EFFICACY OF SUBCUTANEOUS IMPLANTS TO PROVIDE CONTINUOUS PLASMA TERBINAFINE IN HELLBENDERS (CRYPTOBRANCHUS ALLEGANIENSI) FOR FUTURE PROPHYLACTIC USE AGAINST CHYTRIDIOMYCOSIS

Authors: Hardman, Rebecca H., Cox, Sherry, Reinsch, Sherri Doro, Schwartz, Heather Coarsey, Skeba, Sandy, et al.

Source: Journal of Zoo and Wildlife Medicine, 52(1) : 300-305

Published By: American Association of Zoo Veterinarians

URL: https://doi.org/10.1638/2020-0158

DOI: 10.1638/2020-0158 *Journal of Zoo and Wildlife Medicine* 52(1): 300–305, 2021

EFFICACY OF SUBCUTANEOUS IMPLANTS TO PROVIDE CONTINUOUS PLASMA TERBINAFINE IN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS*) FOR FUTURE PROPHYLACTIC USE AGAINST CHYTRIDIOMYCOSIS

Rebecca H. Hardman, DVM, MS, PhD, Sherry Cox, MS, PhD, Sherri Doro Reinsch, BS, Heather Coarsey Schwartz, DVM, Sandy Skeba, AAS, LVMT, Dale McGinnity, BS, Marcy J. Souza, DVM, MPH, DABVP (Avian), DACVPM, and Debra L. Miller, DVM, MS, PhD, CWB

Abstract: *Batrachochytrium dendrobatidis* (*Bd*) is an important fungal pathogen present in wild hellbender (*Cryptobranchus alleganiensis*) populations that appears to cause disease during novel exposure and acute stress. Hellbender repatriation efforts are ongoing to combat declining populations, but mortality by chytridiomycosis (disease from *Bd*) after release has been reported. The goal was to determine whether a safe antifungal agent could be administered and provide prolonged plasma concentrations without repeated handling. A subcutaneous implant impregnated with 24.5 mg of terbinafine was tested in three juvenile eastern hellbenders (*C. a. alleganiensis*) raised in human care, and plasma terbinafine concentrations were recorded from weekly to biweekly for 141 days. Plasma concentrations were variable, with peak plasma concentrations of 1,610, 112, and 66 ng/ml between 28 and 56 days postimplant. Although all hellbenders achieved plasma concentrations above the published minimum inhibitory concentration for terbinafine against *Bd* zoospores (63 ng/ml) at several time points, only one individual remained above this threshold for more than two consecutive time intervals. Results show the potential for these implants as a prophylaxis for chytridiomycosis in captive-to-wild hellbender releases. However, further investigation will be needed to determine the plasma concentrations required to achieve prophylaxis in vivo and implant reliability.

INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*) is a globally important amphibian fungal pathogen that causes severe, often lethal, disease.[13] Motile zoospores infect keratinized epithelium and resulting disease (chytridiomycosis) occurs from disruption of skin function and osmoregulation of the amphibian host.[15] In highly susceptible frog species such as the Panamanian golden frog (*Atelopus zeteki*), death occurs within 2 mo postinfection.[2] In contrast, several salamander species appear to be tolerant where free-ranging populations are observed to maintain subclinical infection.[8,18] However, many of these apparently tolerant species can succumb to disease and experience acute mortality from chytridiomycosis when brought into human care or after experiencing an apparent stress event. For instance, wild populations of the California slender salamander (*Batrachoseps attenuatus*) can have a high prevalence of *Bd* with no observable pathology, but after wild *Bd*-positive individuals were brought to indoor facilities, these same individuals experienced 100% mortality from chytridiomycosis.[16] A case report of Doflein's salamanders (*Bolitoglossa dofleini*) described a similar phenomenon, wherein apparently healthy wild individuals developed chytridiomycosis within a week of transfer to human care facilities.[11] Presumably, these individuals maintained subclinical infections in the wild, only to be overcome by this pathogen during a time of stress and apparent decreased immunocompetency. Although the pathophysiology of this phenomenon of "chytridiomycosis in salamanders under stress" is currently only speculative, prophylactic measures could still be taken to reduce disease incidence.

Hellbenders (*Cryptobranchus alleganiensis*) are the only cryptobranchid species native to North America and have experienced considerable declines over the past few decades.[17] Like other salamander species, *Bd* is present in wild hellbender populations with little clinical disease[18] but is an important cause of mortality during times of novel exposure and acute stress.[5,6] Chytridiomycosis has been a particular challenge for repatriation efforts with reports of mortality rates as high as 100% in head-started individuals after captive-to-wild translocation.[5] Prevention of chy-

From the Center For Wildlife Health, Joe Johnson Boulevard (Hardman, Souza, Miller), and Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, Riverside Drive (Hardman, Cox, Souza, Miller), University of Tennessee, Knoxville, TN 37996, USA; and Nashville Zoo at Grassmere, 3777 Nolensville Road, Nashville, TN 37211, USA (Reinsch, Robertson, Schwartz, Skeba, McGinnity). Correspondence should be directed to Dr. Hardman (lavalizard17@gmail.com).

tridiomycosis needs to be a priority for hellbender conservation because juvenile head-starting is critical to maintain current declining populations. Specifically, a release protocol needs to be developed that will minimize animals succumbing to chytridiomycosis.

Antifungal prophylaxis has been successfully used in immunocompromised human cancer patients that are at higher risk for opportunistic infections.[1] A similar prophylactic treatment protocol could be applied to hellbenders slated for release with an increased risk to develop disease. However, treatment route presents a logistical problem. In human care settings, consecutive daily water bath treatments are effective for treating *Bd* infection in amphibians,[3,12] but this method is not an option for hellbenders that have been released into the wild. This large aquatic species uses large boulders for cover, and boulders are often too large to move, and present a risk of habitat destruction or injury to the animal if disturbed. Furthermore, retrieval of a hellbender in a natural stream can cause additional unwanted stress. A long-acting, safe, antifungal treatment resulting in minimal disturbance to animals and habitat during acclimatization would be greatly beneficial for head-starting programs where risk of chytridiomycosis is high.

Subcutaneous implants or injections can be a minimally invasive method for extended release drug delivery, especially in situations where patients cannot be handled daily. In ferrets (*Mustela putorius*) needing daily hormonal therapy for adrenal tumors, a small subcutaneous implant was created to provide continuous, long-acting absorption for successful consistent and long-term melatonin delivery for 6 mo.[9] This same technology could be used to deliver other drug types, including antifungal agents.

Terbinafine is a lipophilic antifungal agent known to accumulate in the dermis in people even when given orally.[10] It has been used in the treatment of chytridiomycosis in amphibians with no reports of toxicity,[12] as has been reported with other antifungals such as itraconazole.[3] Subcutaneous terbinafine implants have been tested in snakes and did provide therapeutic plasma concentrations for weeks in cottonmouths (*Agkistrodon piscivorus*), a species at risk for developing snake fungal disease.[7]

This study evaluated the efficacy of a long-acting, terbinafine-impregnated subcutaneous implant with the goal of preventing chytridiomycosis in hellbenders incorporated into repatriation programs. The objectives were to determine 1) any clinical changes in animals postimplant, 2) temporal changes in plasma terbinafine concentrations, and 3) average number of days postimplant that provide plasma terbinafine concentrations above the minimum inhibitory concentration (MIC) for *Bd* and, therefore, potential protection from developing chytridiomycosis.

METHODS

Animal housing

This study was performed from 22 May 2019 to 03 October 2019. Juvenile eastern hellbenders of unknown sex ($n = 3$) were maintained according to strict animal husbandry guidelines already in place and under zoo supervision (Nashville Zoo at Grassmere (TN); Institutional Animal Care and Use Committee Protocol 1902). Animals were produced and reared in a hellbender breeding research facility and were 3 yr old and at least 70 g at the time of the study. Animals remained in their home tank at a water temperature of 18°C (±1°C) separate from all other animals for the duration of the study and were given unique study IDs (known as individuals B, C, and D). Individuals were monitored daily for signs of clinical disease such as behavioral changes and gross skin lesions. To monitor for any weight loss, biometrics were collected before phlebotomy.

Implant placement

Implants ($4 \times 2 \times 1$ mm) were custom created with a base silicone elastomer impregnated with 24.5 mg of terbinafine by Melatek LLC (Middleton, WI 53578, USA). On day 0 of the trial, terbinafine implants were injected subcutaneously over the musculature lateral to the spine near the tail base. Hellbenders were sedated before implantation with benzocaine (0.2 g gel dissolved in 10 ml warmed water) added to 1 L of tank water, resulting in a 200 mg/L solution. Surgical glue was placed at the injection site, and individuals were monitored until full righting reflex and proper mentation had returned.

Plasma collection

Approximately 0.25 ml of peripheral blood via phlebotomy from the ventral tail vein was collected at time periods 0, 7, 14, 20, 28, 42, 56, 77, 92, 111, and 141 days postimplant, resulting in 11 samples per salamander. Animals were wrapped in a towel soaked in tank water with only the tail exposed to provide restraint with minimal stress

or injury. The original goal was to perform phlebotomy weekly; however, a notable decline occurred in individual packed-cell volume (PCV) after 1 mo, and phlebotomy was reduced to biweekly to monthly to prevent further anemia. Syringes were preheparinized before collection.

Terbinafine assay and analysis

Samples were collected in lithium heparin tubes and placed on ice for immediate transport to the laboratory on-site. Within 30 min, tubes were centrifuged and plasma stored at 80°C until ready for processing. Frozen samples were transported on dry ice in 1 day to the University of Tennessee Pharmacology Laboratory, where plasma terbinafine concentrations were measured by high-pressure liquid chromatography (HPLC).[4]

RESULTS

Animal health and metrics

All hellbenders gained weight over the course of the 141-day study with no development of gross lesions or behavioral abnormalities. Starting and ending weights were as follows: individual B (130–185 g), individual C (97–130 g), and individual D (125–153 g). As mentioned previously, PCV initially decreased (from 33% to 23% on average) at day 28, which was attributed to weekly phlebotomy that consisted of 0.25 ml (range 0.1%–0.3% body weight). This transient anemia resolved on switching to a maximum biweekly frequency of collection.

Plasma terbinafine concentrations

All three hellbenders achieved plasma terbinafine concentrations at or above the previously published MIC for *Bd* zoospores (63 ng/ml)[19] for at least two time points. Individual B had plasma concentrations at each sampling point well above this value, beginning from the first time point (86.0 ng/ml, 7 days postimplant) until the end of the study (270.1 ng/ml, 141 days postimplant), with a peak of 1,610 ng/ml on day 56 (Fig. 1, Table 1). Individuals C and D maintained much lower concentrations with less defined peaks. Individual C had a peak concentration of 66.2 ng/ml on day 56, whereas individual D had a peak of 112.0 ng/ml on day 28, with a second rise of 64.2 ng/ml on day 77 (Fig. 1, Table 1). For individual C, plasma concentrations were above the *Bd* zoospore MIC on days 56 and 77 (Table 1). For individual D, concentrations above the MIC were achieved on days 28 and 77 (Table 1). Day 77 was the only time point for which all three individuals had plasma terbinafine concentrations at or above the published MIC for *Bd* zoospores. No plasma concentrations achieved the previously published MIC or minimum lethal concentration (MLC) for *Bd* zoosporangia (2 and 100 μg/mL, respectively).[12]

DISCUSSION

The subcutaneous implants tested in this study have the potential to provide prolonged therapeutic concentrations of a safe antifungal agent for the prevention of chytridiomycosis. Although results varied widely across individuals, all achieved plasma concentrations above the MIC for *Bd* zoospores for at least two time points, with several concentrations lingering near this value. Even with very high concentrations, as observed in individual B, no animals displayed any adverse side effects.

Although concentrations in all three animals went above the MIC for *Bd* zoospores, no individual had concentrations that also qualified as above the MIC or MLC for *Bd* zoosporangia. This result could be seen as a potential implant failure; however, it is unknown what concentrations were achieved in the skin, which would be a more appropriate metric for the ability of these implants to affect a dermal pathogen. In fact, plasma concentrations may greatly underestimate concentrations achieved in the skin. Terbinafine is a lipophilic drug and is known to accumulate in skin and adipose tissue when given over a long period of time.[10] Terbinafine has also been shown to accumulate in frog skin and, with continuous daily prolonged water bath treatments, reach concentrations capable of clearing infections,[12] which provides promise that a continuous-release subcutaneous implant could also provide therapeutic concentrations of terbinafine at much higher concentrations than reported in plasma. Furthermore, these implants are meant as a prophylaxis and not as a treatment once disease has developed; concentrations may only need to reach the MIC as opposed to the MLC to prevent organism overgrowth during this critical time point.

Why there was such a discrepancy in plasma concentrations between individual B and individuals C and D is unknown. Unexplained wide variability is reported in use of these implants in other wildlife species.[7,14] Although individual B was the largest study animal, it was not much larger than the other two animals, and weight alone cannot fully explain the extreme difference

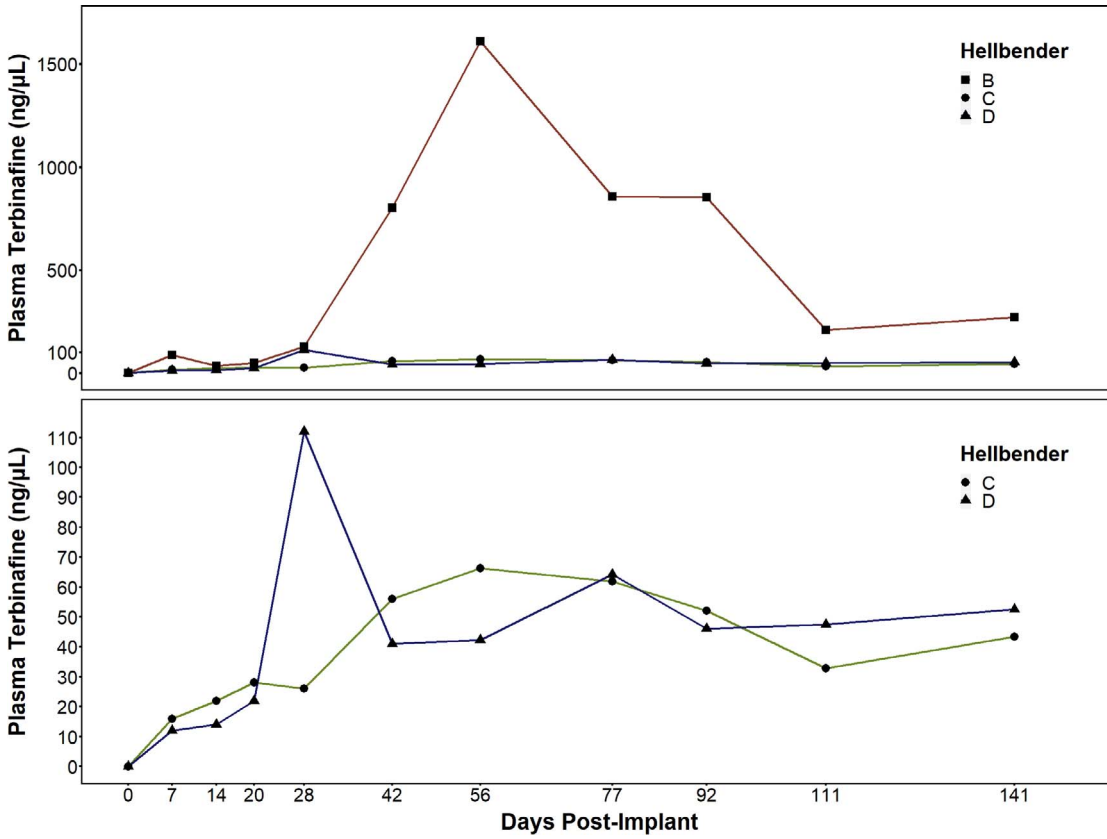

Figure 1. Line graphs displaying change in plasma terbinafine concentrations determined by HPLC in three juvenile hellbenders over the course of 141 days postinjection of subcutaneous implants. Each line represents plasma concentrations for a single individual over time (individuals A, B, and C). All three individuals are represented in the top graph. Because concentrations for individuals C and D were much lower than for individual B, a separate line graph without individual B is displayed in the bottom panel.

Table 1. Plasma terbinafine levels determined by HPLC in three juvenile hellbenders over the course of 141 days postinjection of subcutaneous implants. Plasma terbinafine concentrations are listed for each time point for each individual (Indv B, C, D).

Days postimplant	Plasma terbinafine (ng/ml)		
	Indv B	Indv C	Indv D
0	0.0	0.0	0.0
7	86.0	16.0	12.0
14	34.0	22.0	14.0
20	48.0	28.0	22.0
28	128.0	26.0	112.0
42	802.0	56.0	41.0
56	1610.1	66.2	42.2
77	857.0	61.9	64.2
92	852.8	52.1	46.1
111	208.0	32.8	47.5
141	270.1	43.4	52.6

in plasma concentrations. Variability of systemic absorption may be difficult to control, or conversely, there may be more variability in mobilization of terbinafine into other tissues from plasma, especially if there are marked differences in percent adipose tissue. Future studies comparing plasma vs skin concentrations paired with body condition are recommended to better understand terbinafine absorption, storage, and clearance from implants. An additional caveat to this study was a lack of sufficient plasma available to perform blood chemistry analysis on these individuals to evaluate biochemical markers of liver and other tissue damage after prolonged terbinafine exposure.

Although these implants certainly cannot be assumed to prevent chytrid overgrowth on hellbenders, this study illustrated they do achieve plasma concentrations at or above the MIC for *Bd* zoospores for at least 2, ranging up to 15, wk

consecutively. The use of these implants to clear already infected individuals, especially those with high infection burdens, is not recommended, but the hope is to adapt them to reduce mortality in hellbenders at risk for *Bd* exposure. These implants should be tested in wild releases to confirm efficacy before their use in hellbender translocation programs can be fully recommended. If successful, these implants could provide an economical and easy option for antifungal prophylaxis in juvenile hellbenders as individuals are exposed to *Bd* and a new environment. These implants may also be broadly applicable to other emerging fungal diseases of amphibians, including the related salamander chytrid, *Batrachochytrium salamandrivorans*, a potential global threat to salamanders.

There is an increasing demand for development of novel ways to combat complex wildlife health issues as humans continue to encroach on the native habitats of wildlife populations. It is hoped that these implants can serve as such a tool to increase success of future amphibian conservation programs.

Acknowledgments: The authors thank the American Zoo Association Amphibian Taxon Advisory Group (ATAG) Small Grant program for funding and the Nashville Zoo at Grassmere for project support.

LITERATURE CITED

1. Baden LR, Swaminathan S, Angarone M, Blouin G, Camins BC, Casper C, Cooper B, Dubberke ER, Engemann AM, Freifeld AG, Greene JN, Ito JI, Kaul DR, Lustberg ME, Montoya JG, Rolston K, Satyanarayana G, Segal B, Seo SK, Shoham S, Taplitz R, Topal J, Wilson JW, Hoffmann KG, Smith C. Prevention and treatment of cancer-related infections, version 2.2016, National Comprehensive Cancer Network clinical practice guidelines in oncology. J Natl Compr Canc Netw. 2016;14(7):882–913.

2. Becker MH, Harris RN, Minbiole KPC, Schwantes CR, Rollins-Smith LA, Reinert LK, Brucker RM, Domangue RJ, Gratwicke B. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. Ecohealth. 2011;8(4):501–506.

3. Brannelly LA. Reduced itraconazole concentration and durations are successful in treating *Batrachochytrium dendrobatidis* infection in amphibians. J Vis Exp. 2014;2014(85):51166.

4. Cox, S, Hayes J, Hamill M, Martin A, Pistole N, Yarbrough J, Souza M. Determining terbinafine in plasma and saline using HPLC. J Liq Chromatogr Relat Technol. 2015;38(5):607–612.

5. Dean N, Ossiboff R, Bunting E, Schuler K, Rothrock A, Roblee K. The eastern hellbender and *Batrachochytrium dendrobatidis* (*Bd*) in western New York. In: Proc 65th Int Conf Wildl Dis Assoc; 2016. p. 151.

6. Dusick A, Flatland B, Craig L, Ferguson S. What is your diagnosis? Skin scraping from a hellbender. Vet Clin Pathol. 2016;46(1):183–184.

7. Kane LP, Allender MC, Archer G, Leister K, Rzadkowska M, Boers K, Souza M, Cox S. Pharmacokinetics of nebulized and subcutaneously implanted terbinafine in cottonmouths (*Agkistrodon piscivorus*). J Vet Pharmacol Ther. 2017;40(5):575–579.

8. Muletz C, Caruso NM, Fleischer RC, McDiarmid RW, Lips KR. Unexpected rarity of the pathogen *Batrachochytrium dendrobatidis* in Appalachian *Plethodon* salamanders: 1957–2011. PLoS One. 2014;9(8): e103728.

9. Murray J. Melatonin implants: an option for use in the treatment of adrenocortical disease in ferrets. Exot Mammal Med Surg. 2005;3(1):1–6.

10. Newland JG, Abdel-Rahman SM. Update on terbinafine with a focus on dermatophytoses. Clin Cosmet Investig Dermatol. 2009;(2):49–63.

11. Pasmans F, Zwart P, Hyatt AD. Chytridiomycosis in the Central American bolitoglossine salamander (*Bolitoglossa dofleini*). Vet Rec. 2004;154:153.

12. Roberts AA, Berger L, Robertson SG, Webb RJ, Kosch TA, McFadden M, Skerratt LF, Glass BD, Motti CA, Brannelly LA. The efficacy and pharmacokinetics of terbinafine against the frog-killing fungus (*Batrachochytrium dendrobatidis*). Med Mycol. 2019;57(2):204–214.

13. Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukem A, Acevedo AA, Burrowes PA, Carvalho T, Catenazzi A, De la Riva I, Fisher MC, Flechas SV, Foster CN, Frias-Alvarez P, Garner TWJ, Gratwicke B, Guayasamin JM, Hirschfeld M, Kolby JE, Kosch TA, La Marca E, Lindenmayer DB, Lips KR, Longo AV, Maneyro R, McDonald CA, Mendelson III J, Palacios-Rodriguez P, Parra-Olea G, Richards-Zawacki CL, Rodel M-O, Rovito SM, Soto-Azat C, Toledo LF, Voyles J, Weldon C, Whitfield SM, Wilkinson M, Zamudio KR, Canessa S. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science. 2019;363(6434):1459–1463.

14. Souza MJ, Redig P, Cox SK. Plasma concentrations of itraconazole, voriconazole, and terbinafine when delivered by an impregnated, subcutaneous implant in Japanese quail (*Coturnix japonica*). J Avian Med Surg. 2017;31(2):117–122.

15. Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, Cook D, Webb R, Alford RA, Skerratt LF, Speare R. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science. 2009;326(5952):582–585.

16. Weinstein SB. 2009. An aquatic disease on a terrestrial salamander: individual and population level effects of the amphibian chytrid fungus, *Batrachochy-*

trium dendrobatidis, on *Batrachoseps attenuatus* (Plethodontidae). Copeia. 2009;2009(4):653–660.

17. Wheeler BA, Prosen E, Mathis A, Wilkinson RF. Population declines of a long-lived salamander: a 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. Biol Conserv. 2003;109(1):151–156.

18. Williams LA, Groves JD. Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in eastern hellbenders (*Cryptobranchus alleganiensis*) in western North Carolina, USA. Herpetol Conserv Biol. 2014;9(3):454–467.

19. Woodward A, Berger L, Skerratt LF. In vitro sensitivity of the amphibian pathogen *Batrachochytrium dendrobatidis* to antifungal therapeutics. Res Vet Sci. 2014;97(2):364–366.

Accepted for publication 12 November 2020