UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

USE OF TRI-SOLFEN TO CONTROL PAIN DURING TREATMENT OF HOOF LESIONS IN DAIRY COWS

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Dedicatória

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ABSTRACT

Use of Tri-Solfen to Control Pain During Treatment of Hoof Lesions in Dairy Cows

Hoof lesions in dairy cattle have a great impact, either in production as in animal welfare. Trimming may cause severe pain resulting in violent reaction with risk for humans' safety as well as affecting the animal's immediate welfare. These interventions are usually carried out by non-veterinarian technicians, without any kind of pain management training. An efficient pain management is not only an ethical obligation, as also allows a better manipulation and meticulous treatment.

The present study had the main purpose to test the efficiency of Tri-Solfen®, with a combination of local anaesthetics – lidocaine and bupivacaine - adrenaline and cetrimide in a topical gel form. The efficiency of this formulation has already been tested in other procedures, such as mulesing, castration, disbudding and tail docking in lambs and calves, significantly reducing pain related behaviours. Being dairy cows a second objective was to assess lidocaine, bupivacaine and metabolites, as well as cetrimide residues in milk to determine the safety of use in milking animals.

The selected cows were in the drying off period and lameness scoring was performed when entering the chute. Before trimming, each animal was randomly distributed to two groups: C – usual trimming with no pain control; T – trimming with local anaesthetics being applied once live corium was exposed. Lesions' characteristics were registered. Algometry measurements were performed before and after intervention, to assess animal reaction to pressure. During corrective trimming, behaviour observation was done by two persons blind to treatment. Lameness scoring was again performed at the end of the intervention.

Non-parametric tests and analysis of variance were performed. Analysis of data showed that treatment significantly influenced reaction to trimming and lameness score after trimming on the treated group, when compared with the not treated group. Algometry values showed increased pressure threshold after application of Tri-Solfen. Anaesthetics residues are below the LOQ value in all animals after the first milking, except in one sample at the fourth milking. This study suggests that the use of topical local anaesthetics with lidocaine and bupivacaine helps reducing pain inflicted during corrective trimming of severe lesions, enhancing animal welfare and providing trimmer safety due to diminishing pain related behaviours. We also demonstrated that the levels of anaesthetics and/or metabolites residues are very low in all animals in the four milkings after treatment.

Keywords: Lameness, animal welfare, pain management, hoof lesions.

RESUMO

Uso de Tri-Solfen no Controlo da Dor Durante o Tratamento de Lesões Podais em Bovinos de Leite

As afeções podais em vacas leiteiras têm um enorme impacto quer sobre a produção, quer sobre o bem-estar animal. O desbridamento das lesões pode causar dor intensa, levando a reações do animal que dificultam o maneio e a segurança do mesmo e do operador. Por norma, estas intervenções são realizadas por técnicos não médicos veterinários sem formação no controlo da dor. O maneio eficaz da dor não só é uma obrigação ética, como permite uma mais fácil manipulação e um tratamento mais minucioso. O presente estudo teve como principal objetivo avaliar testar a eficácia de um medicamento, Tri-Solfen®, que tem na sua composição uma associação de anestésicos locais – lidocaína, bupivacaína – adrenalina e cetrimida, na forma de gel tópico. A eficácia desta formulação já foi avaliada noutros procedimentos, como *mulesing*, castração, descorna e amputação de cauda em borregos e novilhos, tendo reduzido significativamente os comportamentos de dor. Sendo animais leiteiros, um secundo objetivo foi detetar a presença de lidocaína, bupivacaína e cetrimida, em amostras de leite após aplicação do produto. As vacas selecionadas encontravam-se no período de secagem e foram classificadas quanto ao grau de claudicação quando conduzidas ao tronco. Antes do início da aparagem cada vaca foi aleatoriamente alocada a um de dois grupos: C – aparagem sem aplicação do medicamento; TriS – aparagem com aplicação do anestésico tópico sobre a lesão do córion. As características das lesões encontradas foram registadas. Foram efetuados testes de algometria antes e após a intervenção, para avaliar a reação do animal a diferentes graus de pressão. Durante a aparagem curativa, dois observadores cegos ao tratamento avaliaram os comportamentos de dor. O grau de claudicação foi novamente avaliada no fim da intervenção. Na análise estatística dos dados foram aplicados testes não paramétricos e análise de variância. A análise dos dados demonstra existir uma redução da reação à aparagem e do grau de claudicação à saída do tronco, no grupo tratado comparativamente com o grupo não tratado. Os valores de algometria demonstram maior resistência à pressão após aplicação do medicamento. Resíduos anestésicos encontraram-se abaixo do LOQ em todos os animais após a primeira ordenha, exceto numa amostra da quarta ordenha. O estudo parece sugerir que a utilização da combinação de anestésicos locais tópicos reduz a dor durante a aparagem curativa, melhorando o bem-estar animal e aumentando a segurança do operador por redução dos comportamentos associados à dor. Os valores de resíduos anestésicos e/ou metabolitos foram consideravelmente baixos em todos os animais nas quatro ordenhas após aplicação.

Palavras-chave: claudicação, bem-estar animal, maneio da dor, lesões podais.

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LIST OF ABBREVIATIONS, INITIALS AND ACRONYMS

ADI: Acceptable daily intake
BW: Body weight
MRL: Maximum residue limits
NSAID: Non-steroidal anti-inflammatories
LOD: Limit of detection
LOQ: Limit of quantification
TTC: Threshold of toxicological concern

PART I - DESCRIPTION OF THE TRAINING PERIOD

The following report has the purpose of presenting the activities which took place during the sixth year of the masters in Veterinary Medicine. The main goals of the internship were to reinforce the previous knowledge acquired during the first five years of academic education, developing clinical reasoning and getting in touch with farm animal's reality. This period of training was divided in two separate parts, the first one in Lisbon Veterinary Medicine Faculty and the second one in the *Associação Agrícola de São Miguel*.

The first part took place between September 2017 and February 2018, integrated in Farm Animals Clinic curricular unit, under the supervision of Professor George Stilwell. Multiple sides of clinical practice were approached, including internal medicine, preventive medicine, surgery, obstetrics and reproduction. Clinical field work was developed in farms in the region of Lisbon and Ribatejo, namely:

- One semi-intensive beef farm, where the main focus was on this type of animal production problems, such as obstetrics, parasitic diseases or fattening period diseases (e.g. Bovine Respiratory Disease).
- Three intensive dairy cow farms, being the main clinical cases: obstetric and reproduction related pathologies, lameness or problems concerning milk production.
- The national zootechnic station, concerning a much large spectrum of species, including beef, dairy cows, pigs, sheep and goats.
- One intensive goat farm, for milk production, where contagious ecthyma or orf and caseous lymphadenitis took a substantial role.

In most of the cases, the patients followed were submitted to physical examination, complementary exams, elaboration of a differential diagnostics lists, proceeded by clinical decision and institution of appropriate therapeutics. Several samples were collected for further analysis on Lisbon Veterinary Medicine Faculty laboratory, from faecal samples for parasite search, to encephalon samples with suspect bacterial meningitis. In approximately 90 field service cases, we performed two C-sections, two surgical corrections of abomasum displacement, one resolution of vaginal prolapse, seven necropsies, one calf dehorning, three abscess drains, six obstetric interventions, seven reproductive interventions, one sanitation of a herd, approximately fifty cases of lameness and about sixty seven internal medicine cases. Animal welfare was always taken in consideration, making sure that no animal would be submitted to unnecessary procedures or suffer pain.

Over this first period of training and has additional knowledge, I attended the *XIX Jornadas da Associação Portuguesa de Buiatria* and to the *V Jornadas Técnico-Veterinárias do Campo Branco*, getting to know new approaches in ruminants medicine and meeting colleagues working in the field. Also, and arising from the field service performed during these first months, I was co-author of the scientific poster "*Caso clínico atípico de* *besnoitiose (Besnoitia besnoiti) num touro limousine*" (Appendix I) presented in the *XIX Jornadas da Associação Portuguesa de Buiatria*; author of the scientific poster "*Aplicação tópica de anestésicos locais para controlo da dor durante a aparagem curativa de lesões podais de vacas leiteiras – dados preliminares*" (Appendix II) presented in the *8º Encontro de Formação da Ordem dos Médicos Veterinários*; and co-author of an abstract on "Use of topical anaesthesia to control pain during and after trimming hoof lesions in dairy cows" (Appendix III) accepted for oral communication at the 2018 World Buiatrics Congress.

On the second part of my training, from April to June 2018, I followed the field service team of *Associação Agrícola de São Miguel* in Azores, supervised by Dr. João Vidal. Along this period I got in touch with several clinical cases in dairy cows, the main animal production in this island. I was involved in the diagnosis and treatment of cases related to internal medicine, surgery, herd health, milk quality services and reproduction management. Getting in touch with field service reality was a major gain to my academic education and a very fulfilling experience.

PART II – EXPERIMENTAL STUDY: USE OF TRI-SOLFEN TO CONTROL PAIN DURING TRIMMING OF HOOF LESIONS IN DAIRY COWS

1. LITERATURE REVIEW

1.1. DIGITAL ANATOMY

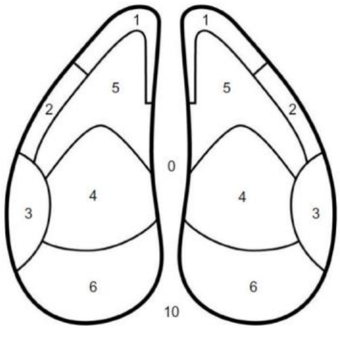
Bovine digital anatomy is similar in both thoracic and pelvic limbs, only changing the designation of the ventral surface from palmar to plantar respectively. In the extremity of both limbs, phalanges form two functional medial digits (III and IV), separated by the interdigital space, and two vestigial lateral paradigiti or dewclaws (II and V). Only digits III and IV have three phalanges, the proximal (P1), the middle (P2) and distal (P3) phalanges. The functional digits are the ones with surface contact and responsible for weight bearing. Branches of the axillary arteria and vein are responsible for the thoracic limb irrigation and drainage. In the pelvic limb, branches of the external iliac arteria send their flux to the homonym vein, creating a complex venous-arterial flux with huge capillarity.

In the thoracic limb, palmar innervation comes mainly from the medial and ulnar nerves and the dorsal innervation from the superficial branch of the radial and dorsal branch of the ulnar nerves. In the pelvic limb, plantar nerves derives from the tibial nerve, and the dorsal nerves originates from the superficial and deep peroneal nerves (Sisson & Grossman, 1986; Budras & Habel, 2003).

The distal extremity of the digit includes three primarily structures: ungula, corium and bones with their associated structures (vessels, nerves and ligaments).

The ungula is the corneal structure giving distal external protection to the digits, covering the skeletal and soft tissue parts. Three distinctly modified layers are present in the hairless skin area in compassing with the haired skin: subcutis, dermis and epidermis. This three layers will have different modifications along the hoof forming five segments: periople, corona, wall, sole and bulb (Budras & Habel, 2003). In the palmar/plantar aspect of the claw it is possible to distinguish the heel, the sole, the white line (area that connects the wall with the sole), and the toe (Figure 1).

Figure 1 – Claw zone diagram: (1) white line at the toe, (2) abaxial white line, (3) abaxial heel - wall junction, (4) sole – heel junction, (5) apex of the sole, and (6) heel (adapt from Risco & Retamal, 2011).



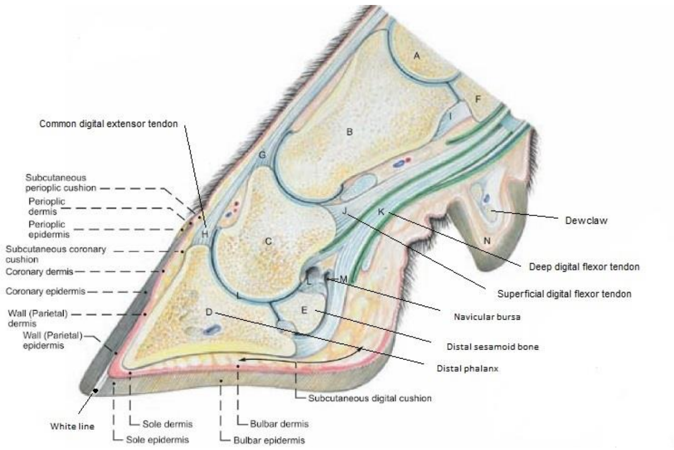
Growth is done along the dorsal wall, abaxial and axial from the corona, and ventrally, growing approximately 5 mm per month. The horn tissue will also surround and protect the corium (Stilwell, 2013).

Corium is a live tissue responsible for producing and giving support to the keratinise tissue and is divided in: papillary corium, lamellar corium and digital cushion. It is supplied by nerves and vessels. The lamellar corium is the primary suspensory tissue for the suspensory apparatus of P3 (Figure 2), by virtue of a series of laminar folds anchored on the abaxial, dorsal, and axial surface of P3, and extend outwards to interdigitate with the lamellae of the wall. Beneath P3 there is a support structure composed of loose connective tissue from the solar and perioplic corium, and caudally by the digital cushion. The digital cushion is an important support structure composed of loose connective tissue and varying amounts of adipose tissue (Shearer & van Amstel, 2001).

The internal structures of the digit includes: the distal phalanx (P3), tendons (the common digital extensor tendon and the deep digital flexor tendon), the distal sesamoid bone (navicular) and the navicular bursa (Sisson & Grossman, 1986; Budras & Habel, 2003).

The distal phalanx is supported only by connective tissue, ligaments and tendons, creating a 30º to 45º angle with the soil (Stilwell, 2013).

Figure 2 - Sagittal section of a bovine digit (adapted Budras & Halen, 2003).



1.2. LAMENESS

Lameness is considered an abnormality of the normal gait in consequence of lesions, defects, injuries, diseases and/or other factors located somewhere in the limb or the rest of the body, inducing pain or discomfort, serving as a ‘being bothered’-signal given by the animal and as a strategy used by the animal in order to maintain a certain state of comfort or even welfare (Beusker, 2007). Van Nuffel et al. (2015) also describes lameness as a clinical manifestation of painful disorders, mainly related to the locomotor system, resulting in impaired movement or deviation from normal gait or posture, with severity variations. Diseases concerning the hoofs of dairy cattle are presented as one of the main multifactorial disorders that affect these animals (Rabelo et al., 2013), mostly in those maintained in intensive productions (Stilwell, 2013). Pain and discomfort related with foot lesions are predominantly seen as behaviour alterations – lameness, more lying time, less social behaviours etc. (Manske, Hultgren, & Bergsten, 2002a; Capion, Thamsborg, & Enevoldsen, 2009).

Factors such as type of flooring, bedding, calving, management, behaviour or lack of functional trimming can enhance the risk of hoof diseases causing lameness (Holzhauer, Hardenberg, & Bartels, 2008). Due to lameness, severe welfare problems and production losses, like reduced milk yield, weight loss, culling, deaths, replacement cost, infertility, prolonged calving interval, veterinary expenses, drugs and additional stockman's time will occur (Weaver, St. Jean, & Steiner, 2005). Whay et al. (1997) suggested that parturition and the associated husbandry changes were also critical for the development of lesions in the claws in heifers. Biomechanical causes concerning gait on dairy cows and contamination due to dirtiness leads to hoof lesions are more commonly present on the outer claw of the pelvic limbs, and when in the thoracic limbs, on the inner claw (Stilwell, 2013; Rabelo et al., 2013). In the pelvic limb, the inner claw both the heel bulb and the axial wall are less developed, and the sole is more concave and sloped axially; as for the outer claw, the sole is flatter and create a more stable weight bearing surface. These anatomic differences between the outer and inner claw will result in a less stable weight bearing surface in the inner claw where more weight is naturally displaced to its abaxial wall, especially when cows are housed on hard surfaces. On the other hand, the thoracic limb claws have similar shape and size and there is greater flexibility due to the anatomic arrangement of the shoulder, having a more stable weight distribution (Shearer & van Amstel, 2001).

The diagnosis of lameness is mainly made by observation of the animal standing and walking, since hoof lesions frequently cause primary and secondary chronical pain and a hyperalgesia state (Stilwell, 2013).

Lameness in cows are usually identified by the farmer, hoof trimmer or veterinarian detecting changes in cow gait, posture or behaviour or the presence of hoof lesions during routine trimming (Van Nuffel et al., 2015). Although early stages of lameness are difficult to identify in cows, since cattle tend to show little behavioural response until injuries are advanced due to their stoic nature, several authors tried to create subjective and objective measuring systems to stablish a more accurate and precise scoring and staging of lameness status. Flower & Weary (2009) evaluate gait assessment methods, discussing the reliability and validity of measures used. They considered that subjective methods provide immediate, on-site assessment and do not require technical equipment, however the results can suffer from poor inter and intra-observer reliability. Subjective scores can be consistent within and among observers, especially if the gait assessment scoring system provides detailed classifications of each category and the observers have been trained. For objective methods of gait assessment, they concluded that although it helps overcome subjective methods gaps, providing accurate and reliable data, often require sophisticated technology, for kinetic and kinematic measures, limiting their use on farms. Sprecher et al. (1997) developed a subjective 5-point lameness scoring system that assess gait, emphasising back posture as an important parameter of evaluation, trying to determine if the system could predict the reproductive future performance, risk of culling and detect early recognition of lame dairy cattle (Table 1). This system categorized cows into normal or mildly lame (1 and 2) contrasted with moderately to severely lame groups (>2), predicting that cows over group 2 would experience extended intervals from calving to first service, to conception, requiring additional services to become pregnant and be 8.4 times more likely to be culled. Currently, this scoring system is the most used and reliable to assess lameness (Schlageter-Tello et al., 2014), and was the basis in our study.

Table 1- Lameness scoring system (Sprecher et al., 1997).

Lameness Score	Clinical Description	Assessment Criteria
1	Normal	The cow stands and walks with a level back posture. Her gait is normal.
2	Mildly lame	The cow stands with a level back posture, but develops an arched back posture while walking. Her gait is normal.
3	Moderately lame	An arched back posture is evident both while standing and walking. Her gain is affected and is best described as short striding with one or more limbs or feet.
4	Lame	An arched back posture is always evident and gait is best described as one deliberate step at a time. The cow favors one or more limbs or feet.
5	Severe lameness	The cow additionally demonstrates an inability or extreme reluctance to bear weight on one or more of her limbs or feet.

In a study comparing different methods of lameness detection based on characteristics visually identified, Van Nuffel et al. (2015) described signs of lameness as: changes in gait patterns, considering the speed of walking, stride duration or weight distribution; changes in posture or body movement patterns, like arched-back posture or head movements; changes in weight distribution patterns; and changes in behaviour, such as longer duration spend at the resting, shorter duration at feeding places or grazing due to pain.

Lameness in dairy cattle is a major concern for animal welfare and productivity, needing a serious and intense intervention, despite cause-and-effect relationships have been difficult to determine (Capion et al., 2009). Lameness score of all the population is extremely important to control, determine and properly correct the risk factors (Stilwell, 2013; Schlageter-Tello et al., 2014). In summary, to find and treat properly lame animals it is crucial to periodically assess cattle based on locomotion or behaviour alterations (Van Nuffel et al., 2015).

1.3. HOOF PATHOLOGIES

Hoof diseases/lesions are normally divided in two categories: hoof/horn tissue pathologies and digital skin pathologies, frequently coexisting in the same animal and in the same limb (Stilwell, 2013). The most common non-infectious causes of lameness affecting the bovine digit are toe or solar ulcers, white line disease and traumatic lesions of the sole. Some of these illnesses are predisposed by metabolic disorders, like rumen acidosis and laminitis along with other physiological factors that can affect the integrity of the suspensory apparatus of the third phalanx. Mechanical factors, as hard flooring surfaces, overgrowth and altered weight bearing, or traumatic lesions of the sole, exacerbated by abrasive flooring conditions, will contribute to lameness complications (Weaver et al., 2005).

Diseases affecting the ruminant digital skin represent some of the most common and important causes of lameness in cattle, however, unlike lesions that specifically affects the claw, these diseases affect the skin of the interdigital space, heel bulbs and interdigital cleft. Although there are some differences between the way these conditions develop and appear, they are all caused by infectious agents capable of inducing inflammation and lameness (Risco & Retamal, 2011). The type of lesion, anatomic location and hoof structures involved that induce a more painful sensation or a more severe state of lameness still brings questions and disagreement among authors (Rabelo et al., 2013).

Next, a summary description of the most relevant podal pathologies will be presented.

1.3.1. Horn Tissues/Hoof pathologies

Laminitis

By definition, laminitis is a diffuse acute, subacute, subclinical or chronic inflammation of pododerm, usually in several digits. In acute stages, blood and serum exudation are present, followed by later (chronic) grooves on hoof wall, concave profile, widened white line and flat sole. Inherited factors, parturition, feeding stress (subacute ruminal acidosis or SARA) from change of dry cow concentrate diet to high production rations, exacerbation by trauma, as in excessive standing due to reluctance to use cubicles, are some of the predisposing factors considered for this disease (Weaver et al., 2005). Subclinical laminitis can have a serious negative impact since it leads to solar ulcers, shedding sole and white line disease (Stilwell, 2013).

Solar ulcer

This type of lesion is a circumscribed limited reaction of the pododerm often characterised by an erosive defect at the sole-heel junction (Weaver et al., 2005). The affected area will develop haemorrhage and necrotic tissue, reaching the solar surface and exposing the corium with cease growth of the horn tissue (Stilwell, 2013). Damage of the pododerm creating horn defects may appear as a secondary result of laminitis, poor trimming, hormonal factors and heel horn deformity (Weaver et al., 2005; Stilwell, 2013). As a consequence, a laxity of the suspensory apparatus of the third phalanx will lead to a continuous impact of the phalanx on the solar corium (Stilwell, 2013). Shearer & van Amstel (2001) considered that the excessive growth of the toe area can be one cause for the rotation of the third phalanx, creating corium lesions associated with solar ulcers. The caudal border of distal phalanx where the deep digital flexor tendon attaches, is usually in the point of pressure (Weaver et al., 2005). In most cases the lateral posterior claw is the more affected one possibly because of excessive weight-bearing following horn overgrowth (Weaver et al., 2005). Granulation tissue appears where the lesion is in an attempt of healing which can prolapse, increasing the pain, and serve as an entrance to secondary bacterial infections, leading to osteomyelitis, arthritis, septic interphalangeal arthritis and podal abscess (Stilwell, 2013). Hard floor surfaces, hoof overgrowth or loss of the impact protection mechanisms, such as the digital cushion, will contribute to an aggravation and more severe states of these lesions. Therefore, dairy cows are more likely to develop solar ulcers due to housing conditions and type of flooring (Stilwell, 2013).

<u>Toe ulcer</u>

This lesions appear on the anterior extremity of the claw, leading the animal to bear the weight in the heel area. Traditionally was correlated with laminitis, when downward displacement of the apex of the third phalanx caused pressure necrosis of the corium in the toe region with toe ulceration as a consequence. However the development of this lesion is still not fully known (Shearer & Amstel, 2009). Some studies present the relationship between subclinical laminitis with lesion of the toe arteries as a cause, when other authors considered the excessive trimming in association with hard surfaces or floors with too much inclination (Stilwell, 2013). Toe ulcers are a very painful claw lesion with high production cost, as milk production decrease and weight loss is significant (Stilwell, 2013).

<u>White line disease</u>

Being the white line an area with less resistance, predisposing factors such as permanent humidity and laminitis can induce and exacerbate lesions (Stilwell, 2013). White line disease is characterized by an abaxial, or less commonly axial, wall separation from laminae at sole-wall area extending proximally, with cavity impacted with mud and faeces leading to the eventual development of an abscess. The development of the lesions follow the sequence: necrosis of wall laminae caused by pressure, and possibly also of solar laminae, followed by under-running and septic laminitis tracking progressively more proximally after entry of purulent micro-organisms, with absence of natural drainage distally due to impacted material (Weaver et al., 2005). Tissues of the white line giving in will allow foreign bodies to penetrate, with separation of the fibre connections and entrance of micro-organisms (Stilwell, 2013). When an abscess is present the primarily responsible micro-organism is *Trueperella pyogenes* (Weaver et al., 2005), possibly creating fistulas though the corona area (Stilwell, 2013). This lesion is predisposed by abnormal horn production resulting from laminitic insult;

insufficient hoof trimming; or related with previously events of the peripartum (Weaver et al., 2005).

Corkscrew claw

A corkscrew claw is a claw twisted throughout its length in a configuration that displaces the abaxial wall by up to 360°, with one or both lateral posterior claws affected. Although bone molding is present, it is not known whether this is a matter of cause or effect. Periarticular exostoses develop around the distal interphalangeal joint, probably from strain of the distal abaxial collateral ligament. Pressure from the exostosis on the dermis of the wall possibly accounts for the excessive growth of the abaxial wall (Greenough, 2012). Normally this condition only presents itself in animal over three year old and a heritable component is considered (Stilwell, 2013).

Heel erosion

This disorder is an irregular loss of bulbar horn in form of multiple blackish pit or pock-like depressions or later deeper oblique grooves, usually affecting posterior digits more severely than anterior (Weaver et al., 2005). Since dairy cows hoofs are permanently exposed to humidity with low pH levels due to manure, typical of the intensive production system, maceration and destruction of heel tissues takes place easily (Stilwell, 2013). In addition to these chemical and physical actions, *Dichelobacter nodosus* and *Fusobacterium necrophorum* can be involved in the erosion process (Weaver et al., 2005).

1.3.2. Digital Skin related pathologies

Interdigital necrobacillosis

Interdigital necrobacillosis is an acute inflammation of subcutaneous tissues of interdigital space and adjacent coronary band, spreading to dermis and epidermis, caused by an interdigital microtrauma and post infection with *Fusobacterium necrophorum, Bacteroides melaninogenicus* and other organisms (Weaver et al., 2005). Advanced cases can develop to digital septic arthritis and eventually release septic thrombi which can trigger endocarditis (Weaver et al., 2005; Stilwell, 2013). The interdigital space is more prone to this infection, providing perfect anaerobic conditions and trauma location.

Digital and interdigital dermatitis

Digital dermatitis is a circumscribed superficial ulceration of skin bordering coronary margin at heels, occasionally more dorsally, being the major lameness problem in some farms. Although not completely clarified, some authors believe there is an involvement of *Treponema genus spirochaete, Borrelia burgdorferi, Dichelobacter nodosus* and *Campylobacter spp.* (Weaver et al., 2005). It is a contagious disease, with high humidity and poor hygiene predisposing dairy cattle to develop this condition (Stilwell, 2013). Interdigital dermatitis is an inflammation of interdigital skin without extension to deeper tissues, and variable associated disturbance of horn growth (Weaver et al., 2005). There is no consensus through the scientific community if the difference between digital and interdigital dermatitis is only the localization and extension of the lesions or if the ethology also differs (Stilwell, 2013).

Interdigital hyperplasia

A proliferative reaction of interdigital skin and/or subcutaneous tissues forms a firm mass, developing a skin hyperplasia with secondary ulceration (Weaver et al., 2005). The main causes are chronic infection, such as interdigital necrobacillosis; repeated trauma/irritation of the interdigital space; poor conformation; and is also inherited in some breeds. Most clinical cases are in adults of four to six years and in the posterior limbs (Weaver et al., 2005; Stilwell, 2013).

1.3.3. Functional and corrective trimming

Claw pathologies affect animal welfare and have economic implications, due to costs of treatment, earlier culling, and production losses, being influenced by management and genetics (van der Linde et al., 2010). Regular claw trimming can be beneficial to claw health and animal well-being. Bell et al. (2009) stated that good claw condition is achieved by proper attention to breeding for conformation, proper foot trimming and good foot hygiene prior to first calving has being one of the most important critical control point for the management of lameness in dairy heifers.

Studies on the pathogenesis of sole ulcers and white-line disease clearly show that claw overgrowth leads to disproportionate weight bearing and eventual claw disease. Restoration of appropriate weight bearing within and between claws can be achieved by hoof trimming (Shearer & van Amstel, 2001). Manske et al. (2002b) studied and described the effects of regular claw trimming on the claw health of dairy cows. These authors compared two groups of cows, one of which received autumn trimming, while the other did not. Claw trimming in autumn proved to be associated with a significantly positive effect on the prevalence of lameness, and the risk of claw lesions requiring veterinary treatment between scheduled trimmings was reduced in trimmed relative to non-trimmed cows. These results are in favour of at least two claw trimmings per year, whether due to therapeutic or prophylactic effect. However, the optimal frequency of claw trimming is likely to be determined by factors specific to each farm and each animal. Holzhauer et al. (2008) also proved that dairy cows trimmed preventively at the end of the housing period had a significantly lower risk of getting solar ulcers than cows trimmed at the end of the grazing period.

Proper application of claw trimming methods provides correction of hoof overgrowth that, if left uncorrected, leads to overburdening of the affected claws and eventually to claw disease. When trimming is performed, claws are reduced to their normal shape and proportions, thereby returning the foot normal function and helping avoid lameness caused by improper trimming. Although claw trimming should be performed routinely one or two times per year, in most dairy farms cow's claws are trimmed only when they become lame or at the dry off period (Shearer & van Amstel, 2001).

To properly apply claw trimming procedures restraining method are extremely important. The best restraint method provides immobilization of the foot for good viewing of the claw and interdigital space and at the same time allows the operator to have free range of movement for trimming procedures, whether done with simple or more complex restraining systems (Shearer & van Amstel, 2001).

Claw trimming can be done by the veterinarian, a claw trimmer or the farmer himself. There are two different trimming technics: functional trimming and corrective trimming. When performing a functional trimming the aims are to restore appropriate weight bearing within each claw; to correct hoof overgrowth that leads to the overburdening of claws and to balance weight bearing between the claws of each foot; and to identify and correct claw lesions at an early stage. If claw lesions are detected, a corrective trimming procedure is required. The proposes of these procedures are to provide rest to the damaged or diseased claw by transferring weight to the healthy claw and to remove loose horn and thin, hard ridges that may cause damage to the underlying corium, always making sure that the corium is not damaged during the procedures. In severe painful cases or if the corrective procedures are unable to create sufficient difference in height between the two claws, application of a block attached to the sound claw is recommended. Cases with severe haemorrhage of the corium or in claw amputation should be addressed with topical treatments under a bandage, taking in account that it has to be removed in order to prevent environmental contamination and further complications (Shearer & van Amstel, 2001).

In digit skin pathologies, foot washes and topical treatments are the regular approaches. Different methods of foot wash in water have been introduced to improve claw hygiene without causing environmental disadvantage and health problems for cows or humans. Fjeldaas et al. (2014) showed that $CuSO_4$ footbaths had a preventive effect on heel horn erosion lesions, as stationary automatic flushing of the hind feet with only water had no beneficial effect on interdigital dermatitis or heel horn erosion.

1.4. PAIN

The International Association for the Study of Pain describes pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. Thus, pain is not only a physical sensation, but is usually associated with emotional and mental alterations, more or less extended, potentially leading to suffering (Stilwell, 2017).

Absence of pain is of course an essential feature of animal welfare. That is the reason the Farm Animal Welfare Council (FAWC, 1993) included 'freedom from pain, injury or disease –

by prevention or rapid diagnosis and treatment' as one of the 'Five Freedoms' as guidelines for the welfare of all species of livestock.

However, recognition of pain in ruminants is not easy. Wild cattle, from which domesticated animals descend, were preys, submitted to a strong evolutionary pressure to mask signs of pain and its implied weaknesses (Huxley & Whay, 2006 citing Phillips, 2002). Even if not displaying obvious pain behaviours, this does not mean that cattle do not experience it. To recognise signs of pain is a significant challenge for veterinarians, where they are expected to be able to diagnose, grade and treat pain in cattle (Huxley & Whay, 2006; Gleerup et al., 2015).

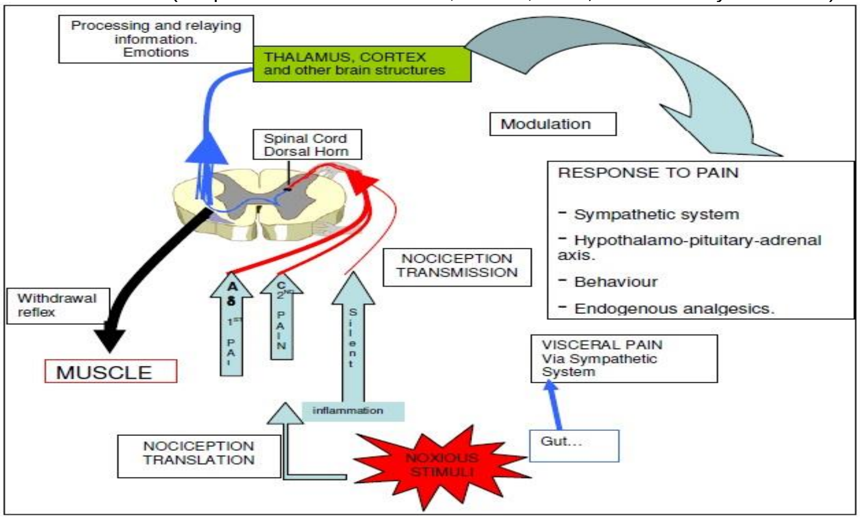
1.4.1. Nociception

Nociception is the unconscious afferent activity produced in the peripheral and central nervous systems by noxious stimuli, with potential tissue damage, being described by many authors as the first and basic part of the pain mechanism (Figure 3) (Stilwell, 2009). The nociceptor is the primary sensory neuron that is activated by those stimuli, with characteristics thresholds or sensitivities that distinguish them from other sensory nerve fibres (Julius & Basbaum, 2001).

Nociception mechanism rests essentially on two stages – transduction and transmission. In transduction, occurring at the sensory endings of the nociceptors, the noxious stimuli (mechanical, temperature or chemical) is translated into electrical activity. Transmission is the propagation, by membrane depolarization, of the electrical impulses along the sensory nervous fibre to the central nervous system. It is here that occurs the first synapsis between neurons, with glutamate being the predominant excitatory neurotransmitter in all nociceptors.

Modulation is also an important component of pain and by which transmission of pain impulses through the spinal cord are inhibited by descending reflexes that originate in the noradrenergic neurons (Stilwell, 2017).

Figure 3 – Pain system in mammals (adapted from Manual da Dor, Stilwell, 2006; authorised by the author).



When a noxious stimuli is first induced it causes what is called “physiologic pain” or “first pain”, serving as a protective biological function to active a response and repair potential tissue damage. This almost instant transmitted sensation travels through thinly myelinated A δ fibres, generating the defensive activity. “Chronic pain” happens as a result of C fibres activation, interpreted by the central nervous system as a dull, diffuse, aching or throbbing sensation. “Neuropathic pain” results from injuries to the nerve fibre, being important in animal welfare since it might be the cause of enduring pain after mutilations and for which treatment is very difficult. “Visceral pain” is an unique type of pain for which there are no first and second components, often being poorly localized, deep and dull and usually triggered by other kind of stimuli, namely stretching, compression or ischemia (Stilwell, 2009; Stilwell, 2017).
All nociceptors (except those coming from the head) have their first axons synapse in the dorsal horn of the spinal cord, namely on laminae I, II and V. It is here, by way of interneurons connecting to the ipsilateral ventral horn, that the reflex arch is produced allowing for rapid muscle contraction and body withdrawal from the stimuli source reducing further damage. The signals then travel through a spinothalamic tract or a spinoreticular tract of the spinal cord to several structures of the brain, namely the mesencephalon, thalamus, reticular formation, hypothalamus, limbic system and cortex (Stilwell, 2009). It is in the central nervous system that perception or consciousness of pain occurs, leading to an emotional response and eventually to suffering.

1.4.2. Pathological pain

Pathological pain can be triggered in different types of tissues and classified as inflammatory or neuropathic (Klaumann et al., 2008). Pain resulting from inflammation is very frequent in animals and may cause a great deal of suffering, reducing animal welfare and performance. It is much more difficult to manage than acute pain (Stilwell, 2009).
After tissue damage an inflammatory reaction usually occurs, with vascular components, fibroblastic components and tissue cell components being activated: blood vessels carry circulating precursors that are released into the area of injury and are activated by enzymes; mast cells release histamines and other substances; macrophages activate fibroblasts, which in turn release interleukin and Tumor Necrosis Factor (TNF); cyclooxygenase activates prostaglandin and leukotrienes and more. Pain may be exacerbated and triggering thresholds are reduced, when nociceptor terminals are exposed to these products of tissue damage and inflammation, called “inflammatory soup”. The acidic pH level of the inflammatory soup is also important in nerve sensitization. This will trigger the terminals to sensitize or excite the nociceptor by interacting with cell-surface receptors expressed by these neurons (Julius & Basbaum, 2001; Klaumann et al., 2008).

Another type of nociceptors, the silent nociceptors, become hyper-excitable when sensitized by the inflammatory soup. This leads to a “primary sensitization” or “primary hyperalgesia”, in which almost any stimulus is felt as pain, generating a constant state of pain. The secondary peripheral hyperalgesia occurs when local vasodilatation, plasma extravasation and extension of the inflammatory soup results in an additional amplification of the inflammatory response by reducing other nerve endings threshold to stimuli, producing pain even without tissue damage (Stilwell, 2009). Peripheral nociceptors’ activation also results in a use dependent neuronal plasticity in the spinal cord that modifies the subsequent performance of the nociceptive pathway by hyperalgesia (an increased or prolonged response to noxious inputs) or allodynia (pain caused by a stimulus that does not normally inflict pain) (Stilwell et al., 2009; Tranquilli et al., 2013).

Prolonged noxious stimuli produces greater sensitivity to subsequent stimuli. This hypersensitivity status is probably what occurs in cows with chronic lameness with pain continuing for a long time even after treatment of the primary hoof lesion (Whay et al., 1998; Stilwell, 2009).

1.5. ANIMAL WELFARE IN FEET LESIONS IN DAIRY COWS

1.5.1. Welfare impact

When under stressful production conditions dairy cows can more easily acquire production diseases, leading to pain or death, raising both ethical and economical concerns (Hultgren, Manske, & Bergsten, 2004). Hoof diseases and lesions causing lameness are one of the most important.

The Farm Animal Welfare Council (FAWC, 1997) stated that “lameness is an extremely painful condition and steps must be taken, as a matter of urgency, to reduce the incidence”. Gait alterations are usually a manifestation of discomfort or pain, caused mainly by claw lesion in dairy cows (Van Nuffel et al., 2015). Lameness, for reasons of prevalence and individual suffering, is considered to be the most severe welfare problem for dairy cows (Stilwell, 2013). Lesions of the hoof horn tissue are the source of most cases of painful lameness. Interdigital or digital skin lesions have a short duration period if properly treated, in contrast with claw lesions that can be long lasting even when treated (Hultgren et al., 2004).

We know little of how much cattle suffer during a lameness episode. Pain is a subjective experience drawing on both physiological and emotional components (Whay et al., 1997). The absence of obvious signs of pain or lower locomotion scores in certain hoof lesions do not necessarily means the absence of pain sensation, indicating either that these lesions are causing different severities of lameness, or that the case definitions used is not sensitive enough to detect all lesions (and possibly discomfort) (Tadich, Flor, & Green, 2010).

Whay et al. (1997) tried to associate locomotion, claw lesions and nociceptive threshold in dairy heifers during the peri-partum period, describing the development of claw lameness in heifers at the time of their first parturition and the relationship between claw pathology, gait score and hyperalgesia, as indicated by nociceptive thresholds. They determined that as lameness increases the nociceptive threshold significantly decreases, demonstrating sensitization to the stimulus. The increased sensitivity to a mechanical stimulus indicates that the limb is in a hypersensitive or hyperalgesic state. Even if not possible to distinguish between peripheral sensitization or spinal sensitization, this study confirms that the locomotion changes were likely to be due in part to the animal's hyperalgesic state and not only as a result of biomechanical restriction of movements.

In a study performed by Bruijnis et al. (2012) assessing the welfare impact of a hoof lesion, a specific impact of foot disorders on dairy cow welfare was shown, mirroring differences in foot disorder painfulness, duration and incidence. Pain induced by this type of lesion causes negative effects in all three domains, causing impaired health and functioning, suffering and affects the ability to perform natural behaviour, as well as compromising the longevity of cows, as the associated lameness and poor performance are important reasons to cull cows prematurely.

To reduce lameness cases, farmers need to be aware of the number of lame cows and the severity of lameness in their herd (Van Nuffel et al., 2015). The usually accepted methodologies to classify lameness stand on detect changes in gait, posture or behaviour of the cows, done using subjective methods such as visual observations for locomotion, which is easy to apply and inexpensive, making the implementation of regular and systematic assessment of gait an ethical obligation. Improvement in education and training, either in recognition of subtle signs of pain exhibited by dairy cows as well as in the acknowledgment of the benefits of analgesia, can minimise lameness negative consequences on animal welfare and health (Becker et al., 2013).

1.5.2. Pain assessment and management in feet lesions

Pain causes harmful effects in interconnected areas: animal welfare, arising ethical challenges; animal physiology, with biological effects; and productivity, having negative economic impact (Stilwell, 2017).

Cows change their way of walking to relieve pain, so an abnormal locomotion is considered an indicator of an underlying problem that induces pain (Flower & Weary, 2009). Due to dairy cattle stoic nature, many authors have been trying to properly assess pain in these animals by detection of several changes in their behaviour as signs of pain. A single sign or measurement cannot be used as an accurate information of how the animal is felling in a particular moment, since animals react differently to stress and pain (Stilwell et al., 2009; Stilwell, 2017).

A study performed by Gleerup et al. (2015) tried to assess bovine pain in general using the most significant signs of pain by creating a Cow Pain Scale. Attention towards the surroundings, head position, ears position, facial expressions, response to approach and back position were the six signs considered as the more reliable and accurate. The resort to this scale showed substantial inter-observer agreement between the two observers and an easier evaluation of the progression of each clinical case.

Shearer et al. (2013) summarized the assessment of pain in lame cattle relaying on seven primary key-points:

- Locomotion or Lameness Scoring Systems

An arched back, already defined as the key behavioural change evaluated in the Sprecher lameness scoring system, is frequently associated with lameness, as is “bobbing” of the head during locomotion. Shortening or lengthening of the stride and the degree of abduction or adduction of the limbs also can be an indicator of problems related with the limbs. Others are: changes in claw placement, the alignment of the pin bones when walking, reluctance in the animal’s willingness to move (being frequently associated with lameness affecting multiple claws) and changes in the stance phase, resulting in the animal maintaining its weight on the sound limb for as long as possible to minimize weight-bearing time on the lame limb.

- Pressure Mats

This type of devices help to determine the contact pressure, contact area, and stance phase duration in the affected claws, and therefore to detect lameness when present.

- Weighing Platform

The use weighing platform was described to measure the redistribution of weight of cattle limbs that occurs in response to pain associated with lameness. Cattle redistribute weight to avoid uncomfortable surfaces and distribute weight away from a limb with discomfort primarily toward the contralateral limb.

- Nociceptive Threshold Tests

The hyperalgesia caused by lameness, can create a more sensitive and exaggerated reaction to stimuli compared with sound animals. When a mechanical pneumatic blunt pin is pressed on the dorsal aspect of the digit with gradually increasing pressure, the pressure at which the reaction occurs is recorded as the nociceptive threshold. This method allows to quantify regional sensitivity and, potential pain objectively. A pressure device, an algometer, can be used in these cases, showing that the animal retracts the limb when the pressure reaches the pain threshold.

- Heart Rate

Heart rate means tends to be less in lower lameness scoring, in comparison to higher lameness scorings.

- Cortisol Response

Measurement of cortisol levels is used to quantify response magnitude and duration to acutely painful states and procedures and these seem to correspond to the predicted noxiousness (McCarthy et al., 2016).

- Accelerometers

Continuously measuring gravitational force in multiple axes, accelerometers' values can be processed to determine activity and postural behaviours occurring on lameness situations.

To effectively manage pain in cattle, reducing primary and acute pain and preventing secondary (central or peripheral) hypersensitivity should be combined (Nolan, 2000 cited in Stilwell, 2009). The main concern of practitioners and farmers is usually the first or acute pain, often neglecting the control of pathologic or chronic pain, since it is less obvious, the control is more expensive and it does not pose safety problems for the operator (Stilwell , 2009).

Pain management in lame cattle can be approached according to the following principles (Shearer et al., 2013):

- Corrective Trimming

Pain relieve and a faster recovery period from claw lesions can be accomplished by reducing the possibility of further complication associated with abscess formation, post-procedural pain minimization and adjust weight bearing on diseased or damaged claws.

- Anaesthesia of the Lower Limb and Foot

Anaesthesia is indicated whenever it is necessary to perform procedures that may be very painful to the animal. There are at least two methods to achieve this: intravenous regional anaesthesia under a tourniquet or ring block, both easy to perform under field conditions. These methods not only alleviates discomfort, but also lessens movement of the foot associated with corrective trimming adjacent to sensitive tissues of the corium, facilitating trimming procedures and reducing the potential to accidental damage of healthy tissues.

- Analgesia

A multimodal approach using analgesics, such as local anaesthetics, NSAIDs, and sedative-analgesics, may be beneficial when treating lame cattle. The use of local anaesthetics, such as lidocaine, reduces gait scores and effects distribution of weight acutely. Non-steroidal anti-inflammatories (NSAID) (e.g. flunixin meglumine) have demonstrated substantial analgesia in induced lameness models illustrated through modifications of gait and improved pressures placed on the affected foot and claw. However, in field trials, NSAID use have yielded variable results with only mild improvement in locomotion score and in nociceptive thresholds (Chapinal, de Passillé, Rushen, & Wagner, 2010; Shearer et al., 2013). Animals treated under sedative-analgesics, such as xylazine, demonstrated lower levels of cortisol and stood longer and had reduced gait scores in the first hour (Shearer et al., 2013).

The two most important reason for not using proper analgesia in cattle are the ability to identify signs of pain, sometimes suppressed by these animals, or because the economic consequences of not using analgesia are negligible (Stilwell , 2009). On a survey performed by Becker et al. (2013), concerning attitudes taken against pain and painful procedures in dairy cattle, it was showed that reducing pain to the lowest possible level when treating lameness cases was much more important to farmers than to bovine practitioners. Also common painful procedures, such as corrective trimming of solar ulcers, were frequently performed without any kind of anaesthesia by practitioners, farmers and claw-trimmers, even if the cost was not a limitation to most of them. The level of pain caused by lameness treatments and the sensitivity of dairy cows to pain was not unanimous, showing lake of education and training, even as the awareness on the benefits of proper pain management.

The reasons pointed out by practitioners for not properly manage severe pain in some pathologic conditions are reduced cost-effectiveness, low practicability (few long acting drugs available), long withdrawal periods and lack of legal license (Stilwell , 2009). However, it is the veterinarian ethical obligation to sensitize and encourage towards the implementation and importance of pain recognition and control.

1.6. LOCAL ANAESTHETICS

In animal production, local or regional anaesthesia are preferred methods for pain management due to practicability and economic reasons.

Local anaesthetics reversibly bind to sodium channels and block impulse conduction along nerve fibres, by inhibiting the generation and conduction of ionic fluxes responsible for membrane depolarization (Lomax, Sheil, & Windsor, 2013; Tranquilli et al., 2013). The interruption of neural transmission in sensory afferent nerves or tracts by a local anaesthetic drug after local tissue infiltration, regional nerve blocks, or epidural injection effectively prevents or reduces pain or nociceptive input. Analgesia in the desensitized area also removes the immediate secondary (central) sensitization to pain and reduces the central facilitation of the nociceptive pathway, preventing or reducing pain escalation response (Lomax, Sheil, & Windsor, 2008). Absorption of local anaesthetics may also be accomplished through mucous membranes or damaged skin to reach the nerve fibres (Tranquilli et al., 2013). Systemic absorption is generally slow, keeping the active substances concentrated at the site and slowing its metabolism, prolonging intensity and duration (Lomax et al., 2013). The duration of effect of local anaesthetics at the site is inversely related to the rate of systemic absorption. Vascular absorption rate varies directly with the vascularity of the injection site and the physicochemical and pharmacological properties and dose of the local anaesthetic. Lidocaine (being a good vasodilator) removal from the site of injection is faster, making lidocaine a shorter-acting anaesthetic (60 to 120 minutes). On other side, bupivacaine is slowly “washed out” from isolated nerves *in vitro*, and it is not readily removed by the bloodstream from nerve membranes, making its duration of action long (180 to 480 minutes) (Tranquilli et al., 2013).

In cattle the anaesthesia of the foot may be accomplished by: ring block, consisting in a local anaesthetic infiltration of the tissues around the limb; intravenous regional anaesthesia, with local anaesthetic injection into an accessible superficial vein in an extremity isolated from circulation by placing a tourniquet on the animal's leg; regional analgesia, desensitizing specific nerves; or by general anaesthesia especially in aggressive animals or for procedures requiring complete immobilization for asepsis and safety during operation (Tranquilli et al., 2013).

Two local anaesthetics are included in a topical gel, called Tri-Solfen. This product has been tried in several different medical procedures in different species, with the aim of providing local anaesthesia in an easy non-invasive way. Tri-Solfen is a spray-on topical anaesthetic, haemostatic and antiseptic gel agent that contains lidocaine, bupivacaine, adrenaline and cetrimide (Lomax et al., 2008, 2013). Application of this gel in open wounds leads to a rapid and prolonged anaesthesia of the area, as well as prevented subsequent pain escalation response. Also, the presence of a gel base can act as a barrier, attenuating pain by coating damaged nerve endings and providing a barrier against ongoing environmental exposure and tactile stimulation (Lomax et al., 2013). The efficiency of this anaesthetic combination was the subject of our study and more detailed explanation will be given in the next topic.

1.6.1. Tri-Solfen

Tri-Solfen (Bayer Animal Health, Australia) is a commercially available only in Australia as a topical anaesthetic, haemostatic and antiseptic formulation for the alleviation of pain in farm animals. Its composition includes lidocaine hydrochloride (40.6 g/L), bupivacaine hydrochloride (4.5 g/L), adrenaline acid tartrate (24.8 mg/L), and cetrimide (5.0 g/L) in a gel base (Australian Pesticides and Veterinary Medicines Authority [APVMA], 2005). The association of the two local anaesthetics was selected to deliver rapid onset of wound anaesthesia action of lidocaine with the prolonged duration effect of bupivacaine. Adrenaline is included as a vasoconstrictor, increasing local anaesthetic compounds at the site of administration and reducing absorption of the compounds away from the traumatised nerve tissue and also reducing the risk of systemic toxicity, and increasing local activity. The vasoconstrictor properties of adrenaline also reduces bleeding, and slows the blood flow to the wound, thereby suppressing the inflammatory cascade and resulting in a reduction on associated pain caused by accumulation of inflammatory mediators. Cetrimide, a topical antiseptic compound, provides antisepsis conditions for procedures performed in non-sterile farm environments. Tri-Solfen is a non-sterile liquid solution, designed for on-farm use, supplied in multi-dose backpack containers with a metered dose spray applicator. The application does not involve the use of injections or needles, which means that may be applied by non-veterinarians in a safe way (Lomax et al., 2008, 2013).

This veterinary formulation is based on similar topical local anaesthetic preparations developed for use in human medicine to provide pain relief in treatment of open wounds and lacerations, burns, skin harvest sites, minor surgical procedures, catheter placement and plastic surgery. The topical formulations are commonly used in pediatric medicine to avoid the pain and fear associated with the administration of infiltrative local anaesthesia in children (Lomax et al., 2008).

Some procedures, such as mulesing in lambs, dehorning and castration in calves, are still performed in farm animals without appropriate pain management and acknowledgment of its importance for animal welfare. These procedures can inflict severe pain in animals and arises very serious ethical dilemmas: whether they should be allowed and under what conditions.

Several clinical trials have been performed, resorting to Tri-Solfen as a local anaesthetic, demonstrating its effectiveness in pain alleviation and recovery. Lomax et al. (2008) performed a study to determine the effect of topical anaesthesia on pain alleviation and wound healing in lambs after mulesing. Results of wound sensitivity testing and behavioural observation suggested that topical anaesthesia has the availability to dramatically improve the welfare of lambs undergoing mulesing. Pain alleviation and improved recovery was achieved in animals under topical anaesthetics, within one minute of application and for at least 24 hours after mulesing, when in non-anaesthetised lambs a significant and increasing hyperalgesic wound response was observed over 24 hours.

In scoop dehorning procedures in calves, wound sensitivity seemed to be reduced until 1.5 hours after application of the local anaesthetics, thus likely reducing the post-procedure acute pain, suggesting some anaesthetic effect even if short-term (Espinoza, Lomax, & Windsor, 2013).

In calf castration, reduction of pain-related behaviours and reduced sensitivity of the wound and surrounding surfaces was reported when using topical anaesthesia up to 24 hours after castration (Lomax & Windsor, 2013). Likewise, studies with topical anaesthesia application demonstrated significantly reduced plasma cortisol concentration and the integrated cortisol response for the first 6 hours and improved lying behaviour in the first 12 hours, suggesting lower pain response (Paull, Lee, Colditz, & Fisher, 2009).

1.6.2. Topical anaesthetics residues in animal products

Lidocaine and bupivacaine are included in the *WHO's model list of essential medications* as needed in basic human healthcare.

Previous studies prove that it is widely and quickly distributed throughout the body following administration and with a rapid metabolism. In guinea pigs lidocaine is metabolised by the liver, with 97% of the dose excreted in urine within 48 hours (Keenaghan & Boyes, 1972). Half-lives have been calculated for non-pregnant, 42 minutes, and pregnant sheep, 62 minutes (Bloedow et al., 1980), and for foetal, 33 minutes, and neonatal lambs, 51 minutes (Morishima et al.,1979). Bupivacaine is similarly distributed rapidly then metabolised in the liver and excreted in urine, with half-lives in pregnant, 102 minutes, and non-pregnant sheep, 118 minutes (Kennedy et al., 1990). The rapid metabolism and complete elimination from the body of both compounds strongly suggests that significant residues are unlikely to occur in lambs or, by cross-species comparison, in cattle following topical application (such as in Tri-Solfen) (Windsor, 2014). The European Agency for the Evaluation of Medicinal Products (EMEA) has concluded that cetrimide use in food producing species should not result in residues in food of animal origin at concentrations that are toxicologically relevant for the safety of consumer, since is poorly absorbed from the gastrointestinal tract, rapidly excreted, nor significantly absorbed after percutaneous administration, with a long history of safe use in human medicine (European Agency for the Evaluation of Medicinal Products [EMA], 1999).

The biotransformation of lidocaine and bupivacaine produces 2.6-xylidine, as a minor metabolite, that has been shown to be a weak mutagenic compound *in vitro* and to have genotoxic characteristics *in vivo*. Recent research indicates 2.6-xylidine has the main metabolite produced by primary hepatocytes and liver microssomes, from pigs and cattle, exposed to lidocaine (Thuesen and Friis, 2012). This metabolite produced nasal tumours in a 2 year oral toxicity study in rats receiving daily doses of the metabolite equivalent to 150 mg/kg BW/day. The results of the rat study using 2.6-xylidine have been extensively reviewed and it is accepted that this metabolite of lidocaine and bupivacaine does not represent a hazard to human health (McLean, 2014).

The European Committee for Medicinal Products for Veterinary Use (CVMP) could not establish a maximum residue limit (MRL) in food producing species other than horses for lidocaine, since the metabolism in these species was unknown. In line with the provisions of Article 11 of Directive 2001/82/EC (under the so-called “cascade”), lidocaine can only be used in *equidae* or exceptionally in other species. As well as bupivacaine products licensed for use in humans are commonly used under the veterinary cascade provisions. There is no MRL set for bupivacaine, however it is classified as an essential medication for horses, according to Regulation (EC) No 1950/2006.

The “cascade” is a legal provision that, in general terms, allows veterinarians under specified conditions to use products that are authorised for another species if no medicinal product has been authorised for the treatment of a specific condition in the concerned animal species. Several products containing lidocaine have been reported to be widely used in major food producing species, such as cattle and pigs, under this specific condition due to the lack of authorised anaesthetics in those animal species (European Medicines Agency's [EMA], 2015).

The CVPM reviewed the toxicological and carcinogencity data regarding lidocaine and 2.6-xylidine, and also undertook a comprehensive risk analysis assessment. The Committee used the Threshold of Toxicological Concern (TTC) approach and pharmacokinetic modelling to predict when "no molecule of lidocaine would be left in the animals' body", concluding that there is negligible risk to consumer safety from therapeutic use of lidocaine in food producing species – including cattle and pigs – with the use of a 28 days for withdrawal period for meat and ofalls, which is established as the minimum under cascade provisions.. Even if an entire carcass could be ingested by a single consumer, exposure to residues would remain below the TTC of 0.15 µg. Regarding milk, to ensure that the total residues in the cow's body are below this level and there is no risk to the consumer, an interval of 15 days between use of lidocaine and the taking of milk for human consumption is required (EMA, 2015).

Sellers et al. (2009) evaluated the pharmacokinetics of lidocaine in Holstein cows following an inverted L and caudal epidural nerve block, by determination of its plasma and milk concentrations. No detectable lidocaine concentration was present in the milk and serum samples at any time following caudal epidural administration. Following administration via inverted L, lidocaine had a half-life in blood of 4.2 hours. Lidocaine was not detected in plasma after 10 hours in any animal. Three of nine animals still had detectable milk residues 48 hours, but not 60 hours after treatment.

In the light of these earlier studies and by species comparison, local anaesthetics administration, such as Tri-Solfen, are very unlikely to lead to significant residues. Although there is minimal published information on the pharmacokinetics of lidocaine and bupivacaine in cattle, there is considerable comparative information indicating the risks of a residue violation from using Tri-Solfen in cattle and sheep for procedures other than mulesing is very low. Due to the small doses used, minimal absorption due to topical application and use of adrenalin and known rapid excretion of the actives in Tri-Solfen, expectation is that all relevant residues are below detection levels very soon after treatment (24 hours) in food animals (Windsor, 2014).

The Australian Pesticides and Veterinary Medicines Authority (APVMA), in order to keep residues of lidocaine, bupivacaine and cetrimide as low as practicable, originally used the limit of quantification (LOQ) for these substances constituents to set the MRL for Tri-Solfen. The regulatory methods actually used to measure the amount of residues in meat and offal set LOQ at 0.02 mg/kg for bupivacaine, 0.5 mg/kg for lidocaine and 0.1 mg/kg for cetrimide. Presumably caution prevailed and the product was registered with a conservative withdrawal period of 90 days when used in lambs in mulesing (Windsor, 2014). The Australian Office of Chemical Safety has reviewed the toxicology and occupational safety of Tri-Solfen and established an acceptable daily intake (ADI) for its active ingredients: 0.009 mg/kg BW/day for lidocaine, 0.001 mg/kg BW/day for bupivacaine and 0.01 mg/kg BW/day for cetrimide (McLean, 2014).

In Portugal, the *Direcção Geral de Alimentação e Veterinária (DGAV)* approved the experimental utilization of Tri-Solfen for the present study according to article 99, DL 148/2008, altered by DL 384/2009, and required a withdrawal period of 6 months for meat and 60 days for milk after application, according to nº2 of the article 100.

Nevertheless, the duties of veterinarians in provision of therapeutics to food animals should be a balance between different regulatory acts, relieve suffering of animals and the right to prescribe unregistered topical anaesthetic products under the cascade (Windsor, 2014).

2. EXPERIMENTAL STUDY

2.1. OBJECTIVES

The main purpose of the study was to measure the efficacy of Tri-Solfen in reducing pain during hoof trimming of lame dairy cows, through behaviour assessment during and after trimming and reaction to pressure with an algometer. Being dairy cows the population of this study a second objective was to assess lidocaine, bupivacaine and metabolites, as well as cetrimide residues in milk to determine the safety of use in milking animals.

Some lame cows with foot skin lesions were also treated with the product to evaluate its anaesthetics and antiseptic properties in these cases.

In summary, an innovative combination of local anaesthetics were tested in order to evaluate the efficacy in controlling pain during treatment of various hoof pathologies, as well as to determine the presence of its residues in milk samples after administration.

2.2. MATERIAL AND METHODS

2.2.1. Ethics Statement

All procedures were approved under the *Direcção Geral de Alimentação e Veterinária*, nº 49/ECVPT/2017 (Appendix IV) and the Ethics and Animal Welfare Committee of University of Lisbon – *Faculdade de Medicina Veterinária*, Ref. 003/2018. All recommendations were taken into account.

2.2.2. Description of the farms

The studies were performed in two separate periods. Initially from 2014 to the beginning of 2017 (without the author cooperation), and then from September 2017 to February 2018 (as part of the author's field study).

Four different dairy cattle farms located in the Ribatejo and Lisbon areas were included. All farms have intensive milk production has the main production purpose, performing two or three milkings a day. Pure Holstein-Friesian or Holstein crossbreeds are the leading population in these farms. The largest farm has around 1,100 animals in lactation and the smallest around 250 milking cows. Animal installations, nutrition, handling and management are similar across farms.

2.2.3. Study 1 - Residues' search in milk after the use of Tri-Solfen

2.2.3.1. Experimental group

Five lame cows were included in this study with different Tri-Solfen dosages applied (Table 2). The cow's weight range varied from 550Kg to 700Kg and the dosage of product from 3mL to 14mL depending on the extension and severity of the lesion. The five cases included: one interdigital necrobacillosis, one toe ulcer and three sole ulcers each in different stages, one of them with a concomitant abscess in the inner white line area. Forty milk samples were collected in total.

Table 2 – Specification of cases, including weigh, type of lesion and dosage used.

ID number	Weigh	Pathology	Tri-Solfen Dosage
4423	600Kg	Interdigital Necrobacillosis	9 mL
4460	700Kg	Bleeding deep sole ulcer	10 mL
4828	550Kg	Superficial sole ulcer with concomitant abscess	3 mL
395	650Kg	Toe ulcer	14 mL
9071	650Kg	Deep sole ulcer	6mL

2.2.3.2. Protocol

a) Inclusion criteria: lame cows at the end of lactation. All cows presenting hoof (e.g. sole ulcers, toe ulcers, white line disease) or digital skin (e.g. digital dermatitis and interdigital necrobacillosis) lesions were considered for the study.

b) Exclusion criteria: chronic lameness cases or those presenting deep infected lesions. No animal destined to produce milk for human consumption in the following 60 days, were used.

c) Lameness scoring before treatment: lameness was scored from 1 to 5 (according to Sprecher et al. [1997] lameness scoring system) when cows were driven to the chute.

d) Lesion assessment and product application: cleaning and superficial trimming were then performed on the affected hooves. If any lesion was found it was described, classified and graded accordingly to dimension. Lesions were also photographed. The lesion area was then further trimmed and Tri-Solfen was applied on the wound once corium was exposed. The quantity of Tri-Solfen used, in milliliters, was registered. Corrective trimming was then performed. The amount of tissue and debris removed from the lesion was also registered. If additional treatments (for example, applying a block on the other hoof) were performed, they were registered.

e) Algometry and behaviour assessment: A Wagner FORCE TEN^{TM} FPX algometer, in Kgf (a digital instrument that measures the pressure that elicits a withdrawal reaction) was used to assess reaction to pressure on the site of the lesion before deep trimming or any further procedure, and one minute after application algometry was again performed on the lesions. Behaviour (reaction, struggling, falling and vocalization) during trimming was assessed.

f) In digital skin lesions: Tri-Solfen was applied after cleaning (thorough washing and drying) the area. The quantity of Tri-Solfen used was registered and after one minute algometry was performed on the wound. In these cases, behaviour assessment is not applicable since no further intervention was done on the lesion.

g) Lameness scoring after treatment: lameness was again scored when the cow left the chute.

h) Sample collection: approximately 10mL of milk from each cow were collected from all four quarters into a sterile container, on the subsequent four milkings. To increase reliability and prevent risk of losses, two samples per cow per milking, were collected. Milk samples were immediately frozen at minus 20ºC until shipment to a laboratory in Australia. During shipment and until laboratory analysis the samples were kept at minus 70ºC.

i) Residues analysis: chromatography analysis was performed by Eurofins Agroscience Testing Pty Ltd (Appendix V – Complete residues search analytical methods protocol). All samples were analysed for concentrations of lidocaine and its metabolites, monoethylglycinexylidide (MEGX), 3-hydroxylignocaine, 2,6-dimethylaniline (2,6-DMA), Lignocaine-N-oxide, 4-OH DMA and GX (glycinexylidide), bupivacaine and its metabolites 3-hydroxybupivacaine, pipecolic acid and desbutylbupivacaine as well as 2,6-dimethylaniline (2,6-DMA), if present, and cetrimide accordingly to the procedures of the analytical test site. Limit of Detection (LOD) and Limit of Quantitation (LOQ) were used as numerical values to describe residue findings. The efficiency of the method was monitored by the analysis of control (untreated) samples and fortified control (recovery) samples analysed concurrently with the test samples.

2.2.4. Study 2 - Use of Tri-Solfen for pain management during treatment of hoof lesions in dairy cows

2.2.4.1. Experimental group

Sixty two dairy cows were included in this study and submitted to the full protocol.

Twenty nine lame cows were included in the Control group (C) showing the following hoof lesions: two cases of white line disease, one of them with a concomitant abscess; twenty three cases of sole ulcer; and four cases of toe ulcer.

On the Tri-Solfen group (TriS), thirty three lame cows were considered for study, with the following hoof pathologies: three cases of white line disease; twenty four cases of sole ulcer; five cases of toe ulcer; and one case of white line disease concomitant with a sole ulcer.

Although, this study was performed in two separate periods, the protocol and the responsible veterinarian were the same.

The sample for algometry testing is smaller since it was only performed in the second stage of this study, therefore only in twenty seven cases. Both hoof lesions and digital skin lesions were included. Algometry values were collected before any treatment ('Before treatment'), after application of Tri-Solfen or the control gel ('After treatment'), and at the end of all treatments ('End of treatment'). Comparisons within the three periods were made in order to determine the difference between them. For each animal, values by period were subtract in order to evaluate the variance between them and obtain a more accurate value. Assessment during this three periods was not possible for all twenty seven animals due to logistic reasons. Only hoof lesions had a Control group, since Tri-Solfen was always applied on the digital skin lesions.

2.2.4.2. Experimental group extra

When performing the study on hoof horn lesions, a considerable number of lame cows presented digital skin lesions. These were not included in the previous trials but even so were treated to investigate the effect of the local anaesthetics on different type of lesions. The purpose was to determine if there was any pain relief when Tri-Solfen was applied on skin lesions.

For that reason, cases including interdigital necrobacillosis and digital or interdigital dermatitis were submitted to the same protocol as in the previous experiment, except for the selection of treatment group, since all cases were treated with Tri-Solfen. Being soft tissue lesions, trimming was not performed and only cleaning was done. The same criteria of inclusion and exclusion were used. The analgesic effect of Tri-Solfen was solemnly established by algometry before cleaning, one minute after application and before leaving the chute.

Ten lame cows were considered for study, including one case of interdigital necrobacillosis and nine cases of digital/interdigital dermatitis.

2.2.4.3. Protocol

a) The inclusion and exclusion criteria: the same criteria as in the previous study were applied. Dairy farmers were asked to select lame cows needing trimming at the end of lactation.
b) Two different working teams were formed: one included two behaviour observers, blind to the treatment; the other team included two operators that perform the trimming and randomly selected the treatment. Trimming was performed by an experienced veterinarian. Both teams recorded their activity on two different forms: the observer's form (Appendix VI) and the operator's form (Appendix VII).
c) Cows showing lameness were scored from 1 to 5 (according to Sprecher et al. [1997] lameness scoring system) when walking to the chute by both teams independently.
d) The affected hoof was submitted to the algometer test as soon as the limb was lifted.

e) Cleaning and superficial trimming were performed until a lesion was clearly identified and there was exposure of the corium, granulation tissue, bleeding or signs of pain. The affected limb and hoof were registered and the lesion was described, photographed, classified and graded accordingly to its dimension.
f) In case the hoof lesion was considered for the study, cows were randomly distributed to one of two groups: Tri-Solfen Group (TriS) and Control group (C) by taking a code-paper from a bag. The lesion area was then further trimmed, with exposure of corium. Tri-Solfen or the innocuous gel were applied on the wound once corium was exposed. The quantity, in milliliters, of Tri-Solfen used was always registered.
g) One minute after the application of the product, algometry was again performed and more invasive procedures required to complete the treatment took place, such as removal of necrotic, haemorrhagic and/or granulation tissue. Lesions were classified from 1 to 3 accordingly to the amount of tissue removed (Appendix VIII). The application of Tri-Solfen could be repeated if trimming had to be very deep or a new lesion was found. The total amount of Tri-Solfen used was registered.
h) An ethogram was filled during trimming, including the following behaviours: unrest, trying to withdraw limb/kicking, falling on one or more limbs (Appendix IX) and vocalization. Accordingly to the ethogram, pain reaction to trimming was graded as: 1 – absent; 2 – moderate; 3 – severe (Appendix X).
i) In very severe cases, a wooden block on the opposite hoof, a bandage, an antibiotic/antiseptic spray or systemic medication was given and registered.
j) When trimming was concluded a last algometry test was performed, lameness was again scored when leaving the chute and, when possible, also two to three days after trimming.

2.3. STATISTIC ANALYSIS

Data collection was performed resorting to Microsoft Excel®. Statistical analysis were performed with help of SAS System®, using parametric and non-parametric tests. Analysis of variance was achieved the GLM procedure.

The variables submitted under statistical analysis were: lameness score before and after trimming; behaviours during trimming, including reaction to trimming and falling; and algometry at various times from podal pathologies.

Differences from lameness score before and after trimming were compared within Control group (C) and Tri-Solfen group (TriS) separately in order to evaluate lameness progress inside each group in a scale from 1 to 5.

Behaviours during trimming were considered in two variables: ‘Reaction to trimming’ and ‘Falling’, each classified in a scale from 1 to 3. The correlation of data was between the

Control group (C) and Tri-Solfen group (TriS), for further analysis of behaviour changes according to group.

Algometry data was combined from all different podal pathologies assessed during the study, including hoof and digital skin pathologies. Analysis of variance was performed between Control group and Tri-Solfen group from 'Before treatment', 'After treatment' and 'End of treatment' periods, and also for differences between 'After-Before treatment', 'End-After treatment' and 'End-Before treatment'. The GLM procedure and a model with only the group factor were used for statistical analysis.

Throughout the study was always considered a significance level at least of when $p<0.05$, for a confidence level of 95%.

2.4. RESULTS

2.4.1. Milk residues of Lidocaine, Bupivacaine and their metabolites and Cetrimide

All residues found were presented in µg/L and analysed according to Limit of Detection (LOD) and Limit of Quantification (LOQ) established by the laboratory. The LOD and LOQ considered for lidocaine, bupivacaine and their metabolites were 0.07 µg/L and 0.2 µg/L, respectively, as for cetrimide were 3 µg/L and 10 µg/L, also respectively. Table 3 discriminate all values found per animal. All other residues searched for each sample were under LOD values, with no value detected. Residues per animal according to milking are displayed on appendix XI.

Table 3 – Residues' values per animal, according to pathology and treatment. Values marked with (*) represent the values above LOQ; all other values were under LOD, marked as NO.

RESIDUES AFTER TREATMENT WITH TRI-SOLFEN								
ID	Weight (aprox)	Description Lesion	Lesion size (aprox)	Quantity Tri-Solfen	Lidocaine and metabolites	Bupivacaine and metabolites	Cetrimide	Other treat.
4423	600 Kg	Interdigital Necrobacillosis	Large	9 mL	1st MILKING 0.128 µg/L and 0.145 µg/L	2nd MILKING 1.18 µg/L*	3rd MILKING 3.66 µg/L	NSAID (Ceftiofur)
4460	700 Kg	Bleeding ulcer	14 cm^2	10 mL	2nd MILKING 0.393 µg/L*	NO	NO	NO
4828	550 Kg	Abscess; Initial solar ulcer	3 cm^2	3 mL	NO	4th MILKING 0.712 µg/L*	NO	Shoe
395	650 Kg	Toe ulcer; Bleeding	6 cm^2	14 mL	1st MILKING 0.263 µg/L* and 0.398 µg/L* 3rd MILKING 0.0705 µg/L	1st MILKING 0.118 µg/L	2nd MILKING 3.48 µg/L	Shoe
9071	650 Kg	Deep infected solar ulcer	3 cm^2	6 mL	1st MILKING 0.204 µg/L* and 0.247 µg/L*	1st MILKING 0.0787 µg/L	1st MILKING 11.5 µg/L* and 24.4 µg/L*	NSAID (Tolfenamic acid)

In animal 4423, lidocaine residues were detected under LOQ values at the first milking, in both samples; bupivacaine residues were detected above LOQ at the second milking, in only one sample; and cetrimide residues were detected under LOQ values at the third milking.

In animal 4460, lidocaine metabolite monoethylglycinexylidide was detected above LOQ, at the second milking and only in one sample.

In animal 4828, the metabolite N-desbutyl bupivacaine was detected above LOQ values at the fourth milking, in one sample.

In animal 395, lidocaine residues were detected above LOQ values in the first milking, in both samples, and under LOQ values at the third milking, in only one sample. Bupivacaine residues were detected under LOQ values at the first milking, only in one sample. Cetrimide residues under LOQ values were detected at the second milking, only in one sample.

In animal 9071, lidocaine and cetrimide residues were detected above LOQ values in the first milking. Also at the first milking, bupivacaine metabolite 2,6-dimethylaniline was detected under LOQ values, however only in one of the samples.

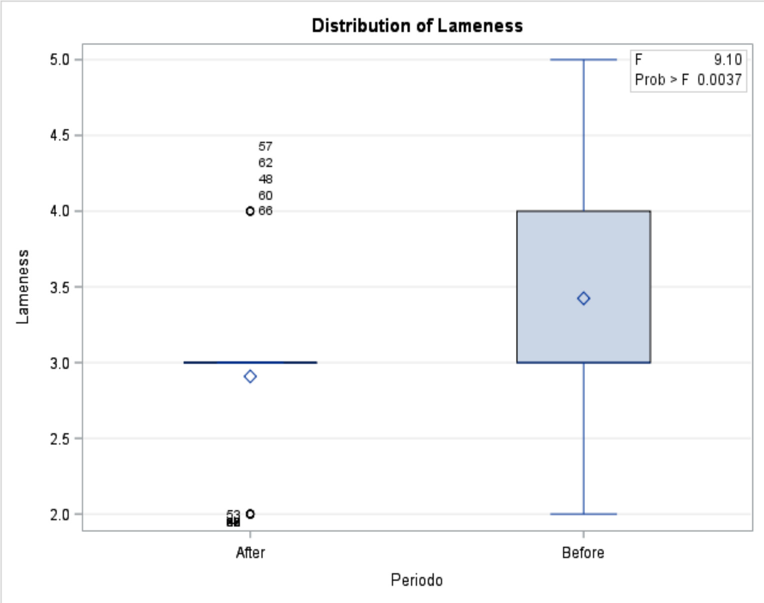
2.4.2. Lameness scores before and after trimming

'Lameness score before trimming' and 'Lameness score after trimming' variables were compared within the Control group, in a total of twenty nine animals (N=29). No significant differences (*p*=0.6919) were found in lameness scoring between the two separate moments of assessment, with means 3.276 +/- 0.122 before trimming and 3.345 +/- 0.122 after trimming (Graphic 1).

Graphic 1 – Boxplot of 'Lameness score before trimming' (Before) and 'Lameness score after trimming' (After) in Control group.

The same variables were compared within Tri-Solfen group, in thirty three animals (N=33). Significant differences (*p*<0.01) were found between lameness scoring before and after the procedure with means 3.424 +/- 0.121 before trimming and 2.909 +/- 0.121 after trimming. (Graphic 2).

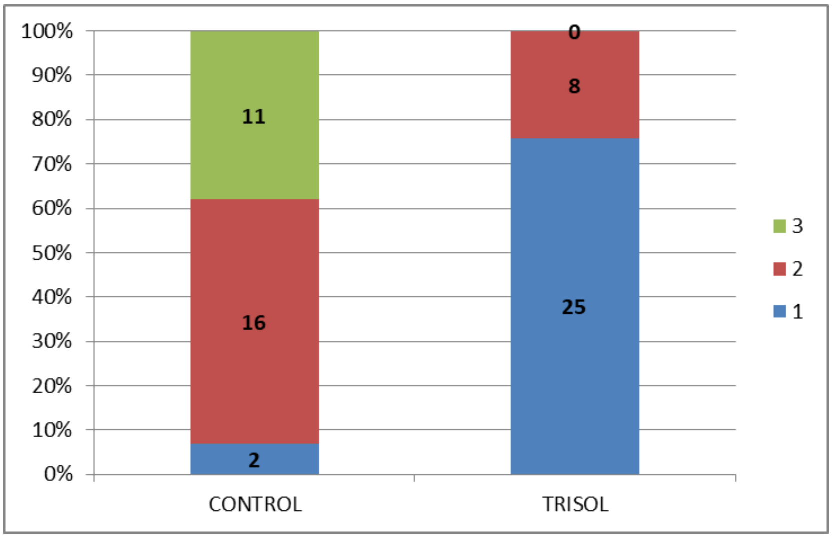
Graphic 2 – Boxplot of 'Lameness score before trimming' (Before) and 'Lameness score after trimming' (After) in Tri-Solfen group.



2.4.3. Reaction to trimming

Sixty two (N=62) animals were included in the analysis of the variable 'Reaction to trimming': twenty nine in Control group (N=29) and thirty three in Tri-Solfen group (N=33). This variable was classified in a scale from 1 (no or mild reaction) to 3 (violent reaction). When comparing classification for this variable between Control group and Tri-Solfen group, significant differences were found (p<0.01) with the TriS animals showing less reaction to the procedure (Graphic 3).

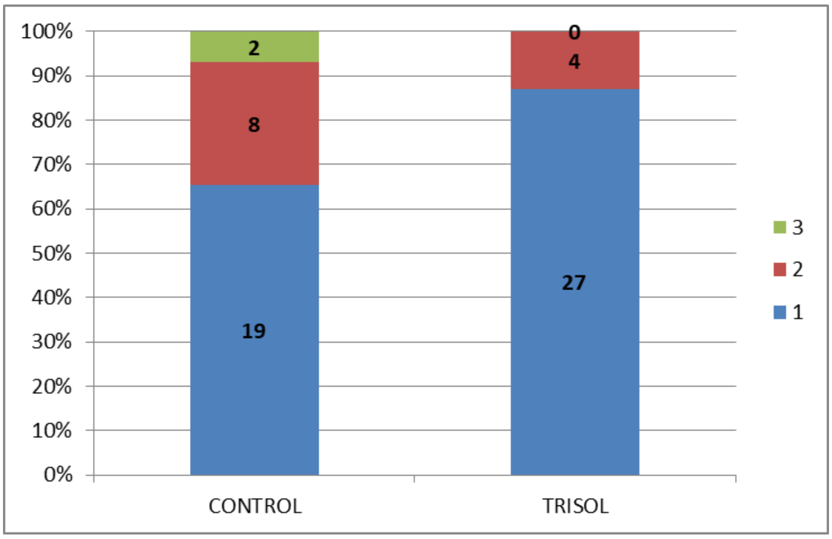
Graphic 3 – Number of animals in each reaction category to trimming in Control (CONTROL) and Tri-Solfen (TRISOL) groups.



2.4.4. Falling

Sixty animals (N=60) were included in the analysis of the variable ‘Falling’: twenty nine (N=29) in Control group and thirty one (N=31) in Tri-Solfen group. This variable was classified in a scale from 1 (no risk of falling) to 3 (cow falls). When comparing values from this variable between Control group and Tri-Solfen group (Graphic 4), significant differences are found along all analytical analysis, with a significance level of $p<0.05$, except for median scores analysis, with $p=0.0503$.

Graphic 4 - Number of animals falling during the trimming procedure in Control (CONTROL) and Tri-Solfen (TRISOL) groups.



2.4.5. Algometry

A total of twenty seven (N=27) animals were included in the analysis of the algometry values, seven (N=7) animals in the Control group and twenty (N=20) in the Tri-Solfen group. All data was combined to determine the differences on algometry values with or without Tri-Solfen application.

Significant differences were found in algometry values between Control and Tri-Solfen groups ‘After treatment’ ($p<0.05$). The values’ variation between ‘Before treatment’ and ‘End of treatment’ (Period 3-1) were also different, with TriS group showing a significant increase for $p<0.05$ (Table 4).

Table 4 – Least squares means from algometry evaluation, on three separate periods and the association of periods, marked bold the differences. Different superscripts indicate significant differences.

	Before treatment (1)	**After treatment (2)**	End of treatment (3)	Period 2_1	**Period 3_1**	Period 3_2
Control Group	1.738±0.389	**1.314^{a}±0.637**	1.280±0.576	-0.424±0.791	**-0.458^{a}±0.563**	-0.034±0.514
Tri-Solfen Group	1.383±0.253	**3.532^{b}±0.600**	2.548±0.395	1.726±0.746	**1.15^{b}±0.386**	0.027±0.550

2.5. DISCUSSION

Pain management is problematic in farm animal practice, especially for countries with large livestock populations and where veterinary services are unavailable to treat individual animals (Windsor, Lomax, & White, 2016)

Hoof pathologies can inflict severe pain on dairy cattle. However, painful procedures are frequently performed without anaesthesia, due to a lack of awareness of the pain caused by lameness treatments as well as to the sensitivity of dairy cows to pain (Becker et al., 2013).

The development of effective pain management must be given high priority to decrease animal suffering, with long term solutions. Yet, currently there is lack of commercially available pain-alleviating options that meet the practical and economic constraints of production animal husbandry (Lomax et al., 2008). Affordable and practical solutions of pain alleviation during treatment of hoof related pathologies is proposed for incorporation into routine farm management practices.

Topical anaesthesia, applied during and immediately after the procedure, has previously been found to be practical and effective for reducing post-operative pain associated with surgical husbandry procedures, such as mulesing in sheep (Lomax et al., 2008 ; Paull et al., 2009), dehorning and castration in calves (Espinoza et al., 2013; Lomax & Windsor, 2013). These findings have major welfare impact for all livestock undergoing such procedures.

In general, local anaesthetics have poor skin absorption, which limits their use for effective pre-procedural skin anaesthesia. However, the benefit in the post-procedural application of local anaesthetics lies in the rapidity and efficacy when they are applied directly to open wounds. (Lomax et al., 2008; Espinoza et al., 2013) Knowledge of this property allowed to suppose and experiment local anaesthetic application on hoof lesion after exposure of the corium.

Results from this study indicates that significant alleviation of pain can be achieved in dairy cows during and immediately after corrective hoof trimming, using a topical anaesthetic and antiseptic spray-on gel preparation. This demonstrated that lidocaine and bupivacaine can effectively block nociceptors after exposure of the corium, resulting in local anaesthesia.

2.5.1. Milk residues of Lidocaine, Bupivacaine and their metabolites and Cetrimide

Since MRL are not established to these active constituents, the LOQ of the developed and validated analytical method, is used to determine the presence and to quantify the residues levels. Values less than LOQ, meaning values between LOD and LOQ, which accuracy and precision cannot be verified, should not be considered as relevant.

According to the LOQ established by Eurofins Agroscience Testing Pty Ltd, no significant milk residues of lidocaine, bupivacaine and their metabolites and cetrimide were found on any of the five animals after the second milking.

In animal 4423, bupivacaine residues were detected at the second milking after treatment with values above LOQ (1.18 μg/L), only in one of the samples. In animal 4828, the metabolite N-desbutyl bupivacaine was detected above LOQ values (0.712 μg/L), at the fourth milking after treatment and only in one of the samples. In animal 4460, lidocaine metabolite monoethylglycinexylidide was detected above LOQ values (0.393 μg/L), at the second milking after treatment and only in one sample. For these three animals, the fact that only one of the samples presented values above LOD, a laboratorial mistake seems to be the more reasonable explanation. Otherwise, both samples collected at the same time should present a similar amount of residues.

In animal 395, lidocaine residues were detected above LOQ values in the first milking after treatment, in both samples (0.263 μg/L and 0.398 μg/L). In animal 9071, in the first milking after treatment and in both samples lidocaine, residues were detected above LOQ values (0.204 μg/L and 0.247 μg/L), as cetrimide residues (11.5 μg/L and 24.4 μg/L). Since values above LOQ were present in both first milking samples, it can be suggested that approximately six hours after treatment residues can still be present. The presence of residues in only two of the five animals can be questioned. This can occur due to the volume of Tri-Solfen applied to the wound, since the largest quantity of Tri-Solfen was in animal 395 (14 mL), the type or extension of the lesion (as a deep sole ulcer in animal 9071), or even individual biological differences of the animal. However, only with a study of residues depletion according official regulations can allow the obtainment of a withdrawal period for milk.

For all purposes, no studies were performed yet to to determine safe residues levels for lidocaine, bupivacaine and their metabolites and cetrimide in milk, and subsequent withdrawal period, after topical anaesthesia application. A study evaluating the pharmacokinetics of lidocaine in mature Holstein cows following an inverted L nerve block suggested a milk withdrawal period of seventy two hours, since detectable lidocaine concentration in milk was present forty eight hours later, but not after sixty hours post-injection (Sellers et al., 2009). This study can only give an idea, since topical application and local injection of lidocaine can have different pharmacokinetics. Nevertheless, milk from exposed dairy cows is diluted with milk from other cows in the same and other farms before reaching the consumer, so it does not seem relevant to calculate exposure through milk from a single treated cow among a herd (Committee of the Norwegian Scientific Committee for Food Safety, 2005).

The findings of this study suggest that the application of this topical anaesthetic formula would not lead to significant presence of lidocaine, bupivacaine, and their metabolites, and cetrimide in milk after six hours after treatment. This suggests that food safety would not be affected when treating topically a dairy cows with a few milliliters of this product.

2.5.2. Lameness scores before and after trimming

Lameness scoring after trimming was significantly lower when compared with lameness scoring before trimming on the Tri-Solfen group (p<0.01). This can suggest that an alleviation of pain is accomplished after trimming procedures with a local anaesthetic application, allowing the animal to put more weight on the affected limb. This improvement on lameness scoring did not happened on the Control group without any pain management, suggesting that trimming alone does not relieve pain or can even inflict and exacerbate pain. Local anaesthetic agents act directly on nerve tissue to reversibly block conduction of signals responsible for the sensation of pain, not only effecting wound anaesthesia, but also preventing or reducing the subsequent pain escalation response (Lomax et al., 2008).

Lameness assessment after three days was initially proposed, not only to assess lameness, but also to try to relate lesion evolution with the antiseptic properties of cetrimide. However, due to the continued dirtiness of the wound site and the difficulties to reassess the lesion by re-examining the hoof, this assessment was abandoned.

2.5.3. Behaviours during trimming

Observation of behaviour responses to an acute painful stimulus is an objective, repeatable and readily measurable form of assessing pain, commonly used for grading pain behaviour and used in many pain trials, allowing the assessor to distinguish between various analgesic interventions (Lomax et al., 2008). More objective assessments of pain can be misleading, such as cortisol levels, that can rise during surgical procedures even when pain is completely abolished by anaesthesia (Lomax et al., 2008). Paull et al. (2009) reported that treatment of lambs with non-steroidal anti-inflammatory drugs resulted in a significant reduction in post-mulesing pain related behaviour, but not in a corresponding reduction in cortisol response. The reliability of heart rate, blood pressure or endorphin release, for estimating pain alleviation can also be dubious in this case, since adrenaline (present on Tri-Solfen) can have confounding effects on such physiological parameters (Lomax et al., 2008).

To assess behaviour responses to pain in this study, reaction to trimming and falling during trimming were evaluated by a group of two observers blind to treatment, to increase repeatability.

The reaction to trimming (in a scale of 1 to 3) was significantly lower on the Tri-Solfen group (p<0.01) when compared with the Control group. Falling classification (in a scale of 1 to 3) during trimming was also significantly lower on the Tri-Solfen group (p<0.05) when compared with the Control group. A less severe and intense exhibition of pain related behaviours during corrective trimming after the application of Tri-Solfen suggests a reduced pain sensation or nociceptive input given by local anaesthetics (Lomax et al., 2008). A significant treatment effect on pain related behaviour after application of Tri-Solfen was already proven in calf castration, expressing significantly less pain than untreated calves (Lomax & Windsor, 2013).

2.5.4. Algometry

Mechanical nociceptive threshold tests, such as algometry, are measures of the responsiveness of animals or humans to a noxious stimulus. These techniques may be used to record changes in the nociceptive threshold associated with physical injury or surgical trauma. The decrease in nociceptive threshold is referred to as a demonstration of hyperalgesia (an exaggerated sensitivity to pain) (Whay et al., 1998).

In this study, algometry values increased on the animals submitted to corrective trimming after topical anaesthetic application (Tri-Solfen group) when compared with animals summited to the same procedure without any pain management (Control group), which maintained algometry values on the same range. This demonstrates a greater nociceptive threshold on the treated group due to diminishing pain sensitivity, since tolerance to pressure was higher. Such conclusions are reinforced when algometry values' variation between 'Before treatment' and 'End of treatment' in the Tri-Solfen group shown an increase, meaning that the hyperalgesic state at the end of all trimming procedures was reduced. In the Control group, such variations of algometry values were not notorious, meaning no alteration on the nociceptive threshold status. Previous studies resorting to nociceptive threshold test to evaluate wound sensitivity after application of topical anaesthetics corroborate these findings. Espinoza et al. (2013) stated that calves submitted to topical anaesthesia were more likely to show no response to stimulation than calves without topical anaesthesia, up to ninety minutes after dehorning. Also in calves, treatment with topical anaesthesia had a significant effect on pain threshold after castration, with response being significantly lower than in untreated calves, indicating that a significant wound anaesthesia was achieved (Lomax & Windsor, 2013).

Since algometry assessment was only implement on the second period of this trial and because skin related pathologies were always submitted to treatment, due to difficulty in measuring pain related behaviours evaluation (no trimming), the Control group was smaller. This means that the same study with a larger control group should be performed to confirm these results.

CONCLUSION

Lameness caused by hoof related pathologies is a complex subject and its incidence is influenced by a number of coexisting factors, like management, genetics, claw trimming, veterinary care and pain control.

The acute pain inflicted by routine procedures, such as corrective trimming, directly influences immediate animal welfare and it is imperative to find new and improved ways to address these procedures, through pain management control. Economic reasons, legal restrains in production animals and availability of drugs may reduce the ability to intervene.

The use of topical local anaesthesia, such as Tri-Solfen, may be well suited and accepted to production animal farming because of its low cost, practicality and easy application. Animal welfare improvement is notorious, with proven reduction of pain related behaviours expressed by the animals during and immediately after corrective trimming of extensive and severe hoof lesions. Not only animal welfare is enhanced, as greater safety for the hoof trimmer is accomplished due to less aggressive behaviour reaction of the animal.

Although substances residues in production animals have a big influence on the implementation of such local anaesthetics on routine procedures, lidocaine and bupivacaine, do not seem to have impact on milk safety after the first milking subsequently to its application, suggesting minimal absorption when applied topically. The safety of animal products for human consumption after the use of topical anaesthetics, such as milk from dairy cows, and its withdrawal periods are probably overestimated and need to be reviewed. Reduction of withdrawal periods not only will constitute an economic advantage for the farmer, also will motivate and stimulate the use of pain management solution on dairy cattle production.

Education and training in the acknowledgement of signs of pain demonstrated by dairy cows, as well as in the benefits of analgesia, needs to be enhanced, and all parties involved in the management of lameness in dairy cattle need to act in a collaborative manner to minimise its negative consequences on animal welfare and health.

One of the main roles of veterinarians of farm animals is to alert and raise awareness among farmers and technicians to better solution in production procedures, always taking into a very serious account the welfare of these animals.

In the future, similar studies should be made with a larger number of animals, where lesion characteristics and the amount of product used could be correlated with the presence of residues, in order to obtain more solid conclusions. The antiseptic properties of Tri-Solfen should also be investigated, through assessment of the lesions and correlation with lameness scores days after the application.

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APPENDICES

Appendix I – Poster "Caso clínico atípico de besnoitiose (*Besnoitia besnoiti*) num touro limousine"

Appendix II – Poster "Aplicação tópica de anestésicos locais para controlo a dor durante a aparagem curativa de lesões podais de vacas leiteiras – dados preliminares"

8º Encontro de Formação da Ordem dos Médicos Veterinários
14 e 15 de Abril de 2018, Lisboa

APLICAÇÃO TÓPICA DE ANESTÉSICOS LOCAIS PARA CONTROLO DA DOR DURANTE A APARAGEM CURATIVA DE LESÕES PODAIS DE VACAS LEITEIRAS – DADOS PRELIMINARES

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Introdução

As doenças podais de vacas leiteiras em regime intensivo têm um enorme impacto, quer sobre a produção quer sobre o bem-estar animal, sendo responsáveis por grande desconforto e sofrimento. Durante o tratamento das lesões, como úlceras da sola ou doença da linha branca, são necessárias intervenções invasivas e dolorosas. Tais procedimentos podem causar dor intensa, não só dificultando o maneio e pondo em risco a segurança do operador, como também afectando o bem-estar do animal. Assim, o maneio eficaz da dor não só é uma obrigação ética, como também permite uma mais fácil manipulação e um tratamento mais minucioso. Por norma, estas intervenções são realizadas por técnicos não veterinários sem formação ou competência para o controlo médico da dor. Um produto em gel de aplicação tópica (Trisolfen ®), composto por lidocaína, como anestésico de rápida acção, e bupivacaína, como anestésico de longa acção, adrenalina, para reduzir absorção dos anestésicos locais e reduzir a hemorragia, e cetrimida, como antisséptico, demonstrou ter efeito no alívio imediato e prolongado da dor noutros procedimentos em ruminantes (e.g. descorna e castração).

Objetivo

Testar a eficácia da formulação de anestésicos locais tópicos, aplicados durante o procedimento, como forma de maneio e controlo da dor durante e após a aparagem curativa de lesões sangrantes dos cascos (Figura 1).

Material e Métodos

Vacas no período de secagem

1. Avaliação do grau de claudicação antes da contenção → escala de 1 (sem claudicação) a 5 (claudicação severa);
2. Avaliação da reação do animal a diferentes graus de pressão → Teste de algometria antes da intervenção;
3. Distribuição aleatória a um de dois grupos – C ou T;

Grupo	Descrição
Controlo (C)	Aparagem sem aplicação de anestésico.
Anestésico local (T)	Aplicação do anestésico local sobre a lesão do cório, seguida de aparagem.

4. Teste de algometria após a intervenção;
5. Avaliação cega dos comportamentos de dor e reação à aparagem curativa → 1- ausente, 2- moderada, 3- severa;
6. Avaliação do grau de claudicação no fim da intervenção.

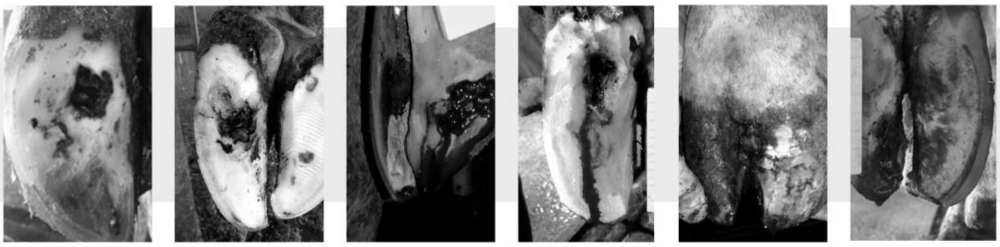

Figura 1 – Exemplos das doenças podais com indicação para tratamento sob anestesia local.

Resultados preliminares

Aplicação de anestésico local

Diminuição da reação à aparagem

Diminuição da claudicação à saída

Diminuição da reação à pressão (algometria)

NOTA: Devido à presença de lidocaína e bupivacaína, está em curso um estudo paralelo para medição da presença de metabolitos excretados no leite.

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Discussão e Conclusão

O estudo demonstrou que a utilização de anestesia local tópica com lidocaína e bupivacaína reduz a dor provocada pela aparagem curativa das lesões do casco extensas e severas, melhorando o bem-estar animal e aumentando a segurança para o operador, por redução das reacções violentas associadas à dor. Considerando a apresentação prática em forma de gel, sem necessidade de procedimentos mais morosos ou mão-de-obra mais especializada, a utilização de um medicamento que favorece conforto e elimina algum do sofrimento induzido durante o tratamento, poderá ser uma excelente alternativa para as práticas realizadas até então, tendo o médico veterinário a função de alertar e consciencializar o produtor para boas práticas que visam o bem-estar animal.

Appendix III – Abstract "Use of topical anaesthesia to control pain during and after trimming hoof lesions in dairy cows"

Use of topical anaesthesia to control pain during and after trimming hoof lesions in dairy cows.

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Treatment of hoof lesions such as sole ulcers in cattle frequently involve trimming of healthy and inflamed tissue (corium). Trimming may cause acute and severe pain resulting in violent reaction with risk for humans' safety as well as potentially affecting the animal's immediate welfare. Specific behaviours are easily identified when pain is caused. Due to technical and practical difficulties and because the procedure is generally carried out by a non-veterinarian, pain management is seldom used during hoof trimming in dairy farms worldwide.
Tri-Solfen® is a "spray and stay" topical anaesthetic formulation containing lidocaine for rapid onset wound anaesthesia, bupivacaine as a long-acting local anaesthetic to provide prolonged duration of effect, adrenalin to concentrate the anaesthetic effect at the wound site and reduce haemorrhage, and cetrimide to provide wound antisepsis, formulated in a viscous gel base. It can be easily applied by non-specialized personnel as a once-only application. Efficacy and welfare studies in mulesing, castration and tail docking in lambs and castration in calves, have shown that Tri-Solfen assists in the immediate alleviation of pain with prolonged effect, reducing both pain related behaviour, and wound pain responses from within 1 minute up to and including 24 hours post procedures.
Our study was designed to determine the effect of Tri-Solfen, applied during the procedure, in attenuating pain during and after trimming bleeding hoof lesions in dairy cows. In treated cows, the gel was applied after removing superficial horn and necrotic tissue and when corium was exposed.
Cows selected for hoof trimming at drying off were graded for lameness (1-non lame to 5-severely lame). The hoofs of those with lameness score above or equal to 3 were carefully examined for lesions by an experienced veterinarian. Lesion site, type and size were registered. Animals with horn lesions requiring trimming were randomly distributed to two groups: C – usual trimming with no pain control; T – trimming with Tri-Solfen being applied once live corium was exposed and on completion of trimming. Tri-Solfen application was repeated if deemed necessary (e.g. extensive or deep lesions) with the total quantity being registered. Trimming was resumed one minute after each application.
Lameness was scored on the way to the chute, when leaving the chute and three days after trimming. Reaction to trimming was scored (1 to 3) based on the display of the following pain related behaviours: kicking, efforts to release limb and vocalization. A digital algometer was also applied on the lesion area before, during and after trimming. Behaviour observation was done by two persons blinded to treatment.
Across several months this study was repeated in three large dairy farms. In total 28 lame cows were trimmed with Tri-Solfen and 22 were used as control. Two types of statistical analysis tests were used: non-parametrics tests and analysis of variance. Treatment significantly influenced reaction to trimming and lameness score after trimming ($p<0.01$), with better results for those animals treated with Tri-Solfen. Algometer values tended to be higher after application of Tri-Solfen. No differences were shown for lameness after three days. Differences between groups were not influenced by the limb (front or hind) or hoof (outer or

inner) affected. Algometry also showed increased pressure threshold in two interdigital skin lesions after application of Tri-Solfen.
In conclusion, this study shows that a topical gel with lidocaine and bupivacaine can reduce pain when trimming severe and extensive hoof lesions, improving dairy cow welfare and eventually trimmer safety.
We acknowledge the funding by Project UID/CVT/276/2013 (CIISA)
We acknowledge Animal Ethics, Australia for providing the product for the trials.

Appendix IV - Direcção Geral de Alimentação e Veterinária approval

REPÚBLICA PORTUGUESA | AGRICULTURA, FLORESTAS E DESENVOLVIMENTO RURAL

dgav Direção Geral de Alimentação e Veterinária

Mensagem nº /Message n.º	Data / Date	Nº de páginas (incl. a capa) /Number of pages (incl. Cover sheet)
1401	15.09.2017	1

Nome do destinatário / Name of adressed (tipe)	Contatos
Ex.mo(a) Sr.(ª) Dr.(ª) George Stilwell	stilwell@fmv.ulisboa.pt

De / From
Direção-Geral de Alimentação e Veterinária

Assunto:

Autorização de Ensaio clínico
UTILIZAÇÃO DO GEL TRI-SOLFEN NO CONTROLO DA DOR DURANTE TRATAMENTO DE ÚLCERAS DA SOLA DE VACAS DE LEITE
N.º de autorização: 49/ECVPT/2017

Cumpre-me informar V. Exa. que nos termos do artigo 99.º Decreto-Lei n.º 148/2008 de 29 de Julho alterado pelo Decreto-lei n.º 314/2009 de 28 de Outubro, é autorizado o seguinte ensaio clínico, com o respectivo n.º de autorização:

UTILIZAÇÃO DO GEL TRI-SOLFEN NO CONTROLO DA DOR DURANTE TRATAMENTO DE ÚLCERAS DA SOLA DE VACAS DE LEITE

De acordo com o n.º 2 do artigo 100.º foi estabelecido um intervalo de segurança para animais utilizados no ensaio:

- Carne e vísceras – 6 meses
- Leite – 60 dias.

A utilização deste medicamento veterinário experimental deve cumprir com o estipulado no artigo 82.º no que respeita ao registo de medicamentos em animais produtores de géneros alimentícios.
Qualquer alteração ao referido ensaio clínico deve ser submetida à DGAV.
Solicita-se o envio de um relatório final conclusivo do ensaio de acordo com os requisitos da VICH GL9, até um mês após a sua conclusão.

Com os melhores cumprimentos,

p/ O Diretor Geral

Fernando Bernardo

GRAÇA MARIANO
SUBDIRETORA- GERAL
Por Delegação de Competências n.º 12601/2016
Publicado no DRE IIª série n.º 201 de 19 de outubro de 2016

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Appendix V – Complete residues search analytical methods protocol

1. ANALYTICAL METHODS

a. Analytical Criteria:

Compound	Substrate	Estimated Limit of Quantification (LOQ) (µg/kg)	Basis on which Results are to be Reported
Lignocaine and metabolites	Ovine tissues, plasma, urine and faeces	0.2*	As received
Bupivacaine and metabolites	Ovine tissues, plasma, urine and faeces	0.2*	As received
Cetrimide	Ovine tissues, plasma, urine and faeces	10	As received

*Note: the LOQ is for each individual analyte and will be refined following validation analysis.

b. Reference Item: An analytical grade standard lignocaine, monoethylglycinexylidide (MEGX), 3-hydroxylignocaine, 2,6-dimethylaniline (2,6-DMA), Lignocaine-N-oxide, 4-OH DMA, GX (glycinexylidide), bupivacaine, 3-hydroxybupivacaine, pipecolic acid, desbutylbupivacaine and 2,6-dimethylaniline (2,6-DMA) and cetrimide will be supplied by the Sponsor or purchased commercially and used as the reference item.

c. Study Residue Definitions: The tissue, plasma, urine and faeces samples will be analysed according to the following:

Compound	Study Residue Definition
Lignocaine	Lignocaine and its metabolites monoethylglycinexylidide (MEGX), 3-hydroxylignocaine, 2,6-dimethylaniline (2,6-DMA), Lignocaine-N-oxide, 4-OH DMA and GX (glycinexylidide).
Bupivacaine	Bupivacaine and its metabolites 3-hydroxybupivacaine, pipecolic acid, desbutylbupivacaine as well as 2,6-dimethylaniline (2,6-DMA) if present.
Cetrimide	Cetrimide

d. Method of Analysis:

"Determination of Lignocaine, Bupivacaine and Cetrimide Residues in Ovine Tissues, Plasma, Urine and Faeces", AATM-R-201, Eurofins Agroscience Testing Pty Ltd.

Changes to the equipment, reagents and chromatographic conditions described in the analytical test method may be necessary due to the equipment and reagents available to the analytical test site. The use of appropriate equivalents is permitted under this Study Plan, suitability being demonstrated by method verification.

e. Method Validation: The analytical method will be validated according to VICH guideline 49 and APVMA guidance document at http://apvma.gov.au/node/723.

The validation will include:

- **Linearity:** Minimum of 5 different concentration points.
- **Selectivity:** Assay triplicate of untreated control in ovine tissues and urine, plasma and faeces (at least from 6 different sheep) in each day for three days.

- **Limit of Detection (LOD)/Limit of Quantitation (LOQ), Within- and Between-Run Assay Precision and Accuracy:** Triplicate recovery assays at estimated LOD, at 3 times the estimated LOD, at 10 times the estimated LOD, at 100 times the estimated LOQ and 1000 times the estimated LOQ in each day for three days.
- **Long Term Freezer Storage:** All tissue matrix types will be fortified in triplicate with Lignocaine, Bupivacaine, and cetrimide and metabolites at LOQ and 100 x LOQ and stored under the same conditions as the samples and analysed at the end of the study when the storage period is known. The APVMA analytical validation advice (Point 2.1.9 at http://apvma.gov.au/node/723) suggests 6 months.
- **Freeze Thaw Stability:** All tissue matrix types will be fortified in triplicate with Lignocaine and Bupivacaine at LOQ and 100 x LOQ and analysed after three freeze thaw cycles.

f. Number of Samples to be Analysed: All primary to be analysed or as advised by the Study Director.

g. Analytical Test Site Location:

Analytical Test Site	Principal Investigator - Analytical	Location	Country
Eurofins Agroscience Testing Pty Ltd	Amy Drewett	Unit F6, Building F, 16 Mars Road Lane Cove West, NSW 2066 Phone: (02) 9900 8442 E-mail: AmyDrewett@eurofins.com	Australia

h. Receipt of Samples: Upon receipt, samples will be stored in a freezer at approximately -18°C until analysed. For each sample, the date of receipt, storage conditions and while awaiting analysis, and the date of analysis will be recorded.

i. Sample Analysis: Samples will be analysed for concentrations of lignocaine and its metabolites, monoethylglycinexylidide (MEGX), 3-hydroxylignocaine, 2,6-dimethylaniline (2,6-DMA), Lignocaine-N-oxide, 4-OH DMA and GX (glycinexylidide), bupivacaine and its metabolites 3-hydroxybupivacaine, pipecolic acid and desbutylbupivacaine as well as 2,6-dimethylaniline (2,6-DMA), if present, and cetrimide following the procedures of the analytical test site.

At least one untreated (control) sample and one recovery must be run with each batch of samples.

Numerical values are to be reported for results between the LOQ and LOD.

At least triplicate recovery assays at the LOQ and 10 x LOQ will be analysed with the test samples at each substrate.

j. Analytical Report: The analytical report will contain at least the following information:

- A summary of the method(s) used for the analysis.
- An explanation of any terms or abbreviations used in the report.
- Details of specimen codes (enabling horizontal traceability for Specimens).
- Details of specimen receipt, preparation and storage.
- Details of the validation for the method.
- The limits of quantitation and detection of the methods.

- Individual results of the analysis of control (untreated) specimens.
- An average of the results from the analysis of untreated fortified specimens (recoveries) including a coefficient of variation (%C.V.).
- Results of individual analyses must be reported (not averages).
- Representative calibration curve(s) applicable to the quantitation
- Representative chromatograms (a minimum of a reference standard, an untreated sample, a fortified untreated sample and a treated sample for each matrix.
- Any deviations, comments or other information relevant to the analysis that is pertinent to the valid interpretation of the results.

The Analytical Report will be provided to the Study Director in the following formats:

- Electronic "Controlled Document" – Signed and Secured Adobe® PDF document.

Appendix VI – Observer's form

Ficha Observador Ensaio Tri-Solfen

Data: ___/___/___

Identificação do Animal:

Nº Identificação: ___________ Raça: ____________ Idade: _______ Peso: ____________

Nulípara ▫ Primípara ▫ Multípara ▫

Avaliação da Claudicação antes da Intervenção:

1- Normal ▫ 2- Irregular ▫ 3- Moderado ▫ 4- Grave ▫ 5- Severo ▫

Avaliação Comportamental à dor durante Aparagem:

1- Ausente ▫ 2- Moderado ▫ 3- Severo ▫

Complementar:

Vocalização ▫ Agitação ▫ Patadas ▫ Queda/Apoio Excessivo na Cilha 1 ▫ 2 ▫ 3 ▫

Observações:

____

____

____

Avaliação da Claudicação após Intervenção:

1- Normal ▫ 2- Irregular ▫ 3- Moderado ▫ 4- Grave ▫ 5- Severo ▫

Avaliação da Claudicação 3 Dias Após Intervenção:

1- Normal ▫ 2- Irregular ▫ 3- Moderado ▫ 4- Grave ▫ 5- Severo ▫

Appendix VII – Operator's form

Ficha Ensaio Tri-Solfen

Data: ___/___/___ Identificação Caso: ___________

Identificação do Animal:

Nº Identificação: _______________ Raça: _____________ Idade: ____________ Peso: _____________

Nulípara ▢ Primípara ▢ Multípara ▢

E.S.G./Tratamentos: ____

Avaliação Pré-Desbridamento:

Claudicação: 1- Normal ▢ 2- Irregular ▢ 3- Moderado ▢ 4- Grave ▢ 5- Severo ▢

Localização: MAD ▢ MAE ▢ MPD ▢ MPE ▢ ; Unha Medial ▢ Unha Lateral ▢ ID ▢

Dimensão: ___________ Algometria Pré Desbridamento: ________ Hiperemia ▢ Temp aumentada ▢

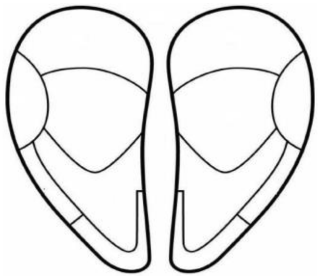
Avaliação Antes Intervenção:

Descrição da Lesão: ____

____

Tecido desbridado: 1 ▢ 2 ▢ 3 ▢ Tipo Ulcera: 1 ▢ 2 ▢ 3 ▢ Dimensão: __________________ Foto: ▢

Esboço da lesão:



Administração:

Gel Inócuo ▢

Tri-Solfen ▢ Lote: __________ Quantidade: ___________ Tempo de Acção: __________

Avaliação Durante Intervenção:

Quantidade de tecido retirado: 1 ▢ 2 ▢ 3 ▢ Algometria 1 minuto após administração: _____________

Taco/Penso: Sim ▢ Não ▢ Algometria fim de intervenção: _________ Tri-solfen durante: _________

Avaliação Após Intervenção:

Claudicação: 1- Normal ▢ 2- Irregular ▢ 3- Moderado ▢ 4- Grave ▢ 5- Severo ▢

Medicação/Procedimentos após tratamento: ____

____

Avaliação 3 Dias Após Intervenção:

Claudicação: 1- Normal ▢ 2- Irregular ▢ 3- Moderado ▢ 4- Grave ▢ 5- Severo ▢

Appendix VIII – Debris classification

Measure		Grades	Description
Debris removed	Amount of horn and other tissue removed by trimming	1	Functional trimming with no bleeding or need to cut deep.
		2	Removal of ulcer's border. Slight bleeding.
		3	Removal of deep tissue including corium. Moderate to severe bleeding. Removal of granulation tissue if present.

Appendix IX – Falling classification

Measure		Grades	Description
Falling	Animal falling in reaction to pain or when trying to remove foot	1	No risk of falling.
		2	Cow slips and nearly falls while trying to pull leg being trimmed.
		3	Cow falls and leg has to be untied.

Appendix X – Reaction to trimming classification

Measure		Grades	Description
Reaction to trimming	Behaviour of animal when ulcer is trimmed/treated - debris, necrotic and other tissue is being removed	1	No or mild reaction when trimming. Animal tries to pull leg once.
		2	Moderate reaction with animal pulling the leg repeatedly.
		3	Violent reaction with continuous pulling of the leg. Tries to kick off the operator. Vocalization.

Appendix XI – Residues per animal according to milking

ID	Residues	1st MILKING	2nd MILKING	3rd MILKING	4th MILKING
4423	Lidocaine and metabolites	0.128 µg/L and 0.145 µg/L	<LOD	<LOD	<LOD
	Bupivacaine and metabolites	<LOD	1.18 µg/L	<LOD	<LOD
	Cetrimide	<LOD	<LOD	3.66 µg/L	<LOD
4460	Lidocaine and metabolites	<LOD	0.393 µg/L	<LOD	<LOD
	Bupivacaine and metabolites	<LOD	<LOD	<LOD	<LOD
	Cetrimide	<LOD	<LOD	<LOD	<LOD
4828	Lidocaine and metabolites	<LOD	<LOD	<LOD	<LOD
	Bupivacaine and metabolites	<LOD	<LOD	<LOD	0.712 µg/L
	Cetrimide	<LOD	<LOD	<LOD	<LOD
395	Lidocaine and metabolites	0.263 µg/L and 0.398 µg/L	<LOD	0.0705 µg/L	<LOD
	Bupivacaine and metabolites	0.118 µg/L	<LOD	<LOD	<LOD
	Cetrimide	<LOD	3.48 µg/L	<LOD	<LOD
9071	Lidocaine and metabolites	0.204 µg/L and 0.247 µg/L	<LOD	<LOD	<LOD
	Bupivacaine and metabolites	0.0787 µg/L	<LOD	<LOD	<LOD
	Cetrimide	11.5 µg/L and 24.4 µg/L	<LOD	<LOD	<LOD