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Model of Hyperalgesia Associated with Lameness in Dairy Cattle

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Abstract: The nociceptive response was evaluated in dairy cattle after injection of a solution of formalin (4%) in the the external claw hoof. The nociceptive response in cows exhibited a biphasic time course behavior to pain stimulus similar to the one described in trials of formalin test in different laboratory animals. The cortisol plasma concentration after injections of formalin was high during the two phases of the pain response showing a correspondence with clinical nociceptive behaviors. The 4 % formalin injections in claw hoof in cows can be used to evaluate the possible mechanisms of anti-nociceptive drugs of central and peripheral actions. Besides, it is a reversible model; it does not need complicated equipment and it is simple to be carried out by personnel with certain experience in cow lameness. This nociceptive model might be useful to research the therapeutic role of analgesic and anti-inflammatory drugs of short half life in the modulation of hyperalgesia associated with lameness in dairy cattle.

Key words: Dairy cattle, lameness, formalin, pain, cortisol

INTRODUCTION

Pain in farm animals associated with different diseases is a difficult concept to identify and measure. Research on acute pain in animals confronts additional problems, such as the lack of a suitable model and ethical considerations associated with inflicting pain[1]. Identification and quantification of pain as well as pain amelioration are crucial in pain alleviation at the farm level. They determine the start and end points of any intervention[2].

The common model used in experimental pain research is laboratory animals or humans. Farmed animals are also used[3,4]. Pain research has been confined to species such as horses and sheep due to their criteria of adaptability or docility of the species. In most cases, results of studies on analgesia are extrapolated from one species to another[5]. Given the wide variation among species and among members of the same species regarding responses to painful stimuli[1], such extrapolation presents problems of validity. The use of cows in pain research is limited. This may be due to an inability to detect the animal response to pain and the difficulty associated with housing and managing large farm animals for research purposes [5] .

None of the nociceptive stimuli (electrical, thermal, mechanical, or chemical) that have been used in different pain models is ideal. Chemical stimuli probably most closely mimic acute clinical pain[4]. Intradermal injections of formalin is a test based on the use of long duration stimuli most commonly used in laboratory animals[6,4]. Less commonly used are hypertonic saline[7], ethylene diamine tetra-acetic acid[8], Freund's adjuvant[9], capsaicin [10] and bee sting[11].

In farm animals hyperalgesia is measured by applying a mechanical stimulus e.g., castration, branding, dehorning, ear notching, teeth clipping, beak trimming and tail docking[12]. Active pain avoidance behaviors, abnormal postures, changes in plasma cortisol and in some cases, lesion scores[13,14] have been studied to assess pain in animals and have been shown to change by varying amounts according to different methods.

In cattle, measurement of nociception is included in stress research to investigate the effects of acute stress[15,16] or chronic pain[17-19] . The pain measures in cattle include radiant thermal stimulation[20,21], contact thermal stimulation[17-22] and mechanical stimulation[23,17].

Lameness is a very common multifactorial disease of dairy cattle and it is defined as a clinical sign of disease or abnormality of the musculo-skeletal system[24]. Many of

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the major claw horn lesions causing lameness are believed to result from so-called laminitis[25]. Laminitis is a recurrent pain pathology of dairy cows and is now considered one of the most urgent health and welfare problems of dairy cattle as well as one of the most significant economic issues for the dairy industry[26,27]. Hyperalgesia associated with lameness in dairy cattle has been measured by means of a mechanical nociceptive test[28,19-22] and alimentary oligofructose overload [29] but few studies on nociception associated with lameness models have been developed in cows by direct injections of chemical compounds.

The goal of this study was to develop a pain model of lameness by direct injection of formalin in the claw horn in dairy cattle adapted to the studies of hiperalgesia modulator drugs.

MATERIALS AND METHODS

Animals: Six healthy Holando Argentino cows (472±80 kg b.w) were used. The cows were 100±17 days postpartum with a mean lactation of 20±5.4 milk yield. The animals were fed twice daily with a total mixed ration (Grass silage, wheat silage, corn silage, concentrates, sun flower pellets, urea and minerals). Sufficient food was given to allow ad libitum intake. The cows were milked twice daily according to the routines of the dairy farm (Las Marias, Tandil Argentina).

Each cow was immobilized in a squeeze chute. All animals were submitted to exhaustive inspection of each foot to detect any manifestations of lameness associated with claw horn pain. In this study, each cow was its own control. The experiment was performed on March 10th 2006, at 7:00 am, with outdoor temperatures of 20.6°C and a relative humidity of 64.1%. Care was taken while handling the animals in order to minimize stress. All experimental procedures in cows were approved by the Faculty Animal Experimentation Ethics Committee.

Treatments and study design: The design of the study was a cross-over trial in which each animal received the following treatments: Ssterile saline followed by 4% diluted aqueous formalin. The treatment order in each animal was determined using a simple randomization procedure. The wash-out period between experiments was 1 week.

Control study: Sterile saline injected: The right leg of cows was immobilized and the claw horn was cleaned and disinfected with a 1% solution sodium of hypochlorite. Each cow received six milliliters of sterile saline injected in different regions of the right external claw horn (three milliliters in interdigital space and three milliliters along abaxial surface of the wall, between skin horn junction and coronary band) using 10cc syringes and hypodermic needles with polypropylene hub 22ga.x 1½". Immediately after injections the animals were released to a pen of flat surface and concrete floor (10×14 m^2) and pain behavior and locomotion scores were recorded by visual observation each hour during 8 h following injection of sterile saline.

Blood samples (10 mL) were taken by venipuncture from left jugular vein at 0, 5 and 30 min and at 1, 2, 4, 6, 8 h post injection of sterile saline. Repeated venipuncture is an established method for obtaining blood for cortisol assays in calves[30]. All the samples were immediately centrifuged for 15 min at 900 g then the plasma was separated and stored at -20°C until assayed.

The clinical degree of lameness was classified from 1 to 5 according to the locomotion scoring of dairy cattle systems adapted by Sprecher *et al.*[31].

Score 1: Normal: Stands and walks normally with a level back. Makes long confident strides.

Score 2: Stands with flat back, but arches when walks. Gait is slightly abnormal.

Score 3: Stands and walks with an arched back and short strides with one or more legs. Slight sinking of dew-claws in limb opposite to the affected limb may be evident.

Score 4: Arched back standing and walking. Favouring one or more limbs but can still bear some weight on them. Sinking of the dew-claws is evident in the limb opposite to the affected limb.

Score 5: Pronounced arching of back. Reluctant to move, with almost complete weight transfer of the affected limb.

Formalin model study: The experimental animals were submitted to exhaustive inspection of each foot to detect any manifestations of lameness associated with claw horn pain (locomotion scoring of dairy cattle: score 1). In this study the cows received an injection of 6 ml of 4% diluted aqueous formalin in the right external claw horn (the 4% formalin solution was prepared by dilution with normal saline of the commercial 40% formalin solution). A series of preliminary experiments was carried out to determine the concentration and volume of formalin solution to be used.

Following injection of formalin, the total number of the leg kicking-lifting as pain response was recorded by visual observation. One experienced person measured the time and another one, dressed like the usual caretakers, carried out the experimental counting during 5 min periods for a total observation period of 8 h using a Hand Tally Counter (WJT-002) (Wenzhou hualong Counters Factory. No. 83, Lingtou Road, Ruoli'Ao Village, Shuangyu Town, Wenzhou, Zhejiang, China).

Analytical method: The changes in plasma cortisol as an indicator of stress and/or pain[14] were measured using a RIA kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA). Serially diluted bovine plasma samples containing high cortisol concentrations produced curves parallel to the standard curve. The sensitivity of the assay was 0.2 μg dL^{-1} and the intra-assay coefficient of variation was below 9 % for concentrations between 1 and 50 μg dL^{-1}. All samples were measured in duplicates and in one single assay.

Conversion Factor: μg $dL^{-1} \times 27.59 \rightarrow$ nmol L^{-1}.

Data analysis: The cortisol plasma concentration from control and formalin treated cows curves as functions of time were plotted and the Areas under Curves (AUC) were measured by the linear trapezoidal rule[32]. Analysis of Variance (ANOVA) was used to compare values of the AUC control and AUC formalin models (Statistical Analysis Systems, Version 9.1.3 SAS, Institute Inc., Cary, NC, USA). Differences were considered to be statistically significant if $p<0.05$.

RESULTS

After sterile saline was injected, any spontaneous behavior as an indicative of pain response was observed. All cows showed a locomotion score of 1 during the experiment.

The spontaneous behavior characterized by kicking/lifting the leg as a response to pain was recorded in all cows following injection of diluted aqueous 4 % formalin in the right external claw horn. The response curves exhibited a biphasic time course Fig. 1. After injection of formalin all cows showed a locomotion score of: 5 during the first 1 h, a score of 4 toward 3 h and a score of 3 after 4 h of experiment and a final score of 1 at 8 h. No animals exhibited clinical sequels of lameness after finishing the trials (8 h) Table 1.

The mean plasma cortisol concentration profiles of control and formalin treated animals are shown in Fig. 2.

The injections of formalin caused a marked increase in mean cortisol concentrations, which peaked at 6.69 μg dL^{-1} 1 h after formalin administration followed by a pronounced decrease nearly 4 h later. The mean concentration returned to control values by 8 h post formalin injections.

Table 1: Locomotion scores (1-5) obtained after treatment with formalin and with sterile saline in cows

Treatment	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
Formalin 4%	5	4	4	3	2	2	2	1
Sterile Saline	1	1	1	1	1	1	1	1

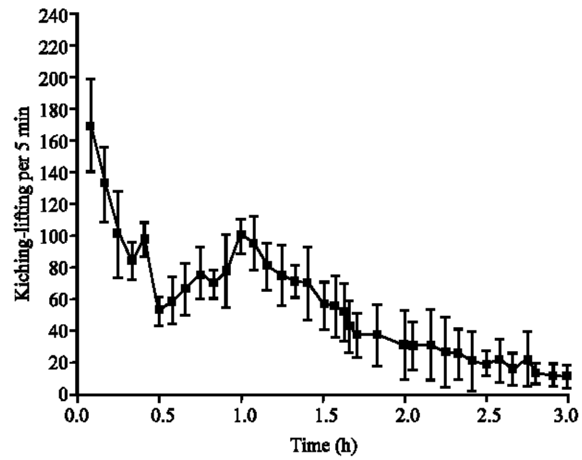

Fig. 1: The intensity of the nociceptive behaviors after injection of six milliliters of solution 4% formalin in different region of the external claw horn. Each point represents the mean±SD (n: 6) number of kicking/lifting during 5 min observation period

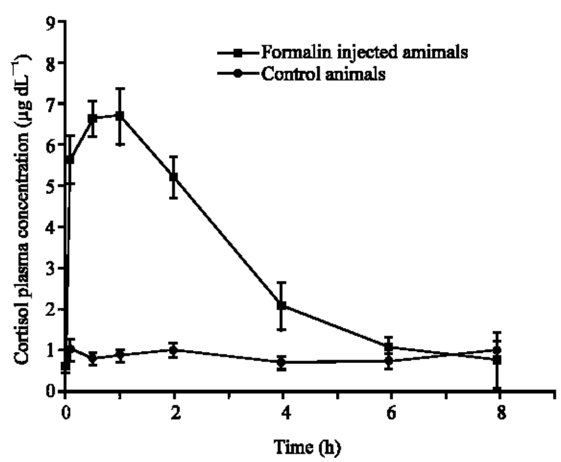

Fig. 2: Changes in plasma cortisol concentration (mean±SD) obtained after injection of sterile saline and injection of 4% formalin injected in different region of the external claw horn

The mean cortisol AUCs were 7.16±0.75 and 25.6±3.09 for control and formalin treated animals, respectively.

There was a significant difference ($p<0.05$) for cortisol AUCs between control and formalin treated animals.

The mean values of kicking/lifting and the mean plasma cortisol concentrations vs time are presented in Fig. 3.

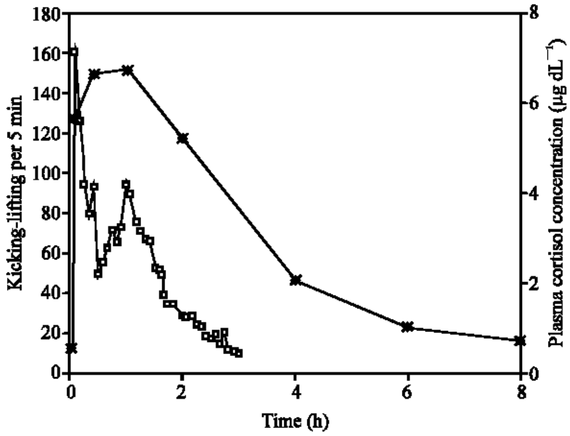

Fig. 3: Relation between the intensity of the nociceptive behaviors represented in number of kicking/lifting during a 5 min and changes in plasma cortisol concentration after injection of six milliliters of solution 4% formalin in different region of the external claw horn

DISCUSSION

In dairy cattle, the inability to evaluate the effectiveness of an analgesic compound and the difficulty in selecting an appropriate painful model where external manifestations of pain are consistent and objectively gradable are the constraints faced by the veterinarians[2].Therefore, the need for analgesia in painful conditions is obvious because it provides immediate pain relief and prevents the development of a hyperalgesic state[2].

There are some models of nociception and locomotion tests associated with lameness in cows[17,19,22,26,28,29]. These studies are designed on the basis of different types of animals with clinical lesions (acute and chronic)[26,7,19] or experimental alimentary oligofructose overload[29] that cause lameness. Therefore to find a homogenous degree of lameness pain with a similar locomotion scoring in each cow may be a problem in determining the experimental protocol. On the other hand, the methods to carry out the nociceptive threshold test use mechanical devices, e.g., pneumatic equipment[28,19] and a laser-based method[22] sophisticated, expensive and of difficult implementation for cows.

The formalin test differs from other nociceptive models, in that it enables evaluation of moderate analgesic activity and continuous pain generated by injured tissue appearing as a valid model for clinical pain[6,33-34,4].

In order to produce a hyperalgesic state the formalin injection was given in the rear lateral (outside) claws of cows on the basis of the studies by Toussaint Raven *et al.*[35] considering that most of the lesions causing lameness occur in the rear lateral claws of cows. Cows normally carry about 60% of their weight on the front legs and about 40% on the rear legs. Looking at the anatomy of the cow it can be seen that when they walk the front feet are placed on the ground more squarely and are more stable. Also, muscles, ligaments and tendons suspend the front legs while the rear legs terminate in a fixed, ball and socket joint at the hip. When the cow walks, it places the medial (inside) claw on the ground when weight is transferred to that leg. The lateral (outside) claw hits the ground but then moves to the outside in a sort of sliding motion. This places more stress on the lateral claw, which also results in more growth compared to the medial claw.

The injection of formalin in cows showed a biphasic response to pain stimulus similar to the one described in trials of formalin test in different laboratory animals [36] . In the rat and the mouse, intraplantar injections of different concentrations of formalin produced a distinct biphasic response to pain stimulus.

The early phase which starts immediately following injection of formalin only lasts approximately 5 min being the result of direct chemical activation of nociceptive primary afferent fibers (neurogenic phase) while the second phase which lasts 20 to 40 min, starts approximately 15 to 20 min following formalin injection and suggests that peripheral, inflammatory processes are involved[34,37-40].

A high nociceptive response was observed in cows during the first 5 min after formalin administration and was followed by a lower response during the following 30 min. Meanwhile, the second phase, of minor intensity, started about 35 min and the pain response decreased and disappeared after 3 h post injection of formalin.

The values of cortisol recorded in this study were higher when compared with other studies in calves where plasma cortisol responses were measured after the initial pain following routine husbandry procedures such as, dehorning and tail docking[41,42]. Besides dehorning caused an increase in mean cortisol concentration followed by a fall to plateau values which were maintained for 3 h approximately[42]. This difference in cortisol plasma profiles may indicate that the formalin injection produces a pain of higher intensity, but for a shorter time. In this study, the cortisol plasma concentrations were not influenced by the amplitude of cortisol circadian rhythm because the cows were completely acclimated to their environment and housed under rigidly controlled conditions. Moreover, the circadian rhythm in dairy cattle is very weak[43].

The observations that cortisol plasma concentrations after injections of formalin were high during the two phases of the pain response are indicating a correspondence with clinical nociceptive behaviors. These results give further support to the hypothesis that cortisol may be used as an index of acute pain-induced distress related to a significant activation of hypothalamic-pituitary-adrenal axis.

CONCLUSION

This study has shown that 4 % formalin injections in claw horn in dairy cattle can be used to evaluate the possible mechanisms of anti-nociceptive drugs of central and peripheral actions. Besides, it develops homogenous degrees of locomotion scores in function of time, it is a reversible model, it does not need complicated equipment and it is simple to be carried out by personnel with certain experience in cow lameness.

This nociceptive model might be useful to investigate the therapeutic role of analgesic and anti-inflammatory drugs of short half life[44-46] in the modulation of hyperalgesia associated with lameness in dairy cattle.

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