The Use of Liposomal Bupivacaine as an Incisional Analgesic and its Effects on Wound Healing

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Abstract:

Application of local anesthetics can improve pain relief post-operatively; however, local anesthetics have been reported to result in impaired wound healing. The objectives of this study are: 1) to determine if administration of a local injection of liposomal bupivacaine (NOCITA®) will provide anesthesia over a surgical incision; 2) to determine if NOCITA will delay wound healing; and 3) to determine if NOCITA alters the inflammatory and growth factor responses at the incision site. We hypothesize that administration of NOCITA will provide local analgesia over a period of days, will not delay healing, and will have a minimal effect on the inflammatory and growth factor responses at the incision site. To test the hypotheses, two skin incisions will be made on either side of the flank of six horses. Immediately after the incision is made, one incision will receive a subcutaneous injection of NOCITA®. The opposing incision will serve as the control and will receive an equivalent volume of saline. Incisions will be evaluated twice a day, and incisional margin biopsies for histology and gene expression determinations will be collected on day 0, 3, 7, and 14. Pain will be assessed using Von Frye filaments. Incisional healing will be assessed subjectively and histologically. The inflammatory response and growth factor production will be determined via RT-qPCR, and the number of neutrophils/macrophages at the incision margin will be determined via immunohistochemistry of MAC387 staining. Results of this study will provide veterinarians with information regarding NOCITA related analgesia efficacy and incisional healing in horses.

SPECIFIC AIM and OBJECTIVES:

Post-operative pain management at the incision site after equine surgeries is a severely under-studied topic in veterinary medicine. It has been shown in humans that liposomal bupivacaine provides excellent analgesia and shortens recovery time.[1] Administration of liposomal bupivacaine intra-operatively could increase patient comfort levels, decrease recovery time, and reduce the need for IV analgesics, thus lowering treatment costs for clients.[1] A prolonged duration of action lasting from 72 hours up to 7 days post-operatively with few side effects has been reported in humans.[2] Therefore, the use of liposomal bupivacaine has been determined to be beneficial after certain surgical procedures in people. However, the efficacy and duration of action is unknown in the equine patient. Furthermore, some studies have demonstrated negative effects on wound healing with the use of local anesthetics at the surgical wound site.[3,4] The objectives of this study are as follows:

Specific Aim 1 (H1): *To determine if liposomal bupivacaine (NOCITA®) can provide an analgesic effect when administered subcutaneously at the incision site. Duration of analgesic effect will also be determined.*

Specific Aim 2 (H2): *To determine if liposomal bupivacaine (NOCITA®) will alter the inflammatory and growth factor responses or wound healing process at the surgical incision.*

Overall, we hypothesize (H1) that bupivacaine will result in significant analgesia at the incision site for a minimum of 48-72 hours when compared with control incisions. We also hypothesize (H2) that bupivacaine will cause no significant alteration to the inflammatory and growth factor responses or wound healing process when compared with control incisions.

INTRODUCTION:

The FDA recently approved liposomal bupivacaine for postoperative care.[2] The liposomal aspect of bupivacaine has a double phospholipid layer that allows the bioavailability of the drug to be longer while slowing the absorption of bupivacaine into the reticuloendothelial system.[1] Release of bupivacaine from the liposome has been reported to occur for up to 72 hours to 7 days post-adminstration.[5] Continuous administration of bupivacaine via a wound infiltration catheter allowed for effective analgesia, reduced morphine consumption, and decreased post-operative ileus in people.[5] The main concern with the use of bupivacaine is the effect it may have on the wound healing process. Bupivacaine has been reported to reduce collagen production and wound breaking strength and increase edema/inflammation scores when administered in surgical wounds of rats.[3] Other local anesthetics such as lidocaine have been reported to impair fibroblast proliferation and expression of dermal collagens; thereby, affecting wound healing.[4] However, clinical studies in people administered liposomal bupivacaine post-operatively have not demonstrated any significant complications in the wound healing process.[6]

Most local anesthetics used for pain management in horses are short-acting and usually must be supplemented with a long-acting analgesic. Equine post-operative pain management typically includes the choices of systemic, intravenous administration of non-steroidal anti-inflammatory medications, lidocaine, and morphine, depending on the severity of the pain associated with the surgical procedure. Due to the large total dosages needed for systemic treatment, the cost of pain management post-operatively in horses can become expensive. Liposomal bupivacaine could provide proper analgesia at the incision site and increase the patient's initial comfort level; thus, requiring less of the intravenous pain medications mentioned above.

Overall, the current study will determine if liposomal bupivacaine has an anesthetic/analgesic effect in horses. The duration of action in horses will also be determined. The wound healing process will be assessed clinically and experimentally through the evaluation of leukocyte migration, fibroblast proliferation, and production of inflammatory mediators and growth factors.

EXPERIMENTAL METHODS:

Experimental design: The protocol for this research project will be approved by the University Animal Care and Use Committee. Six horses will be used from the LSU equine research herd for the study. Two surgical incisions, one in each paralumbar fossa (**Figure 1**) will be made followed by local injection of either a liposomal bupivacaine suspension (NOCITA®) or saline control. Analgesia/sensation and overall pain perception will be assessed using the Von Frey filament system and systemic pain scoring system for horses, respectively. Biopsies will be taken on day 0, 3, 7, and 14. Histological assessment and immunohistochemistry will be performed on the biopsies to evaluate overall inflammation (edema, vascularization, etc), wound strength (via fibroblast quantification), and migration of white blood cells (WBC; via MAC387 staining for neutrophil/macrophage quantification) into the incision margin. Gene expression of inflammatory mediators (IL-1β, IL-6, IL-8) and growth factors (TGF-β and IGF-1) will be assessed from tissue collected from the biopsies.

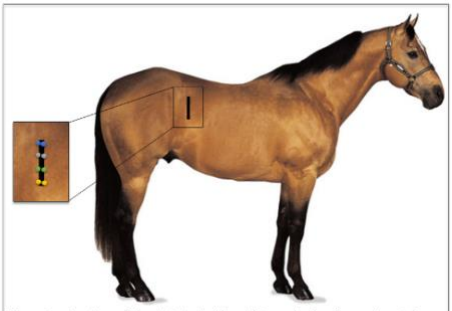

Figure 1. Location of the incision in the right paralumbar fossa. Insert box demonstrates the location of biopsies taken on day 0, 3, 7, and 14. Biopsy sites will be randomized per horse.

Horses: Six healthy horses will be used for the study. The horses will be of varying ages and will be either Thoroughbreds or Quarter horses. A physical exam will be taken on each horse prior to sedation, and if any abnormalities are found, a different horse will be used for the study. After the incision is made, the horse will be allowed to go out into the pasture, but will be brought up twice a day for physical examination and incisional assessment and closely monitored for any incisional complications.

Surgical incisions: Horses will be placed in the stocks and sedated intravenously with detomidine and butorphanol. A short acting local anesthetic (mepivacaine 2%) will be used to perform an inverted L block in the paralumbar fossa. Both the right and left sides will be used. A 12 cm skin incision will be made ½ between the last rib and ventral aspect of the tuber coxae. The incision will continue through the subcutaneous tissue down to the level of the external rectus sheath. NOCITA® treatment (**Figure 2**) will be randomly assigned to either the right or left incision, with the opposing incision receiving an injection of sterile saline to serve as a negative control. Prior to administering treatment, 2 biopsies (8 mm each) will be taken from the wound margin. The skin will be closed in a simple interrupted pattern using 0 prolene suture. An additional 2 biopsies will be taken from the incisional margin on day 3, 7, and 14. One sample will be snap frozen in liquid nitrogen and one will be placed in formalin at each time point for further analysis.

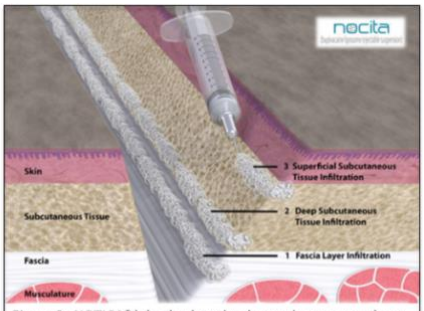

Figure 2. NOTICA® injection into the deep subcutaneous tissue along the incision line. Image from www.nocita.aratana.com

Pain Assessment: To place a numerical value on the amount of analgesia that the liposomal bupivacaine may have surrounding the incision, a Von Frey filament system will be used to assess pain. Briefly, filaments are applied at increasing size (which corresponds to pressure) and the point at which the horse has sensation is recorded.[7] Filaments will be applied to predetermined locations along the incisional line. The readings will be blindly taken by two individuals unaware of the treatment applied to eliminate bias. Filaments will also be applied prior to sedation and placement of the incision to determine baseline values for each horse. Blinders will be used on the horses to prevent the association of these filaments with pain. Filaments will be applied prior to biopsy sampling on days 3, 7, and 14. Overall pain scores will also be assessed daily for 14 days.

Assessment Wound Strength: Fibroblasts play an important role in the wound healing process. During an injury, fibroblasts lay down the extracellular matrix and ultimately promote wound closure.[8] A Texas Red-

conjugated phalloidin stain will be used to observe the quantity of fibroblasts present in the wound at that time. The tissue will be kept overnight in a PBS rinse. The tissue will then be stained with the Texas Red-conjugated phalloidin stain for 40 minutes at 4°C and then counter stained for two minutes with Sytox Green.[9] The number of fibroblasts observed in comparison to the control will allow the wound strength to be quantified. This will determine if liposomal bupivacaine has any effect on the process of wound healing.

Assessment of WBC migration: Migration of neutrophils and macrophages to the incisional margin will be assessed using MAC387 staining. Immunohistochemistry will be performed as previously reported.[10] Briefly, biopsy samples fixed in formalin will be paraffin embedded and 5µ sections will be place on glass slides. After deparaffination and dehydration, slides will undergo antigen retrieval followed by blocking with goat serum for 1 hour. Slides will be incubated in primary antibody diluted per manufacturer's recommendation for 90 minutes at 37°C. Secondary antibody will be applied and DAB used to reveal positive cells. Slides will be counterstained with hemotoxyllin. Positive cells will stain brown and will be quantified at 20x magnification. The number of positive cells between treatment groups will be determined.

Isolation of total RNA, cDNA production, and RT-qPCR: Total RNA will be isolated from biopsy tissue as previously described followed by mRNA isolation using kits per the manufacturer's instructions.[11] Complementary DNA will be made via reverse transcription. RT-qPCR will be performed using a thermocycler and quantified with external standards with the fluorescent format for SYBR Green I dye as previously described.[11] Primers will be designed from reported equine specific sequences.[11] Each gene specific primer set will have a linearized vector containing the cDNA product to use as a template for production of a standard curve.[11] Standard curves (10^5-10^0 copies), water (negative control), and all samples in duplicate will be performed for each gene of interest. The amplification data obtained by RT-PCR for the different genes will be divided by a normalization factor (obtained using GeNorm software) of the housekeeping genes in the same sample as previously described.[11] After normalization, the mean (±SEM) copy number for the selected mediators mRNA concentrations will be compared between control and each treatment group and fold change calculated for the principal groups.

Statistical Analysis: All data will be tested for normality. Data will be analyzed by ANOVA with post-hoc comparisons to compare groups, if data have normal distributions. If data is not normal, transformations will be performed in attempts to achieve normal distributions. If data is not normally distributed, non-parametric testing will be performed to assess differences between groups. Immunofluorescence results will be assessed for cellular and structural location by qualitative description. Significance will be set at $P<0.05$.

ANTICIPATED OUTCOMES:

1. The liposomal bupivacaine injected subcutaneously at the incision site is expected to provide adequate analgesia without the use of other pain medications.
2. It is also expected that the liposomal bupivacaine will cause no alteration in the inflammatory and growth factor responses or the healing time for the surgical wound.

POTENTIAL PITFALLS:

- If delays or impairment of surgical wound healing occurs, it will be unknown if it is a result of the bupivacaine or the liposomal component of the NOCITA®. However, future studies could be designed to evaluate this.

- Biopsy at day 3, 7, and 14 could result in local inflammation and delay wound healing. However, biopsy sites will be randomized and compared to controls; therefore, only differences between the two groups will be noted.
- Dosage and amount of NOCITA® is extrapolated from use in humans. The bioavailability and pharmacokinetics of NOCITA in horses is unknown. To provide more information, samples of jugular blood and wound margin tissue will be collected for future analysis
- It is possible that surgical incision in the upper body will respond differently than surgical incision of the distal limb, as wound healing properties do vary between these locations in the horse. Flank incisions are common in the horse, particularly for laparoscopic spays and cryptorchid surgeries. This site is beneficial because it can be performed standing, and local blocks for making the incisions and performing biopsies can easily be made away from the surgical incision site. Surgical incisions in the paralumbar fossa were chosen for their ease of performing a local block away from the location of incision and their ability to be kept clean while horses are out in pasture.
- Antibody use for laboratory procedures performed with equine samples can always be a concern, as they are not equine specific. However, antibodies to be used in this proposal have been used in our laboratory with success in equine samples. RT-qPCR primer and equine specific standards have also been designed and used within our laboratory.

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