection, and ability to proliferate in culture. In humans, ASCs have been used to promote healing in radiation ischemia and perianal fistulas, and animal models show promise for the treatment of cardiovascular, genitourinary, and musculoskeletal disorders.[31–33] In dolphins, ASCs have been isolated, cultured, and differentiated into multiple cell lineages, suggesting that they may have similar regenerative capabilities as in other mammalian species.[4,5] Because the stem cells were derived from the sire of the patient,

there was less risk of immune rejection of the cells than if they were derived from another, unrelated animal. In addition, adult mesenchymal stem cells are less antigenic than other cells and are rarely associated with allogeneic reactions.[34] Nonetheless, the stem cells were first applied topically, and then in increasing concentrations to monitor for an immune response. No adverse response was noted to either topical or subconjunctival administration. Both topical and subconjunctival injections of mesenchymal stem cells have been shown to reduce inflammation and significantly accelerate corneal wound healing in rats.[35,36]

Autologous serum or plasma is commonly used to treat severe corneal ulceration, as they contribute growth factors, fibronectin, and vitamins that stimulate epithelial cell growth, migration, and differentiation, and also inhibit matrix metalloproteinases such as MMP-9 that lyse components of the corneal epithelial barrier.[17,37,38] Platelet-rich plasma concentrates platelets, which are the main source of these growth factors. Subconjunctival application of PrP to treat severe ocular alkali burns has been shown to reduce corneal epithelialization time and healing time in comparison with traditional therapies.[39] In addition, PrP provides a nutrient-rich bath for the stem cells. Platelet counts on the PrP showed no evidence of platelet concentration using the equine benchtop kit. The likely effect in this case was the use of plasma to treat corneal ulceration; it may have provided growth factors and promoted healing, but likely not at a comparable level as with true PrP. Using PrP may be less practical than using serum, because it must be used shortly after preparation. The preparation process leads to platelet activation, during which time nearly 100% of the growth factors are released within 1 h.[40]

Both systemic and local analgesia, and local anesthesia were used to make the patient more comfortable. Clinicians elected to administer oral diazepam to achieve mild sedation in this patient, although animals that are not accustomed to being out of the water may require more significant sedation to ensure that movement is minimized, especially when performing the regional blocks or subconjunctival injections. Parenteral midazolam is frequently employed in ophthalmic procedures necessitating sedation. In addition, clinicians should be aware of the profound bradycardia that can result from an oculocardiac response to manipulation of the eye and should be prepared to manage this complication and potentially abort the procedure if prolonged.[41]

While relaxation of the upper lid was adequate after the local block, total relaxation of the eyelids was not achieved. This was likely due to the fact that the technique was extrapolated from the technique described in horses, to the known anatomy of the dolphin skull. The technique will need to be refined to optimize the success of the blocks.

There are many factors that play a role in the healing time of a corneal defect, including the severity of the lesion, individual local and systemic immunity, and various environmental factors. Multiple treatments were used during disease progression, and the treatment regimens shifted over time. As such, it is difficult to determine which treatment was most effective. This case alone is not conclusive in proving that stem cells or plasma provided a beneficial effect on corneal wound healing, but the clinical resolution seen here suggests there are benefits worth pursuing further. Further investigation is required to determine whether stem cells and/or PrP can affect corneal wound healing in the dolphin, and the optimal dosing regimen for stem cells and PrP in the treatment of cetacean corneal disease remains to be determined. This case description provides novel techniques that may be supportive in the treatment of complex cetacean ocular pathology.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Robert Harman and the staff of VetStem Biopharma for culture and preparation of stem cells; Dr. Shawn Johnson for his guidance with stem cell collection and administration in dolphins; Dr. Sentiel Rommel for the use of dolphin anatomy illustrations; and to the veterinary technicians and trainers at the National Marine Mammal Foundation and the U.S. Navy Marine Mammal Program for their care of the animals in these cases.

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