Photobiomodulation therapy using a class 3b laser does not cause premature rupture of multivesicular liposomal bupivacaine suspensions in a cadaver model

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OBJECTIVE
To determine if photobiomodulation causes a premature release of liposomal bupivacaine (LB) suspensions.

ANIMALS
A 25-kg mixed breed dog cadaver euthanized for reasons unrelated to this study.

METHODS
In September 2022, a proximomedial tibial incision was made in a dog cadaver, and a tibial plateau leveling osteotomy plate was implanted. A stab incision was made one-half inch distal to the incision, and a tunnel to the plate was created prior to closure of the primary wound. A 3-cc anal sac catheter was advanced through the distal incision until the bulb rested against the face of the plate. Seven treatment groups of treatment power (watts) and total energy (joules/cm²) were defined as: A, 0.5 W, 2.0 J/cm²; B, 0.5 W, 4.0 J/cm²; C, 0.5 W, 6.0 J/cm²; D, 1.0 W, 2.0 J/cm²; E, 1.0 W, 4.0 J/cm²; F, 1.0 W, 6.0 J/cm²; and sham, 0.0 W, 0.0 J/cm². Ten samples per group of 2 mL of LB were infused into a new catheter and treated percutaneously with a class 3b laser. All samples remained in the catheter for 12 seconds to reflect the longest treatment time. Post-treatment free bupivacaine concentrations were identified with high-performance liquid chromatography.

RESULTS
The median free bupivacaine concentration was reported as: sham, 1.89 mg/mL; A, 1.93 mg/mL; B, 2.01 mg/mL; C, 2.05 mg/mL; D, 1.92 mg/mL; E, 2.03 mg/mL; and F, 2.00 mg/mL. There were no differences in median free bupivacaine concentrations between groups (P = .988).

CLINICAL RELEVANCE
Concurrent LB and photobiomodulation are recommended during the postoperative period. The results of this proof-of-concept study suggest that concurrent use of LB and photobiomodulation may be safe, but in vivo studies at similar and stronger photobiomodulation settings are warranted.

Keywords: photobiomodulation, free bupivacaine, liposomal bupivacaine, low level laser therapy, TPLO

Liposomal bupivacaine is a multivesicular liposomal suspension reported to be safe and effective as a long-acting local anesthetic following tibial plateau leveling osteotomy (TPLO) in dogs.1 For up to 72 hours, the outer vesicles of the multivesicular structure degrade at a known rate, releasing a continuous therapeutic dose of bupivacaine into the local tissue. However, premature release of potentially myotoxic doses of bupivacaine have been reported due to exposure of the vesicles to concurrent therapies.2–5

Photobiomodulation (PBM), or low-level laser therapy, refers to the use of red or near-infrared light as a therapy to stimulate biologic processes. Although there is little positive evidence for PBM’s use as an adjunct therapy following TPLO,6–8 PBM’s use has been reported to accelerate the healing of surgical and chronic wounds9,10 and reduce pain in osteoarthritic hips and elbows.11,12 As a result of these findings, PBM has been recommended and implemented in many standard rehabilitation plans following surgical procedures, including TPLO, as a
means to improve surgical wound healing and reduce inflammation and inflammation-related pain.

Though liposomal bupivacaine and PBM are both utilized in the period immediately following TPLO procedures, it is unknown if PBM therapy causes premature rupture of the vesicles that contain liposomal bupivacaine, which may result in the loss of desired efficacy with or without local myotoxicity. The objective of this study was to determine if PBM causes premature release of bupivacaine from liposomal bupivacaine suspensions. Our null hypothesis was that there would be no difference in free bupivacaine in samples treated with or without PBM therapy.

**Methods**

This study was approved by the BluePearl Science Study Design and Review Committee. A client-owned dog with no known current or historical pelvic limb disease was euthanized for reasons unrelated to this study, and written client consent was obtained prior to use of the cadaver. The dog’s remains were ethically disposed of per the client’s wishes after completion of this study.

Power calculations were performed with standard software (G*Power, version 3.1.9.2; Heinrich Heine Universität Düsseldorf). A significance threshold of 0.05 and power of 0.80 were assumed as well as a two-sided test. For simplicity, a Student t test was assumed for the analysis of the primary outcome, which was free bupivacaine concentration. The mean and standard deviation for the control group were assumed, based on preliminary data, to be 1.7 mg/mL and 0.2 mg/mL, respectively. To detect a 20% increase from control in the PBM group concentrations (to 2 mg/mL), a sample size of 9 samples per group was needed. Rounding up to 10 samples per treatment resulted in an actual power of 84%.

**Test site preparation**

A proximomedial tibial incision was made on the cadaver, soft tissues were dissected from the tibia, and a 316L stainless-steel TPLO plate was secured to the proximomedial tibia using Kirschner wires without performing an osteotomy (Figure 1). A stab incision was then made 2 cm distal to the incision, and a tunnel was dissected underneath the fascia using an AO elevator so that a 3-mL silicon bulb catheter (6-French anal sac balloon catheter; Mila International) could be advanced through the distal incision alongside a continuous-read thermometer probe connected to a multiparameter anesthetic monitor until they were overlaying the TPLO plate. The primary incision site was closed routinely.

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**Figure 1**—An approximately 25-kg dog cadaver was used to collect proof-of-concept data regarding the potential impacts of photobiomodulation at clinically relevant power and total energy settings on liposomal bupivacaine suspensions in September 2022. A—A proximomedial approach to the tibia of a dog cadaver was performed, and a 316L stainless-steel tibial plateau leveling osteotomy (TPLO) plate was implanted against the tibia without performing a TPLO. The primary wound was closed routinely (*). B—Subsequently, a stab incision was created distal to the primary incision (arrowhead), and soft tissue was dissected to create a tunnel of adequate size to advance a 6-French anal sac catheter alongside a continuous-read thermometer probe so that they overlayed the implanted TPLO plate. C—Deconstructed incision site showing 2 mL of liposomal bupivacaine infused into the anal sac catheter to fill the bulb (arrow) adjacent to the TPLO stainless-steel TPLO plate prior to photobiomodulation treatment.
Laser protocol

A class 3b, continuous wave, 808-nm wavelength laser (Luminex Vet; Respond Systems) with variable (user-defined) power (watts) and total energy (joules/cm²) settings was used for all treatments. Seven sample groups (1 sham group and 6 treatment groups) of 10 samples each were defined as follows: A: 0.5 W, 2.0 J/cm²; B: 0.5 W, 4.0 J/cm²; C: 0.5 W, 6.0 J/cm²; D: 1.0 W, 2.0 J/cm²; E: 1.0 W, 4.0 J/cm²; and F: 1.0 W, 6.0 J/cm². The sham treatment group received no laser treatment. Due to a void of evidence-based therapeutic laser treatment settings for the treatment of postoperative TPLO surgical sites, these settings were extrapolated from previous PBM/TPLO publications7–10 and anecdotal use recommendations from sales representatives and clinical practitioners that use a laser in postoperative TPLO cases.

Testing procedure

Liposomal bupivacaine (Nocita) samples were extracted in 2-mL aliquots from 20-mL vials with the same manufacturer lot number using a 3-mL syringe and 22-gauge needle. A new anal sac catheter was advanced alongside the continuous-read thermometer probe so that the bulb and probe tip rested against the face of the TPLO plate. The 2-mL aliquot was infused into the anal sac catheter and partially filled the 3-mL bulb, and the liposomal bupivacaine in the bulb was treated percutaneously according to the group assignment. The peak temperature registered on the monitor was recorded for all samples. All samples remained in the anal sac catheters for a total of 12 seconds to reflect the longest treatment time. Following treatment, the liposomal bupivacaine was removed from the anal sac catheter with a new 3-mL syringe and deposited into a cryovial (Simport Scientific) and refrigerated at 4 °C until analysis. Unencapsulated bupivacaine concentrations were compared between groups with ANOVA.

Sample analysis

All samples were stored, prepared, and analyzed as described by Carlson et al.13 Liposomal bupivacaine samples were centrifuged at 3,500 X g for 30 minutes at 4 °C. Two hundred fifty microliters of supernatant were collected and stored at −80 °C until analysis. Unencapsulated bupivacaine concentrations were determined by using HPLC (1260 Infinity HPLC System with a multiwavelength detector; Agilent Technologies). The ultraviolet detector was set at a wavelength of 210 nm. The column was a 4.6 X 150-mm C18 column (Zorbax SB-C18; MAC-MOD Analytical) kept at a constant temperature of 4 °C and a flow rate of 1 mL/min. The mobile phase consisted of 65% water, 35% acetonitrile, 0.02% trifluoroacetic acid, and 0.01% triethylamine. Samples were diluted 100-fold in water. Fifty microliters of diluted sample were injected into the HPLC system. The calibration curve was linear, with an R² value of 0.99 or higher. Limit quantification for bupivacaine was 0.05 μg/mL, which was determined from the lowest point on a linear calibration curve that produced an appropriate signal-to-noise ratio by using guideline.14

Statistical analysis

All analyses were performed using standard software (SAS, version 9.4; SAS Institute Inc). A significance threshold of 0.05 was used. Quantile-quantile plots, probability plots, and histograms of data from each group and linear model residuals were examined to evaluate the assumption of normality. The assumption was reasonable for peak temperature. However, free bupivacaine concentrations were left-skewed and Box-Cox transformed prior to analysis, which normalized the distributions. Transformed free bupivacaine concentrations and peak temperatures were compared between groups with ANOVA.

Results

The mean (± SD) free bupivacaine concentrations were reported as: sham treatment, 1.89 ± 0.46 mg/mL; A, 1.93 ± 0.46 mg/mL; B, 2.01 ± 0.36 mg/mL; C, 2.05 ± 0.19 mg/mL; D, 1.92 ± 0.42 mg/mL; E, 2.03 ± 0.32 mg/mL; and F, 2.00 ± 0.34 mg/mL. The mean (± SD) peak treatment temperatures were reported as: sham treatment, 20.12 ± 0.02 °C; A, 20.13 ± 0.04 °C; B, 20.13 ± 0.04 °C; C, 20.12 ± 0.04 °C; D, 20.16 ± 0.06 °C; E, 20.12 ± 0.05 °C; and F, 20.12 ± 0.04 °C.

There were no differences between groups in free bupivacaine concentrations (P = .988) or peak treatment temperatures (P = .637).

Discussion

We examined the effects of 2 PBM variables (treatment power and total energy) on the premature release of bupivacaine from a liposomal suspension. Our data provide evidence that there was no significant release of bupivacaine from the liposomes when PBM was performed using an 808-nm wavelength at total energies determined to improve wound healing by Wardlaw et al.9 In light of these findings, we failed to reject our null hypothesis.

Recommendations for liposomal bupivacaine use state not to expose the liposomal suspension to anything that may disrupt it due to the possibility of premature release. Premature release, at the least, results in diminished efficacy of an expensive local anesthetic and, at worst, may result in myotoxicity at the injection site with or without elevated serum concentrations. Although the myotoxic dose of bupivacaine is not known, it is recommended that doses should be limited to less than 2.0 mg/kg to minimize the risk of local toxicity.15 The highest concentration of free bupivacaine in any treatment group in this study was 2.05 mg/mL. The normal dosing of liposomal bupivacaine is 0.4 mL/kg, which would result in a tissue concentration of 0.82 mg/kg based on the highest free bupivacaine concentration of our sample groups. This is well below the recommended maximum tissue concentration of 2.0 mg/kg for the prevention of local myotoxicity and the threshold for systemic toxicity.11,12
cardiotoxicity and neurotoxicity (seizures) of 4.3 mg/kg associated with intravenous bupivacaine dosing.\textsuperscript{16}

This study was performed using a class 3b PBM device with a therapeutic wavelength of 808 nm because Hochman-Elam et al\textsuperscript{17} reported the greatest tissue penetration regardless of coat length or color in dogs with a mixed-frequency PBM device using both 810-nm and 990-nm wavelength light. Although their use of a mixed-frequency PBM device obscured the true optimal wavelength for light penetration through cadaver dog coat and soft tissue, the use of approximately 810 nm has been reported in TPLO PBM studies\textsuperscript{6,7} which suggests that it is a commonly prescribed therapeutic dose in dogs. However, it is possible that other wavelengths of PBM may have a greater impact on liposomal bupivacaine.

Manufacturer recommendations suggest avoiding exposure of liposomal bupivacaine to extreme temperatures due to the risk of premature rupture of the liposomes. This was hypothesized to be a potential cause of premature release associated with PBM, especially in groups with increased power or energy settings. The maximum power and energy density tested in our study were 1.0 W and 6.0 J/cm\textsuperscript{2}, respectively, which are greater than the single-session energy density value of 4.0 J/cm\textsuperscript{2} that was shown to improve wound healing by Wardlaw et al.\textsuperscript{3}

However, the peak temperature and free bupivacaine concentrations were unchanged compared to the control sample in any groups. This may be the result of the use of a class 3b PBM device rather than a more powerful class 4 device. Class 4 PBM devices may have different focal spot sizes and increased power (up to 16 W), which would increase the potential heat production. However, although class 4 lasers are used in veterinary medicine, current PBM treatment recommendations for wound healing at nascent surgical sites are consistent with the power and total energy settings tested in this study.\textsuperscript{9}

The lack of peak temperature changes in this study may be attributed to the use of a continuous-read thermometer probe with readings registered on a multiparameter veterinary anesthetic monitor. Although no significant change in temperature were recorded as the result of any PBM treatments, many multiparameter monitors use algorithms to create an average reading over a short period of time to provide a more stable readout. Since the PBM treatments in this study were short in duration, the algorithm may have masked any brief increases in temperature. Additionally, the exact position of the thermometer probe during PBM treatment was obscured by the soft tissues, so it is possible that the probe was not being directly treated by the PBM. Although a recording of the liposomal bupivacaine's temperature during treatment would have been more accurate, the probe could not be inserted into the anal sac catheter with the liposomal bupivacaine without rupturing the bulb and releasing the sample. Future studies may be improved by using a thermocouple instead of a continuous-read thermometer probe.

A limitation of this study was that we tested a single wavelength (808 nm) with a single PBM device. There are numerous other PBM devices available, with wavelengths generally ranging from 600 to 1,200 nm, and it is possible that other wavelengths will interact differently with the liposomes and cause premature release of the bupivacaine. In addition, other laser units have different focal spots and delivery mechanisms (continuous and pulsed, which may alter local energy delivery) and increased power or irradiance capabilities, which may cause premature breakdown of the liposomes.

Further, the clinical significance of our results may be limited for several reasons. First, this study was performed using a dog cadaver rather than a living dog. A cadaver model was chosen because of the risk associated with injecting of large volumes of liposomal bupivacaine into the same site. Even though all samples were housed in an anal sac catheter during treatment, malfunction of the anal sac catheter’s bulb receptacle would have released large doses of liposomal bupivacaine into the surrounding tissue, which may have caused the dog harm. The impacts of using a cadaver instead of living tissue on the results are unknown, but we consider the buildup of heat associated with PBM to be the greatest risk factor for liposomal bupivacaine, and we expect that a living dog’s vascular circulation would help to dissipate heat rather than add to it. The second limitation associated with using a cadaver model was that a single cadaver specimen was used for our testing. Tissue thickness and density vary between dogs and can alter the penetration of the laser. Our cadaver represented an average 20-kg dog, and it is possible that PBM treatments may have better skin/soft tissue penetration in smaller dogs. Pigmentation of the skin also varies between dogs, and it is known that darker skin tones will absorb more of the laser energy. Our single cadaver specimen does not cover all ranges of dog skin tones, and it is possible that patients with darker skin tones or spots may have areas of increased energy absorption. Finally, the placement of the liposomal bupivacaine bulb was in the fascial layer, which represents the recommendations for injection following TPLO. However, injection of liposomal bupivacaine superficially in the subcutaneous tissue may result in different outcomes.

In conclusion, our null hypothesis that there would be no difference in free bupivacaine concentrations in samples treated with or without PBM was accepted. Concurrent use of liposomal bupivacaine and PBM following TPLO, at the PBM device settings described in this report, were safe. However, the impacts of PBM at the tested settings or with a more powerful class 4 laser on liposomal bupivacaine in living tissue were not tested in this study, and concurrent use of PBM and liposomal bupivacaine in living animals should be used with caution.

**Acknowledgments**

Deborah Keys, PhD, performed all statistical analyses. Lab analyses were performed by Dr. Kristen Messenger’s lab at North Carolina State University’s College of Veterinary Medicine. The photobiomodulation device was provided by Respond Systems.
Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

Funding

The research was funded by the authors’ departments.

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