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MANAGEMENT BRIEF

Plasma Bleomycin Concentrations during Electrochemotherapeutic Treatment of Fibropapillomas in Green Turtles *Chelonia mydas*

Kyle A. Donnelly*
Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, 2015 Southwest 16th Avenue, Gainesville, Florida 32610, USA

Mark G. Papich
Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, North Carolina 27607, USA

Bette Zirkelbach
The Turtle Hospital, 2396 Overseas Highway, Marathon, Florida 33050, USA

Terry Norton
The Turtle Hospital, 2396 Overseas Highway, Marathon, Florida 33050, USA; and
Georgia Sea Turtle Center/Jekyll Island Authority, 214 Stable Road, Jekyll Island, Georgia 31527, USA

Anna Szivek
Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, 2015 Southwest 16th Avenue, Gainesville, Florida 32610, USA

Brooke Burkhalter
The Turtle Hospital, 2396 Overseas Highway, Marathon, Florida 33050, USA; and
Whitney Laboratory for Marine Bioscience, The Sea Turtle Hospital, 9505 Ocean Shore Boulevard, St. Augustine, Florida 32080, USA

Joseph A. Impellizeri
Veterinary Oncology Services, 517 Route 211 East, Middletown, New York 10941, USA

Nicole I. Stacy
Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, 2015 Southwest 16th Avenue, Gainesville, Florida 32610, USA

*Corresponding author: kyleadonnelly@gmail.com
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Abstract

Fibropapillomatosis of sea turtles is traditionally treated with surgical debulking techniques that are often associated with prolonged healing and tumor recurrence. Electrochemotherapy was recently described for green turtles *Chelonia mydas* and can be an alternative to surgery and even general anesthesia. The objectives of this study were to replicate an electrochemotherapy protocol from a previous report and add plasma bleomycin analysis to the treatment. After bleomycin injection into similarly sized tumors of two green turtles and immediate electroporation at two time points, plasma bleomycin reached detectable concentrations that were considerably lower than those found in human studies. At 3 months posttherapy, no healing complications or recurrences were encountered and only scar tissue remained. This study adds further support that electrochemotherapy with bleomycin has the potential to be used as an effective alternative treatment for this complex disease.

Fibropapillomatosis (FP) is a debilitating neoplastic disease of sea turtles. Although reported in every species of sea turtle, FP has only reached panzootic status in the green turtle *Chelonia mydas* (Jones et al. 2016). Single or multiple variably sized benign fibroepithelial lesions characterize the disease, and cutaneous lesions appear to precede visceral tumors (Jones et al. 2016). These cutaneous tumors are the traditional target of veterinary intervention.

The treatment for FP generally entails surgical removal of tumors and supportive care. The complications include difficulty in surgical closure of large areas of tissue, risk of secondary infection or anemia, anesthetic risk, and a high recurrence rate. One study found that 38.5% of postoperative green turtle patients regrew tumors at removal sites within 36 d of surgery (Page-Karjian et al. 2014). Electrochemotherapy (ECT) is a technique used in human and veterinary medicine to treat cutaneous and subcutaneous tumors (Cemazar et al. 2008; Tozon et al. 2016; Spugnini et al. 2017). This technique relies on enhancing the effects of intralesional chemotherapy through the use of short, intense electric pulses that increase the permeability of cell membranes (Weaver 1995; Gothelf et al. 2003; Al-Sakere et al. 2007; Impellizeri et al. 2016). Although the cell membrane is a physical barrier that normally prevents the entry of hydrophilic drugs and macromolecules, electroporation significantly increases the cellular uptake of certain chemotherapeutics such as bleomycin (Impellizeri et al. 2016). The benefits of this therapy include expanding the options for hydrophilic drugs such as bleomycin, lowering the total chemotherapeutic dose by promoting the influx of drugs directly into the tumor cells, targeting neoplastic cells over healthy cells due to their increased susceptibility to electroporation, and inducing vasoconstriction, which restricts the drug to the treated area (Impellizeri et al. 2016). This technique was recently described for green turtles, allowing avoidance of surgery and even general anesthesia (Brunner et al. 2014). The objectives of this study were to replicate the ECT protocol from the previous report and to add plasma bleomycin analysis during FP treatment to assess drug safety.

METHODS

One similarly sized fibropapilloma was selected from each of two juvenile green turtles. Turtle 1 weighed 11.0 kg and turtle 2 weighed 5.4 kg. The targeted tumor in turtle 1, located in the right axilla, measured $1.7 \times 1.2 \times 0.6$ cm with a calculated tumor volume of 1.28 cm^3. The targeted tumor in turtle 2, located over the left gular scute, measured $1.3 \times 1.3 \times 0.3$ cm, with a calculated tumor volume of 1.15 cm^3. Both turtles harbored additional untreated tumors that were subsequently removed in a staged approach over the next 4 weeks using a carbon dioxide laser, which is the current technique of choice (Page-Karjian et al. 2014). The turtles were restrained manually and a volume of 0.5 to 1.0 mL of 2% lidocaine (VetOne, Boise, Idaho; 2 mg/kg) was injected intratumorally at the base of the mass for local analgesia; the location of the injection site helped to limit leakage or dilution of bleomycin. This anesthetic was administered to alleviate any discomfort associated with the needle grid electrode as well as the application of voltage. Following this, intralesional bleomycin (Hospira, Lake Forest, Illinois; 5 mg/mL) was injected at a dose of 0.5 to 1.0 IU/cm^3 of lesion (Brunner et al. 2014). The volume of bleomycin used ranged from 0.10 to 0.39 mL based on tumor volume calculations. Electroporation was immediately performed using an anticipated voltage of 1,000 V, a measured voltage of 400 V, a frequency of 5,000 Hz, a pulse width of 100 μs, and a period of pulses at 2 ms, with 8 pulses generated. Electroporation was applied over the entire tumor using an electrode composed of a grid of eight stainless steel needles positioned in two rows of four needles each. The needles were 2 mm apart and penetrated to a maximum tissue depth of 15 mm (Vet-ePorator; Evvivax, Rome [first treatment], Electrovet EZ; LeRoy Biotech, Gameville, France [second treatment]).

Immediately following ECT, 0.25 to 1.00 mL of heparinized whole blood was collected from the dorsal cervical sinus at 0, 1, 6, 12, 24, and 48 h after drug administration. This was a conservative collection schedule based on pharmacokinetic studies performed in humans (Kramer et al. 1978; Hall et al. 1982) that took into consideration the maximum blood volume that can be withdrawn from sea turtles (SEFSC 2008). This procedure was repeated 6 weeks after the first treatment using the same protocol. The treated and untreated tumors were visually examined and measured with calipers once monthly.

Bleomycin sulfate is a mixture of basic glycopeptides produced by the growth of *Streptomyces verticillus.* This bacterial species produces bleomycin A_2, A_5, B_2, B_4, and

other minor components. Since bleomycin A_2 and B_2 represent the major components, the assay was developed to test for these. Bleomycin A_2 and B_2 in plasma samples were analyzed by high-performance liquid chromatography (HPLC) with a modification from a previously published method (Mabeta 2012). The HPLC system consisted of a quaternary solvent delivery system (with a flow rate of 1 mL/min), an autosampler solvent delivery system (Agilent Technologies, Wilmington, Delaware; 1200 Series), and an ultraviolet variable wavelength detector (Agilent Technologies; 1200 Series) set at a wavelength of 295 nm. Chromatograms were integrated with OpenLAB software (Agilent Technologies). The column was a reverse-phase, 4.6-mm × 15-cm ACE 5 C18-AR column that was kept at a constant temperature of 40°C. The mobile phase for HPLC analysis consisted of 10% acetonitrile and 90% distilled water adjusted to a pH of 4.3 with ammonium hydroxide. A fresh batch of mobile phase was prepared, filtered (with a 0.45-μm filter), and degassed for each day's run.

The assay was validated specifically for this study by fortifying blank (control) plasma from untreated turtles. The reference standard of bleomycin was obtained as an analytical reference standard from the United States Pharmacopeia Convention (USP; Rockville, Maryland). The content of bleomycin A_2 in the reference standard was between 55% and 70%, and the content of bleomycin B_2 was between 25% and 32%. The content of bleomycin B_4 was at most 1%, and the content of bleomycin A_2 and B_2 comprised at least 90%. The bleomycin reference standard was used to prepare a stock solution for the blank sample matrix. The calibration curve consisted of eight standard solutions that ranged between 0.05 and 10.00 μg/mL of bleomycin and included a blank (0.00 μg/mL) sample. The blank sample was used to detect interfering peaks that elute into the window of the chromatographic peak of interest and to measure background interference. The calibration curve was accepted if the linear coefficient of determination (R^2) was ≥0.99 and the calibration curve concentrations could be back-calculated to ≤15% of the true concentration of the standard. Fresh calibration curves were prepared for each day's analysis.

The samples were prepared by mixing 500 μL of plasma with 600 μL of methanol. The mixture was vortexed and then dried until only a residue remained, after which it was reconstituted with the mobile phase before injection. The retention time for the peak of interest was approximately 5.5 and 9.5 min for bleomycins A_2 and B_2, respectively. The limit of quantification for bleomycin in turtle plasma in the samples was 0.05 μg/mL, which was determined from the lowest point on a linear calibration curve that yielded an acceptable accuracy and was within accepted guidelines for the signal : noise ratio (ICH Guidelines 2005; U.S. Pharmacopeia 2018).

The treated turtles were housed out of the water in a smaller containment (i.e., "dry-docked") for 48 h following treatment to prevent possible contamination of tank water with excreted bleomycin. This conservative (i.e., long) interval was based on pharmacokinetic information for humans, which demonstrates that 60% to 70% of the drug is eliminated in the urine within 24 h (Hall et al. 1982).

RESULTS

The tumors marginally increased in size in the first 7 to 9 weeks after the initial treatment (1 to 3 weeks after the second treatment), during which the tissue darkened to purple–black. Both treated tumors then shrank and/or partially fell off in the following weeks. Both turtles gained weight, behaved normally, and did not develop biochemical derangements of the blood or plasma during this time period. Electrochemotherapy resulted in the complete remission of tumors at 3 months in both turtles (Figures 1 and 2). No regrowth had occurred at the time of writing (8 months posttreatment). The concentrations of bleomycin A_2 reached a peak of 0.35 μg/mL during the first treatment and 0.81 μg/mL in the second treatment. The concentration of bleomycin B_2 was not detected in the first treatment but reached a peak of 1.20 μg/mL in the second treatment (Figure 3). Plasma concentrations of bleomycin decreased after 48 h posttreatment and were mostly undetectable except in one turtle with an A_2 concentration of 0.10 μg/mL and a B_2 concentration of 0.36 μg/mL.

The untreated tumors were not available for the duration of the study due to the timing of their surgical removal. In one case, an untreated tumor in a distant location (the inguinal space) appeared to exhibit necrosis within a week of ECT and darkened to a deep purple–black. This tumor was surgically excised shortly after its appearance; thus, longer-term follow-up was not possible. Interestingly, the ECT treatment that was given 1 week before this color change did not result in detectable circulating concentrations of bleomycin (Figure 3; the first treatment for turtle 1).

DISCUSSION

This study adds support that ECT with bleomycin has the potential to be used as an effective alternative treatment for FP. The detection of bleomycin components is somewhat inconsistent, as the formulation of bleomycin reportedly contains mostly bleomycin A_2 (55–70%), whereas the amount of bleomycin B_2 is minor (25–32%); however, in turtle 1, treatment 2 there was more B_2 than A_2, and in turtle 2, treatment 1 B_2 was not detected at all. This inconsistency may be related to differences in local

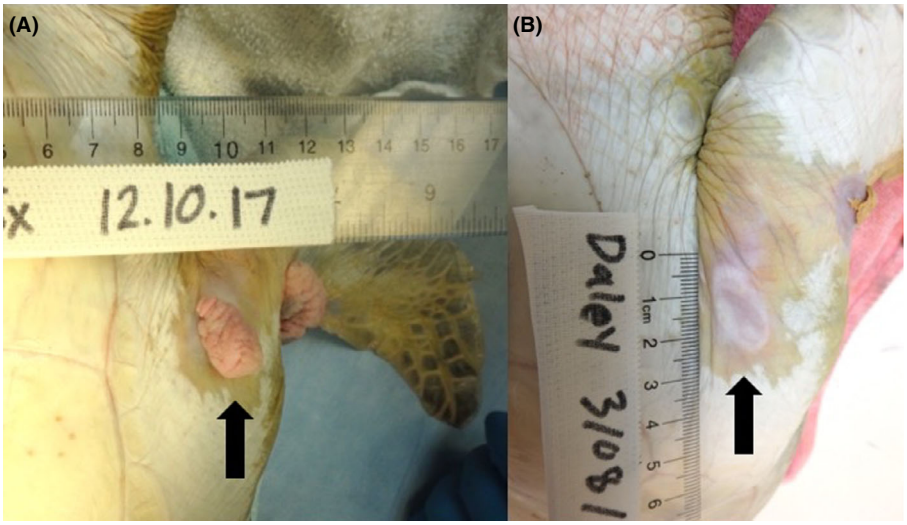

FIGURE 1. The targeted fibropapilloma in the right axilla of green turtle 1 **(A)** on the date of the first treatment, when the tumor measured 1.7 × 1.2 × 0.6 cm, with a volume of 1.28 cm^3, and **(B)** 3 months later, when only scar tissue was visible.

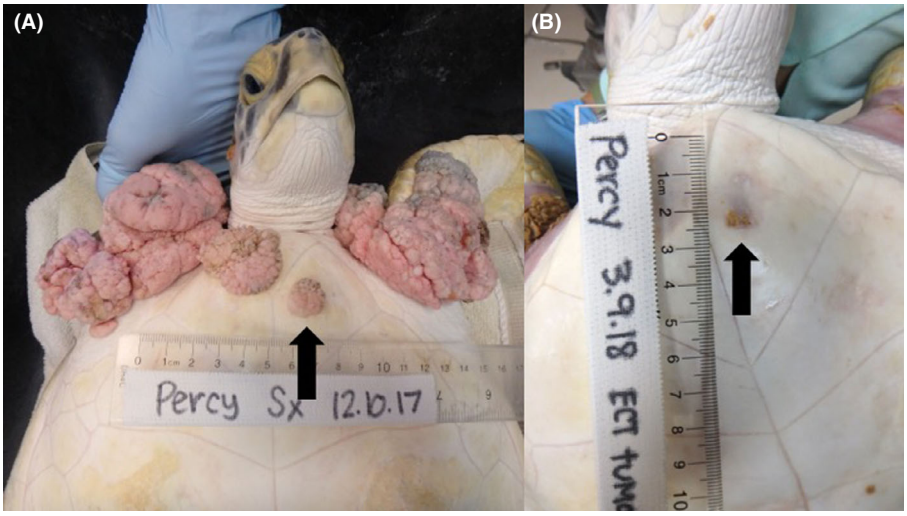

FIGURE 2. The targeted fibropapilloma on the left gular scute of green turtle 2 **(A)** on the date of the first treatment, when the tumor measured 1.3 × 1.3 × 0.3 cm, with a volume of 1.15 cm^3, and **(B)** 3 months later.

versus systemic effects, individual metabolic differences, metabolic variation between reptiles and mammals, or an unpublished change in the formulation of the drug. Bleomycin appeared to have reached detectable plasma concentrations in this study, suggesting systemic circulation, but its concentrations were many orders of magnitude lower than those documented in human pharmacokinetic studies (Hall et al. 1982; Groselj et al. 2016).

In one human study, 30 IU of bleomycin was injected subcutaneously without electroporation and the resulting peak concentration was 900 IU/mL (450 μg/mL after conversion; Hall et al. 1982). Another human study reported a peak of 3.1 μg/mL after an intravenous bolus (consisting of 23,000–30,000 IU) was administered and electroporation was performed (Groselj et al. 2016). In addition, the turtles' peak plasma concentrations in this study were lower than those in a mouse model, in which peaks of 10.9 to 19.9 μg/mL were detected after 0.1 mg of bleomycin was given intravenously to 21-g mice, followed by electroporation (Groselj et al. 2018). The difference in bleomycin concentrations between species may be a function of variable local action and absorption from the

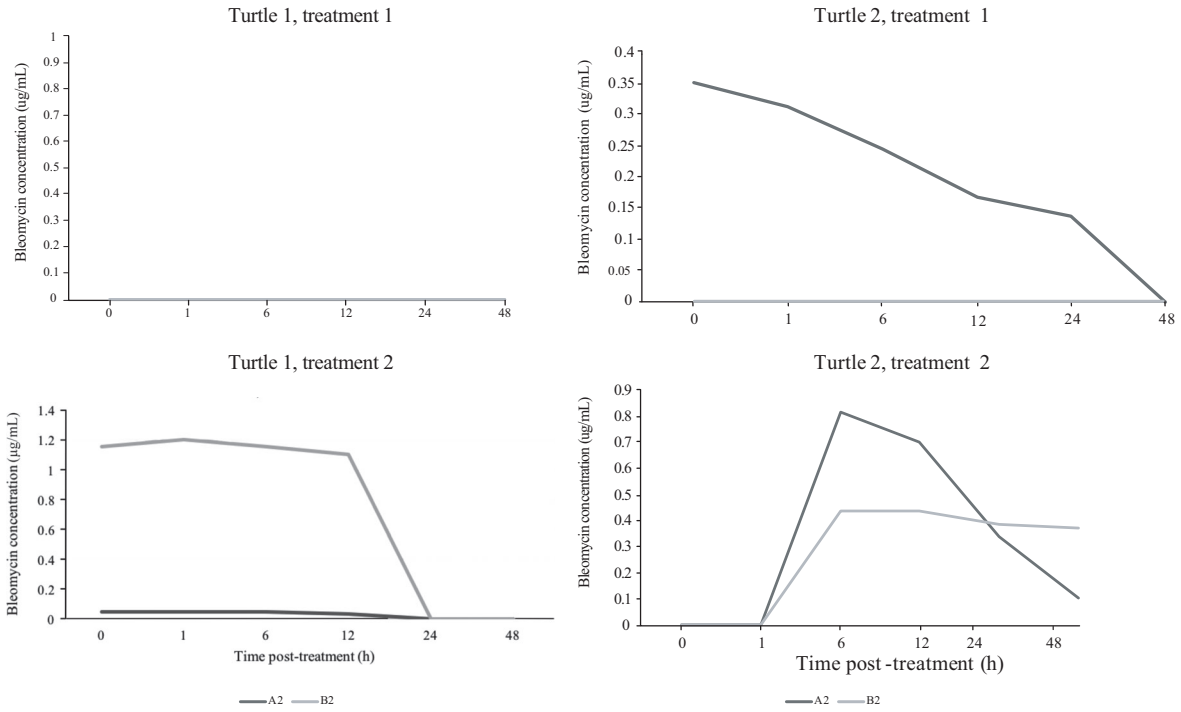

FIGURE 3. Plasma bleomycin A_2 and B_2 concentrations of turtles 1 and 2. Concentrations were measured following each electrochemotherapeutic treatment (treatment 1 at day 0 and treatment 2 at day 41).

electroporation, a lower relative dose being delivered to the turtles than to other species, or differences in methodologies for measuring plasma concentrations (since the radioimmunoassay tends to overestimate concentrations; Kosjek et al. 2016). The tumor microenvironment plays a role in tumor response and systemic absorption; for example, one study reported better success using ECT with well-vascularized tumors such as carcinomas than with less-vascularized tumors such as melanoma (Groselj et al. 2018).

The absence of circulating bleomycin in the case with apparent necrosis of the untreated tumor in turtle 1 (Figure 3; treatment 1) suggests the potential for additional systemic effects of ECT in response to a local treatment. Spontaneous regression of FP in turtles has previously been documented; however, it is unknown why some turtles self-resolve and the disease progresses in others (Herbst 1994; Hirama and Ehrhart 2007; Machado Guimaraes et al. 2013). Other viruses such as human and canine papilloma viruses can evade the host immune response and grow unchecked yet also display spontaneous regression (Nicholls and Stanley 2000; Nicholls et al. 2001; Scott et al. 2001; O'Brien and Saveria Campo 2002). The tumor microenvironment can modulate the overall response to treatment; for example, cytotoxic T cells present within the tumor stroma will kill tumor cells by expressing tumor neoantigens (Hirata and Sahai 2017). Overcoming this immune surveillance is a critical part of tumorigenesis, and additional research is needed to determine whether there is a relationship between electroporation and activation (or re-activation) of cell-mediated immunity outside of the treated tumor.

Bleomycin is highly toxic once inside the cell, but its inability to cross the cell membrane due to its hydrophilic nature limits its full therapeutic potential (Tounekti et al. 1993). Electroporation increases the permeability of neoplastic cells and thus the intracellular concentrations of bleomycin. In humans, the half-life of subcutaneously administered bleomycin is longer than that of a single intravenous injection, and there is no difference in plasma clearance between subcutaneous and continuous intravenous injection (Hall et al. 1982).

Bleomycin can cause pulmonary fibrosis in humans and domestic animals; however, unlike other chemotherapeutics, bleomycin does not typically cause myelosuppression, although thrombocytopenia and leukopenia are described (Plumb 2015). No adverse effects were observed in the clinical condition, complete blood counts, or plasma chemistries in either turtle. These results support that ECT with concurrent local bleomycin administration is a safe and effective treatment for FP. Additional studies with more patients and multiple tumor treatments are needed. This study adds to the known applications for ECT, for which treatments in humans, dogs, cats, mice, rats, yellow-bellied sliders, and green turtles are described (Gothelf

et al. 2003; Brunner et al. 2014; Lanza et al. 2015; Groselj et al. 2016; Impellizeri et al. 2016; Lanza et al. 2017). Disadvantages of this technique include the need for "dry-docking" animals, the need for personal protective equipment for chemotherapy that may not be available in all rehabilitation settings, and the need to take a stepwise approach to animals with heavy tumor burdens until more information on systemic immune responses and logistic feasibility becomes available. Caution should be exercised in the treatment of tumors over sensitive areas such as the cornea until additional research is performed.

Electrochemotherapy has great potential for FP treatment in sea turtle patients since it eliminates the need for surgery and general anesthesia, which are often limiting factors in initiating treatment in sick and debilitated turtles. This technique enhances the effects of bleomycin and spares more normal tissue. Based on the present study and other recent sea turtle studies (Brunner et al. 2014), ECT with bleomycin can be considered an effective alternative treatment for fibropapillomas in green turtles.

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