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Original Research

Dose-Finding in the Development of an LPS-Induced Model of Synovitis in Sheep

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Models of transient synovitis that can be controlled with antiinflammatory and analgesic drugs have been used to study pain amelioration. To this end, we aimed to determine the dose of intraarticularly administered *E. coli* LPS that induced signs of synovitis without systemic signs in clinically healthy male castrated sheep (n = 14). In phase 1, a single dose of LPS (0.5, 1.0, 1.5, or 2.0 ng in a total volume of 0.5 mL) was administered into the right stifle joint. In phase 2, a dose of LPS (1.0 or 2.0 μg) in 0.3 mL was administered to 4 naïve sheep. In phase 3, 4 sheep from phase 1 were inoculated after a 60 d washout period with either 0.5 or 1.0 μg of LPS. During the first 48 h after LPS administration, the following were performed: assessment of clinical parameters; scoring for lameness, pain on limb flexion, and local swelling; and ultrasonography of the joints were performed. The doses tested during phase 1 produced subtle signs. During phase 2, mild to moderate lameness with no evidence of systemic signs occurred at both doses. In phase 3, clinical responses were similar between the 0.5- and 1-μg doses. Signs of swelling were not observed at any time. Therefore, we consider the 0.5-μg to be the most appropriate for this model, because it was the lowest dose tested capable of causing lameness without signs of systemic inflammation in all animals.

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As an experimental model for the study of arthropathies, the aseptic administration of small doses of endotoxin in the joint induces mild to moderate inflammation and the development of clinical signs similar to those of the naturally occurring disease.[6] Some studies have used models of transient synovitis to determine whether the associated pain can be controlled with antiinflammatory and analgesic drugs. The use of an LPS-induced model of synovitis to evaluate the analgesic effect of various therapeutic protocols has mainly been reported for horses.[9,16,27,28] However, sheep are an important model species in biomedical research, particularly in orthopedic studies,[15,20,32] due to their similarity in weight, size, and joint and bone structure with humans, and in cardiovascular[7,11] studies, because they are good models of cardiac anatomy and physiology. Consequently, the development of analgesia protocols for acute pain conditions is greatly needed.

Animal experiments are under increasing focus regarding their ethical and legal aspects. In vivo studies are permitted when methods consistent with the 3Rs principals (replacement, refinement, and reduction) are considered and implemented.[26] This means that experiments have to be performed without animals when possible (replacement) or with as few animals as possible (reduction) and with as little pain and distress as possible (refinement). In this context, species-specific analgesia is considered an important refinement method applicable to the majority of research.[25] However, few studies have been conducted to determine analgesia protocols for different pain conditions in sheep. The standardization of animal pain models is necessary for the reliable evaluation of efficient and different drug protocols.[10,19,24]

To guide standardization of the dose of *E. coli* LPS for intraarticular administration, with the aim of developing a pain model for studies of analgesia in sheep, we here assessed the ability of various intraarticular doses of LPS to trigger synovitis. Our hypothesis was that the dose established for use in horses (0.5 ng/joint) would trigger similar effects in sheep.

Materials and Methods

Ethical permissions. The study was conducted after approval by the Universidade Federal de Goiás Ethics Committee on the Use of Animals under protocol number 063/2016. Our facility is licensed according to Brazilian law number 11.794, dated 10/8/2008, and Normative Resolution number 01, dated 09/07/2010, of the National Council of Animal Experimentation. According to the Council's classification system, the severity was classified as mild, meaning that the animals only briefly experienced slight distress, discomfort, suffering, or pain.

Animals. Clinically healthy male crossbred (Santa Ines × Dorper) sheep (n = 14; age, 9 to 12 mo; weight, 38.9 ± 5.9 kg) were used in this study. During the experimental period, the sheep were kept in covered bays, fed hay and commercial sheep feed twice daily, and provided water ad libitum. Health status was confirmed by physical examination and laboratory tests (CBC count, total protein, creatinine, and GGT). Sheep were also vaccinated (Heptavac P Plus, MSD Animal Health, São Paulo, Brazil) before the experimental phase. The sheep were acclimated to a halter and handling, so that they could be walked without resistance, because resistance could make it difficult to identify lameness.

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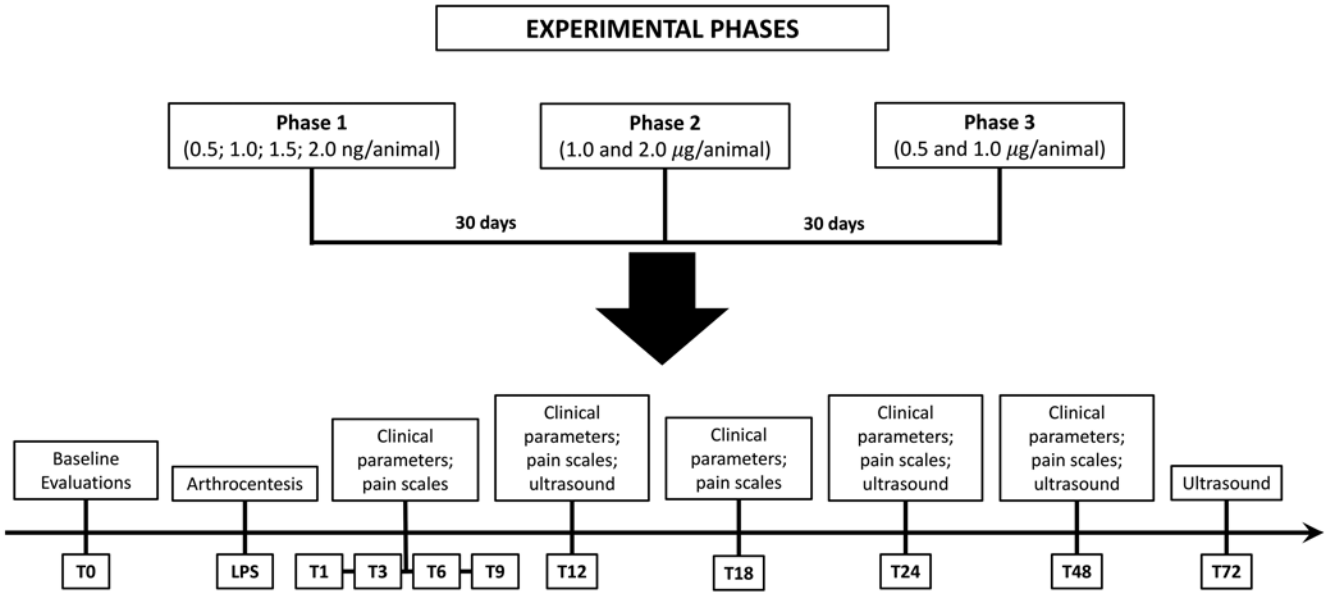

Figure 1. Time line of the experimental period, including all phases. T, time point.

Heart rate and respiratory rate were evaluated and measured by auscultation. Rectal temperature was measured using a conventional thermometer. Mean baseline values were obtained after three evaluations performed in each animal. Then the mean value of all sheep from each group was calculated. Sheep were not tranquilized during any of the evaluations.

The study was divided into 3 phases, with a minimal interphase interval of 30 d. Phases 2 and 3 were performed because phase 1 did not produce typical clinical signs of synovitis, such as mild lameness, increased local temperature, and moderate pain on palpation.

Phase 1. LPS from *Escherichia coli* O55:B5 (catalog no. L2880, LPS 10 mg, Sigma, St Louis, MO) was diluted in sterile PBS to concentrations of 1, 2, 3, and 4 ng/mL; 0.5 mL of each of these final solutions was used for inoculation. Sheep (n = 10) were sedated with 2% xylazine (0.1 mg/kg IV; Xilazin, Syntec, Santana de Parnaíba, São Paulo, Brazil) and restrained while lying on their right side, with the left hind limb pulled dorsally, and displaying the medial region of the right stifle joint. After shaving and antisepsis of the region, ultrasound-guided arthrocentesis was performed for inoculation. The sheep were randomly distributed into 5 dose groups (n = 2 per group): 0.5, 1.0, 1.5, or 2.0 ng LPS or PBS only (control group).

Phase 2. In the second phase, the doses of LPS were increased 1000-fold, based on doses previously used in horses.[19,30] *E. coli* LPS was diluted to the concentrations of 3.34 and 6.67 µg/mL. Arthrocentesis was performed as for phase 1, except that the total volume inoculated intraarticularly was reduced to 0.3 mL. This adjustment was made to avoid morphologic changes, such as an increase in the diameter of the medial joint recess, which had been observed by ultrasonography after administration of the 0.5-mL dose volume. A total of 4 naïve sheep were inoculated, at doses of 1.0 µg/animal (n = 2) and 2.0 µg/animal (n = 2).

Phase 3. Given the results of phase 2, phase 3 was conducted to confirm the effects of the synovitis observed with the 1.0-µg LPS dose and to evaluate whether reducing this dose by 50% would cause lameness. For this purpose, 4 sheep previously used in phase 1 underwent a 60-d washout period and then received doses of 0.5 µg (n = 2) or 1.0 µg (n = 2) per joint.

In all phases of the study (Figure 1), each sheep's heart rate, respiratory rate, and rectal temperature were determined before sedation for arthrocentesis and at 1, 3, 6, 9, 12, 18, 24, and 48 h after inoculation with the LPS-containing solution. Concomitant to the clinical evaluation, scoring systems adapted from a previous study[1] were used to evaluate lameness (0 to 5), joint swelling (0 to 3), and pain on limb flexion (0 to 3). The veterinarians responsible for the subjective evaluations were blind to the dose administered to each animal.

Using previously standardized techniques, ultrasonographic examination of the stifle joint was performed by using a linear transducer with the frequency set to 10 Hz (Logiq E, GE Healthcare, Waukesha, WI).[13,29] Sheep stood during ultrasonography, which occurred prior to sedation for arthrocentesis and at 12, 24, 48, and 72 h after LPS administration. Echotextural and echogenicity changes in the patella, patellar ligament, joint capsule, menisci, muscles, tendons, and ligaments were evaluated. As a control, the baseline examination (before sedation) was compared with each subsequent evaluation.

Sheep that were assigned a lameness score of 4 or greater 4 (scale, 0 to 5), representing altered behavior due to pain, received rescue analgesia with morphine (0.2 mg/kg IM; 1% morphine, Dimorf, Cristália Prod Quím Farm, Itapira, São Paulo, Brazil), as shown in Figure 1.

Data analysis. Statistics were not performed due to low number of animals used. Results are therefore presented individually. Baseline data are presented as mean ±SD.

Results

Phase 1. None of the sheep showed any change in behavior, such as separation from the rest of the group, decreased interest in feeding, prostration, or increased time lying down. The average baseline heart rate, measured before the experimental phase, was 81 ± 10 bpm. Heart rate ranged from 72 to 98 bpm during phase 1. The baseline respiratory rate was 41 ± 7 breaths per minute and ranged from 36 to 47 during phase 1. The mean rectal temperature during baseline was 39.0 ± 0.4 °C and ranged from 38.7 and 39.6 °C during the experimental period.

The 0.5-ng dose did not induce lameness or changes in echotexture or echogenicity on ultrasonographic examination. One animal at this dose showed mild discomfort (score, 1)

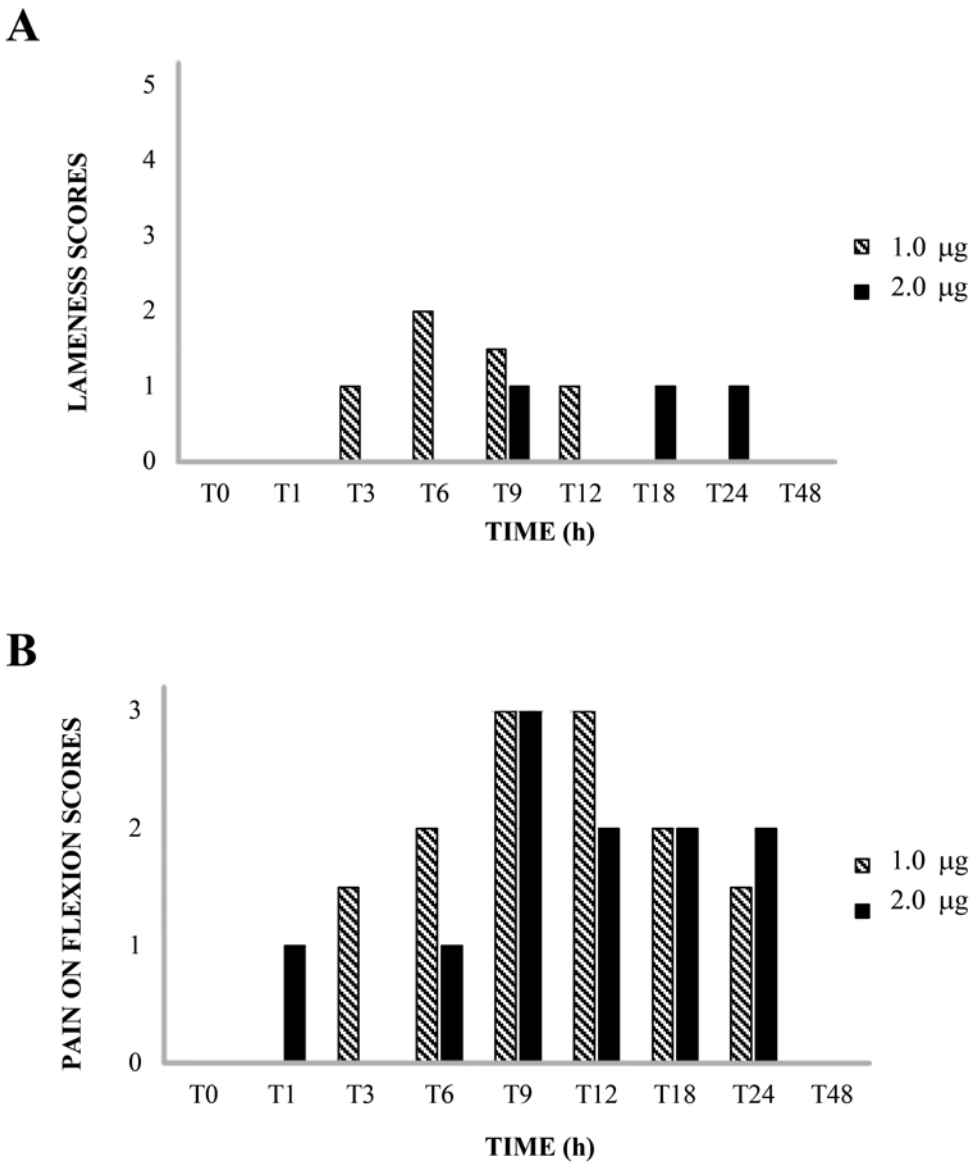

Figure 2. Mean scores of (A) lameness and (B) pain on flexion scores of adult sheep before (0 h) and 48 h after the administration of 1.0 (n = 2) and 2.0 (n = 1) µg of *E. coli* LPS into the medial recess of the right stifle joint. T, time point.

during intense flexion (which represented maximal knee flexion) at 3 to 12 h after inoculation. In comparison, the other doses (1.0, 1.5, and 2.0 ng LPS) induced mild lameness (score, 1), characterized by apparent weight support on all 4 limbs, decreased fluidity of movement, and shortened step length, but sheep remained able to change direction. The time to onset of the signs and their duration was highly variable among sheep, even among sheep that had received the same dose; onset of signs occurred mainly between 3 and 9 h after inoculation. No rescue analgesia was needed.

Pain on flexion was more apparent at the higher doses, but variability among animals was high, as was observed with lameness. Only 2 sheep, one inoculated with 1.0 ng and the other with 1.5 ng, received a score of 2 due to apparent discomfort during intense flexion, but only at the 3-h time point. The remaining animals were assigned a score of 1 at a single assessment point between 1 and 9 h after inoculation. Joint swelling did not occur in any of the animals during phase 2.

Only the doses of 1.5 and 2.0 ng LPS were associated with ultrasonographic changes. Increased synovial fluid and distension of the joint recesses were present, and one animal inoculated with the 2.0-ng dose also had hyperechoic spots dispersed in the synovial fluid, suggesting increased cellularity, at 12 to 48 h after dosing.

Phase 2. The mean heart rate of the sheep varied between 72 and 93 bpm throughout the experimental phase, with a mean at baseline of 83 ± 11 bpm. The mean respiratory rate ranged from 36 to 88 breaths per minute, with a baseline of 73 ± 29. Rectal temperature ranged from 39.2 to 39.8 °C, with a baseline of 39.4 ± 0.2 °C.

In this phase, the doses tested caused an easily detectable change in gait, which resolved in up to 48 h in all sheep. The variability in the onset time, duration and intensity of the signs observed among the individuals in phase 1 was not observed with the doses tested in phase 2. The 2 animals inoculated with 1.0 µg of *E. coli* LPS limped (score, 1) from the 3-h time point until 12 h. The highest lameness score attributed to these animals was 2, characterized by abnormal posture, less fluid movement and decreased step length, perceptible lameness during straight walking, and difficulty changing direction. No rescue analgesia

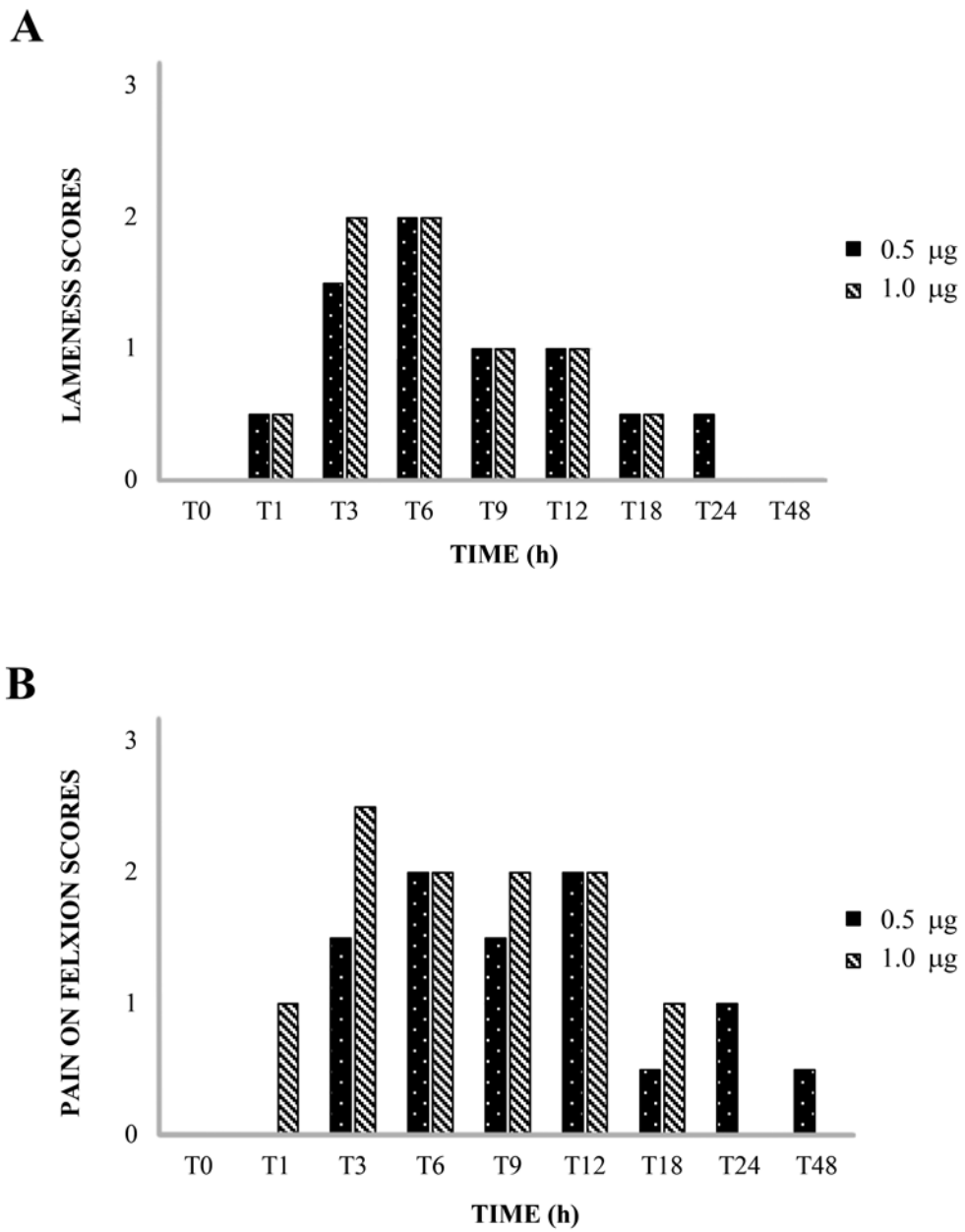

Figure 3. Mean scores of (A) lameness and (B) pain on flexion of adult sheep before (0 h) and 48 h after the administration of 0.5 (n = 2) and 1.0 (n = 2) µg of *E. coli* LPS into the medial recess of the right stifle joint. T, time point.

was needed. For the pain on limb flexion test, both animals showed signs of resistance starting at the 3-h time point and continuing until 24 h. For both animals, the highest scores were recorded at 9 and 12 h, characterized by severe discomfort even during light flexion (when the femorotibial angle was flexed to approximately 90°) and extreme reluctance to flexing the joint (score, 3; Figure 2). Ultrasound examination between the 12- and 48-h time points identified increased synovial fluid and presence of hyperechoic spots in suspension and irregularly shaped hyperechoic structures, suggesting the formation of villi in the synovial membrane.

Of the 2 sheep that received the 2.0 µg LPS dose, the results from one were excluded due to an error in dosing during inoculation of LPS. The other animal presented mild lameness (score 1) at only 2 assessment times (9 and 24 h). This sheep presented signs of pain on limb flexion that started at 1 h after dosing and were present until 24 h afterward. The highest recorded score for this sheep was 3, which occurred at 9 h. As in phase 1, this sheep had swelling in the limb inoculated with LPS. Ultrasound examination showed increased synovial fluid and the presence of hyperechoic spots in suspension between 12 and 72 after inoculation with LPS (Figure 2). Analgesic was not deemed necessary.

Phase 3. The mean heart rate was 56 ± 6 bpm at baseline, and ranged from 47 to 62 bpm during phase 3. The mean respiratory rate was 40 ± 16 breaths per minute at baseline and ranged from 22 to 63 during phase 3. The mean rectal temperature was 37.6 ± 0.3 °C at baseline and ranged from 36.9 to 39.7 °C during phase 3.

In phase 3, as in phase 2, all sheep had easily detectable lameness, and the clinical manifestations of synovitis were similar in all sheep. Of the sheep inoculated with 1.0 µg *E. coli* LPS (n = 2), one showed lameness from 1 h until 12 h after dosing, whereas the other showed lameness between 3 and 18 h. Both animals had their highest lameness scores at 3 and 6 h (score, 2), as in

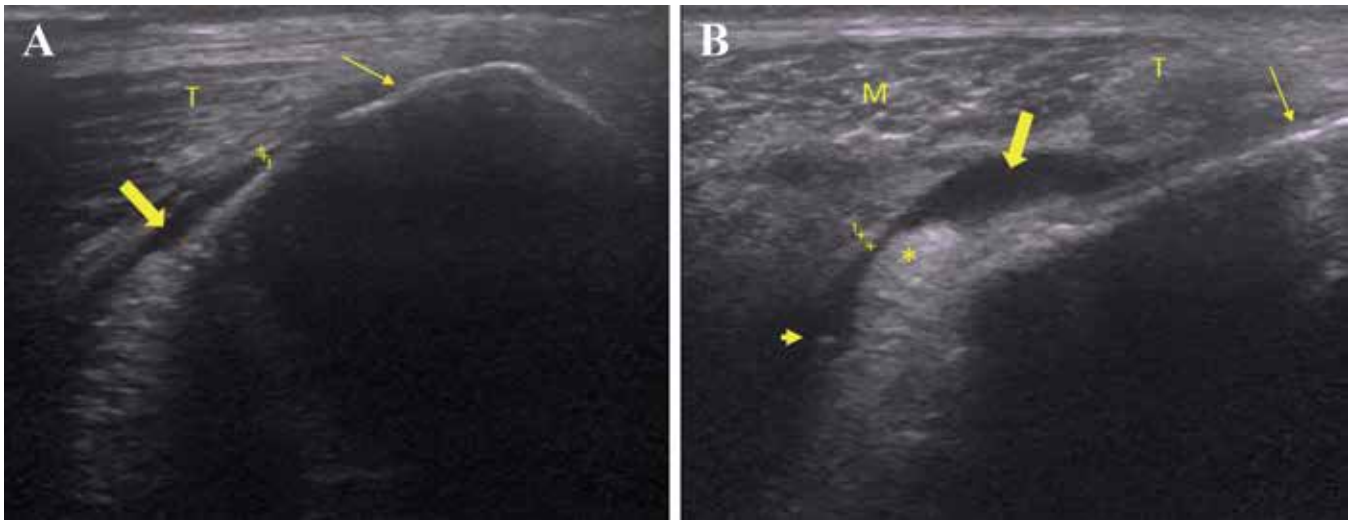

Figure 4. Longitudinal scan of the stifle joint of a 12-mo old sheep (12-MHz linear probe). (A) Ultrasonographic examination at 0 h showed normal appearance of the bone surface of the distal femur (long thin arrow); hyperechoic joint capsule measuring 0.04 cm (between cursors); tendons with normal echogenicity (T); and little synovial fluid between the joint capsule and synovial membrane (solid arrow). (B) Ultrasonographic examination at 12 h after intraarticular administration of 0.5 µg LPS showed increased synovial fluid (solid arrow) with dispersed echogenic material (arrowhead) and an irregularly shaped, hyperechoic structure in the synovial membrane (asterisk). The bone surface (long thin arrow), tendon (T), muscle (M), and joint capsule (0.05 cm; between cursors) are without abnormalities.

phase 2. The pain on flexion of the affected limb started at 1 h for one of the animals and at 3 h for the other, continuing until the 18-h time point in both. Between 6 and 12 h, a score of 2, characterized by clear discomfort during intense flexion, was assigned to both sheep. Only the animal that showed earlier signs of synovitis obtained a pain score of 3 and then only at 3 h. The ultrasound changes observed were similar to those described during phase 2 for the same dose. Rescue analgesia was not deemed necessary.

Of the sheep inoculated with the 0.5 µg LPS dose (n = 2), one showed lameness from 1 h until 12 h after dosing; the other showed lameness from 3 h until 24 h after dosing. The highest lameness score for both sheep was 2 and occurred at 6 h, similar to what was observed at the 1.0 µg dose. Pain on flexion was observed from 3 until 24 h after dosing for one of the animals and until 48 h for the other. The highest observed scores (score, 2) occurred between the 3- and 12-h time points for both sheep (Figure 3). Swelling was not observed in the joints inoculated with LPS. Rescue analgesia was not deemed necessary.

Ultrasound examination revealed increased synovial fluid causing distension of the joint recesses, with increased cellularity and the presence of villi on the synovial membrane (Figure 4). No changes in the joint capsules, lateral and medial menisci, muscles, tendons, ligaments, fat pads, or adjacent vessels were detected during any of the 3 experimental phases.

Discussion

The nanogram-scale doses in sheep failed to trigger clinical signs of synovitis similar to those observed in horses. By increasing the doses into the microgram range, lameness was apparent in our sheep, and pain on flexion of the affected limb was considered moderate.

In the absence of published data in sheep, the doses used in this study were extrapolated from data published for horses.[6,19,27,30] In the current study, a minimal number of sheep were used and the description of the signs was individualized, given that complications such as chronic synovitis[4] or endotoxemia[19] could occur. Our data can guide future research on determining the ideal dose to induce experimental synovitis in sheep.

Mean heart rate values remained within normal limits for sheep in all phases of the study, considering data reported for the species in the tropics.[22] Mean respiratory rates of all sheep also remained within the normal range, considering reference values in Santa Inês and Dorper sheep and their crossbreds from the tropics.[5] Rectal temperature also remained within the reference limits for sheep[17] in all phases of the study. No clinical changes were seen that were suggestive of systemic endotoxemia, which has been reported in other studies.[19,30]

Lameness was considered the most important parameter during evaluations, because visual inspection with minimal handling of the animals is desirable in a model for pain studies. The doses tested in phase 1 did not cause lameness, making the evaluations difficult. In horses, a 0.5-ng dose of LPS achieved grade 2 lameness, increased local temperature of the limb, mild joint swelling, moderate pain on palpation, and changes in synovial fluid compatible with acute synovitis.[19] In sheep, this same dose did not cause noticeable lameness. When doses of 1.0 to 2.0 ng of LPS were administered, individual sheep showed high variability in responses and assessment times. The characteristic behavior of sheep to hide signs of pain may have contributed to difficulty in perceiving subtle changes during subjective evaluations.[3]

In the ultrasound evaluation, only the 0.5ng dose was not associated with changes indicative of an inflammatory response. For the other doses, joint effusion and cellularity of the synovial fluid were observed, compatible with synovitis and accounting for the presence of pain in some animals. After contact with a joint, LPS triggers marked increases in synovial leukocytes and total protein concentration, in addition to inflammatory biomarkers such as prostaglandin E2, interleukin 1β, and TNF, with a peak occurring as long as 8 h after inoculation and a return to the baseline after 168 h.[31] The changes in our sheep were observed as long as 48 h after LPS administration in phase 1.

By increasing the dose to the microgram range in phases 2 and 3, mild to moderate clinical signs became evident in our sheep, with complete remission within 48 h. The signs mainly began at 3 h, as was reported in a recent study in horses using a

nanogram-level dose in which all horses showed clinical signs of pain starting at 3 h after inoculation.[4] The highest lameness score in our sheep occurred at 6 h after dosing, again similar to the previously mentioned study,[4] in which horses inoculated with LPS needed rescue analgesia at 6 h after inoculation due to worsening of the clinical signs. However, none of our sheep reached the minimal score necessary for rescue analgesia. The majority of sheep (n = 5) ceased limping within 24 h. They did not show sensitivity on flexion within 48 h (n = 6), corroborating a previous study[30] in which the authors observed a complete reversal of the clinical signs of synovitis within 24 h after inoculation of a 3-µg dose in horses. Microgram-level doses in horses inoculated via the intraarticular route generated severe lameness within 2 h and were sufficient to induce systemic signs of endotoxemia, such as fever, lack of appetite, depression, increased heart and respiratory rates, and neutrophilic leukocytosis. Resolution of these signs occurred within 36 h.[8,10]

The main sonographic changes observed after inoculation of sheep with microgram-scale doses were more subtle than cases of synovitis in cattle,[12] horses,[2] dogs[21] and humans,[14] involving pathologies such as osteoarthritis. This may explain the observed mild to moderate lameness in sheep. In humans, ultrasonography is more sensitive than clinical examination in detecting synovitis and correlates well with MRI and arthroscopy.[33] The changes in echogenicity and echotexture detected by this exam are also highly correlated with the presence of pain in patients with osteoarthritis.[18]

Animal models of pain contribute to medical advances in pathophysiology and treatment. The ability to mimic the clinical presentation of a disease is important to achieve reliable results.[23] In our study, the potential harm caused by synovitis induction using *E. coli* LPS can be justified by the expected benefit to this species regarding the future development of specific analgesic protocols. In addition, we assessed a minimal number of sheep because no previous study on an LPS-induced synovitis model had been conducted in sheep when we began our study and we could not predict how severe the signs would be.

Among the limitations of this study is that the small group size and lack of statistical analysis reduce the impact of our results. However, possible complications of the technique, such as endotoxemia and the development of a chronic joint disease, were our major concerns. The lack of validated pain scales for use in sheep also limited the interpretation of the results. We used a semiquantitative evaluation system from a previous study in which the authors sought to validate an ovine model of collagen-induced arthritis.[1] The lack of data on changes in synovial fluid and inflammatory markers limited the identification of inflammatory response that could explain the mild discomfort on flexion observed in phase 1.

The continuation of the current study, gradually reducing the dose per animal and increasing the number of subjects tested, is necessary to standardize an experimental model of acute inflammatory pain induced by *E. coli* LPS in sheep. Our observational results indicate that the doses of LPS commonly used in horses were unable to promote lameness or changes in echotexture or echogenicity on ultrasound examination in sheep, which need a 1000-fold increase in dose. These are important starting points to guide future research.

In conclusion, the 0.5-ng LPS dose, defined for experimental development of synovitis in horses, did not induce clinical signs in sheep. In contrast, the 0.5-, 1.0-, and 2.0-µg doses of LPS generated easily detectable lameness and mild to moderate pain on flexion of the limb, without systemic clinical signs of endotoxemia. Therefore, among the doses tested, 0.5 µg/animal was the lowest dose capable of experimentally inducing the clinical signs of synovitis.

Acknowledgments

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