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Dexmedetomidine vs. Xylazine: effects of disbudding and simulated disbudding on plasma concentration of cortisol and substance P in calves undergoing the administration of 2 different alpha 2-agonist sedatives

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Index

CHAPTER 1

Foreward

Veterinary Drugs

Public Health and the Role of Veterinarians

The treatment of animal diseases recognizes in veterinary drugs an indispensable tool for the recovery of physiological conditions and the preservation of Animal Welfare.
There is a clear difference between the treatment of pet animals and that of food animals. Disease treatment in pet animals focuses primarily on drug effectiveness; whereas in food animals the main issue is, paradoxically, Human Health, taking into consideration food residues and withdrawal periods. The second important issue to observe is Animal Welfare (Fossati 2010).
"A food animal must be considered food itself" (Pezza, 2008 personal communication).
This concept is reinforced by the new hygiene package Regulations (EC Regulations No. 178/2002, 852/2004, 853/2004, 854/2004, 882/2004). As a matter of fact, with the EC Regulation No. 178/2002, the European Legislator sets out the principles and requirements of food law and consumers' protection, with a policy increasing awareness for Public Health. This Regulation strengthens the rules applicable to the safety of food and feed circulating in the European market. It establishes a framework for controlling and monitoring the production, prevention and management of risks. This is done by regulating every aspect of food animal life, from feed to farms. This "philosophy" is called "from feed to food."
The EC Regulation No. 178/2002 begins saying*: "This Regulation provides the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food, taking into account in particular the diversity in the supply of food including traditional products, whilst ensuring the effective functioning of the internal market. It establishes common principles and responsibilities, the means to provide a strong science base, efficient organisational arrangements and procedures to underpin decision-making in matters of food and feed safety."*
The goal set by the European Union is to ensure a high level of protection of human life and health through the search of uniformity as far as food safety requirements regards.
The food law dictated by the European Union involves, therefore, all sectors of the food chain, and animal herds in particular. This implies the implementation of good practice measures in any phase of production. This will increase the responsibility of people working at any level of the production chain. In this regard, high professionalism of the staff of farms and food products is required, with the obligation of professional skills development (Ruffo et al., 2005).

So, the main purpose of these Regulations since 2002 is to ensure the quality of foodstuffs intended for human consumption and animal feed and to guarantee the free circulation of safe and secure food and feed in the international market (Ruffo et al., 2009, 2010).
In addition, the European Union's food legislation protects consumers against fraudulent or deceptive commercial practices. This legislation also aims to protect animal health and wellbeing, plant health and the environment.
Veterinarians are the main responsible of this and they have to be conscious about their important role in Public Health. They are the guarantors of public health (Locatelli et al., 2009).
Every single medical act, including the ordinary administration of a drug by injection, has inevitably an impact on Public Health. Veterinarians must consider many aspects before making a medical procedure. The Ethical Code for Italian Veterinarians (Fnovi 2010), recently reviewed, reiterates and reinforces these concepts. Veterinarians are warned about their vital role in food chain and with the registration to the Order of Veterinarians and the reading of the professional Oath (Figure 1) they take officially care of this important responsibility (Ruffo 2010).


Ordine Medici Veterinari
della Provincia di Bergamo

Giuramento Professionale

Entrando a far parte della Professione e consapevole dell'importanza dell'atto che compio

prometto solennemente

di dedicare le mie competenze e le mie capacità alla protezione della salute dell'uomo, alla cura e al benessere degli animali, promuovendone il rispetto in quanto esseri senzienti;

di impegnarmi nel mio continuo miglioramento, aggiornando le mie conoscenze all'evolvere della scienza;

di svolgere la mia attività in piena libertà e indipendenza di giudizio, secondo scienza e coscienza, con dignità e decoro, conformemente ai principi etici e deontologici propri della Medicina Veterinaria.

Dott.ssa Valentina Locatelli

Bergamo, 8 gennaio 2009

Figure 1: Italian Professional Veterinarian Oath

European Directive 2004/28/CE and Italian Legislative Decree n. 193/2006: community code relating to veterinary medicinal products

This Directive and, consequently, the Italian Decree are born in the context of a comprehensive reform in the field of food law. They underline the importance of contextualizing food animals in food chain. These laws are also related to aspects of Human Health protection and to Animal Health and Welfare (Fossati et al., 2007, 2009).
This Directive applies to medicinal products, as well as medicated feeding stuffs.
The main issues of this law are Marketing Authorization, times of suspension and extra-label use of drugs.

The Marketing Authorization of Veterinary Medicinal Products
The marketing of veterinary medicinal products is subject to a Marketing Authorization issued by the Ministry of Health (in Italy, it is called Autorizzazione all'Immissione in Commercio [AIC]).
In case of veterinary medicinal products targeted to food-producing animals, the license is granted if the active substances are included in the EC Regulation No. 470/2009 (which replaces the previous legislation on the determination of residues).
Particular attention is paid to the safety tests and studies on residues, as well as to relevant information for assessing the quality, safety and efficacy of veterinary medicines.
To obtain the Marketing Authorization for a medicinal product, medical firms must write down a complete dossier of administrative, technical and scientific documentation, which must comply with Technical Annex to the Legislative Decree No. 193/2006.

A veterinary medicinal product registration dossier is composed of 4 parts, divided as follows:

- PART I:

administrative data (claims, permits),
printed leaflets (summary of product characteristics, package leaflet, labels),
export reports (reports made by a qualified expert, one for each of the three constituent parts of the technical documentation regarding the veterinary medicinal product).

- PART II (Quality):

technical documentation on the pharmaceutical quality characteristics of the product (composition and quantitative description of the preparation method

and checks carried out on raw materials, controls during manufacturing and the finished product, testing stability and measures to prevent TSE transmission).

- PART IIIA - IIIB (Safety and Residues):

documentation about medicine characteristics (basic pharmacology, toxicology studies, and only repeated dose, reproductive toxicity, teratogenicity, mutagenicity studies for the definition of residual waiting time for food-producing animals to humans, data on the environmental impact.)

- PART IV (Effectiveness):

documentation on characteristics related to preclinical and clinical medicine (basic pharmacology, pharmacodynamics, pharmacokinetics, tolerability in the target species, clinical trials conducted with the test product on the target species according to the requests and dose escalation).

The Ministry of Health has the support of the Technical Advisory Group of Veterinary Medicine, which was established by a Decree of the Ministry of Health.
This Commission is composed of experts, selected on the basis of their specific expertise (analytical chemistry, pharmacology, clinics and ecotoxicology). They evaluate the documentation concerning quality, safety (including the evaluation of data on residues of veterinary medicinal products in foodstuffs intended for human consumption and environmental impact of the product) and efficacy of veterinary medicines. These experts are also asked to comment on general issues concerning the problems related to Veterinary Medicine.
After the procedure, once the veterinary medicinal product has been positively evaluated by technical experts, the Commission grants the Marketing Authorizations through a Decree published in the Gazzetta Ufficiale della Repubblica Italiana (Official Gazette of the Italian Republic) (Fossati et al., 2007).
The Marketing Authorization is valid for five years and can be renewed upon request of the interested parts. After the first renewal, it has no expiry date, unless the Ministry of Health decides to proceed with a further five-year renewal for pharmacovigilance reasons (Ch. 33, paragraph 4 of Legislative Decree No. 193/2006).

Extra-Label Use of Drugs
Veterinarians have the obligation to use and prescribe veterinary medicines that are registered for specific species and for specific diseases. However, because of a lack of sufficient veterinary medicinal products able to meet these conditions any time, the use of other drugs is permitted by law to achieve a therapeutic

solution. This is called extra-label use of drugs (Legislative Decree No. 193/2006 Ch. 11, Sundlof 2008).
Of course, for food-producing animals the rules are more stringent than those required for pets, because of the implications that an extra-label use of drugs may have on human health (Fossati 2007, 2008, 2009).
In the event of an extra-label use of a drug due to the lack of medicines suitable for a specific species or disease, Veterinarians must always justify its use, in order to guarantee Human Health and Animal Welfare. In this regard the responsibility of the choice of medicines falls to Veterinarians, who, in case of official control, must prove their reasons of choice (Fossati 2007).
When a Veterinarian makes an extra-label use of drugs for a food animal, he/she must prescribe an appropriate "time delay" to ensure that foods derived from treated animals do not contain residues harmful to consumers. The waiting time for not registered drugs, with extra-label use in cattle is at least 7 days for milk and 28 for meat and viscera (Legislative Decree No. 193/2006 Ch. 11).
Every Veterinarian can use the drug he/she considers more effective, except those prohibited (EC Reg. No. 470/2009), but he/she must absolutely observe the waiting times to guarantee Public Health (Pezza et al., 2003; Fossati et al., 2006; Fossati 2010).

Withdrawal period
EC Regulation No. 470/2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin
Although veterinary medicines are absolutely necessary to ensure animal health and welfare, their use in food animals may leave residues in food products obtained from treated animals (Bellini et al., 2008).
Starting from this situation, and in order to protect consumers' health, it is equally essential to make an assessment of the safety of these substances taking into account toxicological hazard, environmental pollution, and the undesirable pharmacological and/or microbiological effects from any residue (Locatelli et al., 2009; Ruffo et al., 2009).
Thus, a substance that is pharmacologically active can be used in food animals if it has undergone a risk assessment, based on solid scientific evidence. Maximum Residue Limits (MRL) for this substance must be established.
Definitions:

- Veterinary medicine residue: *"all pharmacologically active substances, whether active principles, excipients or degradation products, and their metabolites which remain in foodstuffs obtained from animals to which their veterinary medicinal product in question has been administered".*

- Maximum Residue Limit (MRL): *"the concentration of residue resulting from the use of a veterinary medicinal product legally permitted in the EU and considered admissible from the point of view of consumer safety of a food product"*.

These MRLs are set according to their toxicological characteristics based on their use according to codes of good veterinary practice, resulting from residue depletion tests, etc. In turn, they serve as a reference to determine waiting time, which means the time elapsing between administering the drug and slaughtering the animal (Locatelli 2008).

Active substances are classified in the therapeutic categories to which they belong: substances with MRL, substances with a provisional MRL, substances with no need to set an MRL, or prohibited substance.

The list of pharmacologically active substances for which no maximum limit can be established that can be considered safe for the consumer, so that their use in animals for food is prohibited includes:

- *Aristolochia spp. and its formulations*
- Chloramphenicol
- Chloroform
- Chlorpromazine
- Colchicine
- Dapsone
- Dimetridazole
- Metronidazole
- Nitrofurans (including furazolidone)
- Ronidazole

National Residue Plan

The National Residue Plan (NRP) is a program of surveillance and monitoring of the presence, in food animals and food of animal origin, of residues of every chemical agent that may be harmful to Public Health.
It is prepared annually by the Ministry of Health, in consultation with Italian Regions and Autonomous Provinces, and the National Reference Laboratory for residues ("Istituti Zooprofilattici", IIZZSS), pursuant to Legislative Decree No. 158/2006 and Legislative Decree No. 232/2007, with a transposition of EU Directives 96/22/EC and 96/23/EC.
The NRP is the result of the analysis of samples taken during the primary production and primary processing of food of animal origin and affects 12 different sectors: cattle, pigs, sheep, goats, horses, poultry, rabbits, fish farming, game, milk, eggs and honey.
The samples are collected at farms, slaughterhouses, egg collection and honey extraction centers.
Normative References
The objective of the NRP, as specified in Annex III of Directive 96/23/EC, is to "examine and highlight the reasons for residue hazards in food of animal origin on farms, slaughterhouses, industries dairy, manufacturing plants and fish processing and collection centers and packing of eggs" (Bellini et al., 2008).
The monitoring carried out pursuant to the NRP aims at detecting the illegal administration of prohibited substances and abusive administration of approved substances and should verify the compliance of residues of veterinary medicines, pesticides and contaminants with maximum residue limits set by the relevant Community legislation.
It should be noted that the NRP focuses only on primary production.

Sampling Strategy
The sampling levels, for example the number of samples for each production sector, are established on the basis of production levels, in accordance with the provisions of Annex IV of Directive 96/23/EC and Commission Decision 97/747/EC.
The total minimum number of samples so determined is then divided among the different groups of substances to search.
The work made to verify the presence of residues in food is carried out using three types of activities, called "Plan", "Extraplan" and "Suspect".
The "Plan" involves a proper sampling planned on the basis of the scale of production in different sectors of national interest.
In addition, the Regions and the Ministry of Health may predispose control plans ("Extraplan") that respond to specific local or national needs, based on

research provided by the NRP. Finally, when there are reasons to suspect the presence of residues, samples are taken on "Suspect".

Planning and Implementation

By the end of the year the Ministry of Health issues the NRP to be implemented during the following year, based on several factors: scientific updates and/or regulatory requirements, specific requirements of the European Commission, changes in local production company, analytical capabilities of laboratories, analytical results from previous years, etc.

Each year, the European Commission collects the control plans of different countries and the results obtained with the implementation of the previous plan.

The NRP data and the related activities are subject to a substantial flow of information that affects the Ministry of Health, the IIZZSS and the European Commission.

The sampling strategy does not take into account drugs used extra-label. In Italy, for example, lidocaine 2% is the most used local anesthetic for cattle. Despite the widespread use of lidocaine 2%, this drug has never been searched during the NRP activity, neither in meat nor in other matrices. Thus, there is a huge gap in the activity of NRP (Locatelli et al., in press).

Recent research studies suggest to expand the sampling strategy, because at the moment it cannot ensure Public Health (Locatelli et al., 2009).

An anonymous questionnaire given to Veterinarians working for NRP raised other problems.

In 2007, for example, on more than 32000 samples for 11 producing sectors, there were only 72 positive samples. 15000 samples were dedicated to cattle and, of these, only 32 were positive.

This fact is interpreted by Veterinarians like a waste of money for the big number of samples and for a lack of Veterinarians' activity (Locatelli et al., 2009)

Animal Welfare and Protection of Farmed Animals

The European Union establishes minimum Welfare standards for farmed animals.
The European Government published a Council Directive 98/58/EC on 20 July 1998 concerning the protection of animals kept for farming purposes. In Italy it was acknowledged by Legislative Decree No. 146/2001.
All Member States have ratified the European Convention for the Protection of Animals Kept for Farming Purposes, the main provisions of which relate to the provision of housing, feed and care appropriate to the animals' needs.
The Treaty of European Union calls on the institutions and the Member States to take full account of Animal Welfare requirements when drawing up and implementing Community legislation, especially when agricultural policy matters are concerned. Furthermore, to ensure the smooth running of the Community market in livestock, common standards must be laid down on the protection of animals kept for farming purposes (Fossati 2010).
This Directive applies to animals reared or kept for the production of food, wool, leather or fur, or for other farming purposes.
The Member States must adopt provisions to ensure that the owners or keepers of animals preserve their welfare and make sure they are not caused any unnecessary pain, suffering or injury (Vettore et al., 2006).
Based on past experience and present scientific knowledge, the rearing conditions relate to the following:

- staff: there should be a sufficient number of staff members looking after the animals and they must have the appropriate ability and professional skills;
- inspections: all animals kept in husbandry systems must be inspected at least once a day. Injured or ill animals must be treated immediately and isolated in suitable premises if necessary;
- records: the owner or keeper of the animals must keep a record of any medical treatment for at least three years;
- freedom of movement: all animals, even if tethered, chained or confined, must be given enough space to move without unnecessary suffering or injury;
- buildings and accommodation: materials used in the construction of buildings must be capable of being cleaned and disinfected. Air circulation, dust levels, temperature and relative humidity should be kept within acceptable limits. Animals kept in buildings must not be kept in permanent darkness or constantly exposed to artificial lighting;

- automatic or mechanical equipment: automatic or mechanical equipment essential for the health and wellbeing of animals must be inspected at least once a day. Where an artificial ventilation system is in use, an appropriate backup system must be in place to guarantee sufficient air renewal;
- feed, water and other substances: animals must be given a wholesome and appropriate diet, fed to them in sufficient quantities and at regular intervals. All other substances are prohibited, unless given for therapeutic or prophylactic reasons or for the purposes of zootechnical treatment. In addition, the feeding and watering equipment must minimize the risks of contamination;
- mutilations: with minimal animal suffering and always with the presence of a Veterinarian.

Rearing methods that cause suffering or injury must not be used unless their impact is minimal, brief or expressly allowed by the national Authorities.
The Member States must take the necessary steps to ensure that the competent national authorities carry out inspections. They must report on these inspections to the Commission, which will use the reports to formulate proposals on harmonizing inspections.
Every five years the Commission must report to the Council on the implementation of this Directive, with proposals for improvement, if appropriate. The Member States have to introduce the legislative, regulatory and administrative provisions (including any penalties) needed to comply with this Directive. They are allowed to keep or introduce stricter provisions.

Related Acts
Report from the Commission of 19 December 2006 on the experience acquired on the implementation of Directive 98/58/EC on the protection of animals kept for farming purposes
In this report the Commission refers to the need for Member States to improve the planning and carrying out of inspections and the recording and transparency of inspection results. It emphasizes the necessity of intensive staff training of the authorities concerned and of a better notification system. It is also important to simplify procedures in order to avoid excessive bureaucracy.
The measures cover the upgrading of standards, the development of research and indicators, information for professionals and consumers, and action at international level.
There is a growing agreement that animals used for food production should be well treated. It is clear that strict welfare standards have an impact on food safety and quality. The difficulty lies in precisely quantifying this impact. Standard improvement has also resulted in costs for producers, which are partly covered

by the additional price that consumers are willing to pay for high-quality products (Fossati et al., 2006; Fossati 2010).

Pain in Cattle

Pathophysiology of Pain in Cattle

One clinically useful definition of animal pain states that "pain is an aversive feeling or sensation associated with actual or potential tissue damage and resulting in physiologic, neuroendocrine, and behavioral changes that indicate a stress response" (Broom, 2000). The International Society for the Study of Pain has defined pain as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage". Tissue damage that may occur through trauma or disease usually leads to what has been described as "pain-induced distress" of animals (Mellor et al., 2005).

In ruminant veterinary practice it is easy to find situations of crisis, which include for example abomasal volvulus, intestinal accidents, cesarean section, fractures, extreme disruption in acid-base balance, and hydration deficit as encountered in cattle with severe enteritis. These and other diseases cause pain.

Animals who perceive pain, as a result of a highly integrated multidimensional system, have a reaction (fight, flight, freeze) which is useful to protect themselves from their environment (Anderson et al., 2005; Craig, 2003; Eicher et al., 2002; Muir et al., 2001; Broom 2000; Rushen Pinheiro et al., 1999; Machado et al., 1997; Taschke et al., 1997).

The normal pain experience is a result of the activation of a specialized group of high threshold sensory nerves innervating skin, muscle, joints and the viscera which only respond to this type of stimuli (Carstens et al., 2000; Scholz et al., 2002; Craig et al., 2010). Nerve endings and fibers responding to noxious stimuli extend from the periphery to the brain via specialized fiber tracts that have both a sensory-discriminative and an emotional-affective function. These systems provide the structural basis for the variability and complexity of pain and are responsible for signaling the intensity of pain on one hand and the aversiveness of pain sensation on the other hand. The acute nociceptive system is ideally suited to serve as a warning system for stimuli that are either damaging or potentially damaging in the environment (Scholz et al., 2002; Julius et al., 2001). This sort of stimulus, whether it is a pinprick, an irritant chemical or a burn (for example disbudding), elicits an acute and unpleasant pain response, which helps protecting the individual (Stafford et al., 2002).

The nociceptive warning system also serves to activate motor reflexes when the individual withdraws from the stimulus to avoid injury or further harm. Moreover, by activating spinobulbar systems, the noxious stimulus alerts the individual from further dangerous stimuli.

By accessing the bulbar systems controlling autonomic functions, acute pain will also elicit cardiovascular and sweat responses. There are clear differences between this acute nociceptive system which is fundamental for survival and the chronic pain which serves no physiological purposes, yet represents a major health and societal problem (Jensen and Baron, 2003; Woolf, 2004).

Pain Perception

The noxious stimulus is translated into electrical impulses that are transmitted to the dorsal horn of the spinal cord resulting in the release of glutamate from presynaptic nerve terminals. Glutamate activates postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Craig, 2003) and kainate (KA) receptors (Craig, 2003). The AMPA and KA receptors are the primary mediators of fast excitatory pain transmission.

In the absence of tissue damage, pain is considered a physiologic warning of potentially harmful stimuli. Pathologic pain is considered to be present when tissue or nerve damage occurs and frequently involves the development of peripheral sensitization, central sensitization, and disinhibition (Muir et al., 2001). Tissue damage and the associated inflammatory response produce various chemicals that function as nociceptor activators or sensitizers and include hydrogen and potassium ions, prostaglandins, histamine, bradykinin, nerve growth factor, cytokines, and chemokines. Together these factors act as a "sensitizing soup," by changing high-threshold nociceptors to low-threshold nociceptors and by activating quiescent "silent" or "sleeping" nociceptors resulting in peripheral sensitization and a zone of primary hyperalgesia (Muir et al., 2001; Woolf et al., 2000).

The activation and modulation of NMDA receptors (NMDARs) by the excitatory neurotransmitter glutamate is believed to be a key component in the development of central sensitization, secondary hyperalgesia and pain amplification (Muir et al, 2001). Sensitization of dorsal horn neurons can last for hours and it is believed to be responsible for pain outside the area of tissue injury (secondary hyperalgesia) and allodynia (pain from nonpainful stimuli).

In several disease settings, increases in dorsal horn sensory neuron excitability is also enhanced by the production of sensitizing substances (for example prostaglandins) by glial cells in response to increases in cerebrospinal cytokines (TNF-α, IL-1) (Watkins et al, 2001; Woolf et al, 2000).

Central sensitization boosts the responsiveness (hyperalgesia, allodynia) of dorsal horn neurons to sensory inputs, expands receptive fields, and is believed to be responsible for the discomfort and agony produced by severe injury. The extension of central sensitization from the spinal cord to the brain leads to the development or modification of memory patterns and is responsible for changes in animal behavior.

Severe or continuous pain leads to biochemical (transcriptional) changes in dorsal horn neurons promoting the development of chronic pain states caused by changes in the neurons (neuroplasticity) phenotype (Muir et al., 2001; Julius et al., 2001; Moore et al., 2002).
The development of peripheral and central sensitization represents a continuum of the pain process, which exists as a consequence of continuous, unrelenting, and untreated pain.
In conclusion, the translation of these pain mechanisms into clinical pain is still a matter of debate (Finnerup and Jensen, 2006), but at present it seems natural to distinguish between acute physiological pain, and 3 types of chronic pain: inflammatory, neuropathic and generalized pain. Pathologic pain represents an aspect in which either nerve damage, long term inflammation or persistent tissue hyperactivity without known cause gives rise to pain that is out of proportion to any tissue damage and often outlasts the duration of tissue damage so that no tissue damage will be visible. (Jensen, 2008).

Diagnosis of Pain in Cattle

Legislation

Italian Legislative Decree No. 146/2001 about Animal Welfare in farms imposes to every Veterinarian to recognize and report every situation in which animal wellbeing is not respected. Consequently, every Veterinarian must be able to identify and diagnose an animal which suffers. Veterinarians are the only people trained to make a diagnosis of animal maltreatment.

Safeguarding Animal Welfare is also a promise that every Veterinarian makes when he/she reads the Professional Oath.

Despite many definitions, Welfare refers primarily to the subjective psychological state of the individual, as related to its internal and external environment (Mormède et al., 2007; Rushen et al., 2003; Fraser et al., 1999).

Since we are not yet able to read directly animals' feelings and emotions, we try to infer those from measurable indices that we know or suppose to be related to them. Most of these measures, including behavior, biology, production traits and pathology, derive from the study of emotions/stress/adaptation psychophysiology and physiopathology (Dantzer et al., 1993).

Physiology: Cortisol as Indicator of Pain

The measure of HPA axis activity is the standard approach to the study of stress, pain and Welfare in farm animals. But is cortisol a valid indicator of pain?

1. The neuroendocrine systems are primarily involved in metabolic homeostasis and particularly in the regulation of energy fluxes (Dallman et al., 1997). HPA axis is able to produce energetic metabolites, not necessarily the response to a stressful stimulus, but it can also reflect their involvement in homeostatic metabolic processes. The best example is the increase in cortisol levels induced by meals that are not usually considered as stressors (Mormède et al., 2007).
2. The duration of the stimulus plays an important role in the 'general adaptation syndrome' as described by Selye, with the three successive phases: alarm, resistance and exhaustion. The immediate biological responses to acute challenges (such as parturition, castration, weaning, mixing of animals from different social groups, restraint, transportation, slaughter) have been studied extensively and activate biological stress systems in a more or less standardized manner (alarm phase). This common pattern of response is at the origin of the stress concept that was defined by Selye as the 'non-specific response of the body to any demand made upon it'. However, if the stimulus is maintained for some time, circulating levels of corticosteroid hormones return to baseline value even

if the sustained activation of the HPA axis can be detected (Korte et al., 2005; McEwen et al., 1998).

3. There exists a huge variability, across species, breeds, and individuals, in the basic functioning of adaptation mechanisms and in their responses to environmental challenges (Mormède et al., 2007).

Although the reference technique is the use of blood plasma to measure glucocorticoid hormones (cortisol or corticosterone), several alternative methods such as the measurement of corticosteroids in saliva, urine or feces have been developed to overcome the stress induced by blood sampling itself (Veissier et al., 1999; Hay et al., 1997; Mormède et al., 1994).

In chronic stress situations, as is frequently the case in studies on farm Animal Welfare, hormonal secretions are usually unchanged but dynamic testing demonstrates functional changes at several system levels, including the sensitization of the adrenal cortex to ACTH and the resistance of the axis to feedback inhibition by corticosteroids (dexamethasone suppression test). (Mormède et al., 2007).

Beyond these procedural aspects, the main pitfall in the use of HPA axis activity is in the interpretation of experimental data. The large variability of the system has to be taken into consideration, since corticosteroid hormone secretion is usually pulsatile, follows diurnal and seasonal rhythms, and it is influenced by feed intake and environmental factors such as temperature and humidity, age and physiological state, just to cite the main sources of variation. The corresponding changes reflect the important role of glucocorticoid hormones in a number of basic physiological processes such as energy metabolism and central nervous system functioning.

In cattle HPA axis reactivity is now insufficient to use response patterns as a reliable indicator of Animal Welfare status. (Stilwell et al., 2010; Stafford and Mellor, 1993; Mellor and Stafford, 1997; Mellor et al., 2000).

Substance P

Substance P is an undecapeptide (H-Arg1-Pro2-Lys3-Pro4-Gln5-Gln6-Phe7-Phe8-Gly9-Leu10-Met11-NH2) involved in pain transmission mechanisms. Its molecular structure was first identified in bovine hypothalamus by Chang et al. in 1971.

Substance P was initially isolated in 1931 as a crude extract from equine brain and rabbit gut, and it was found to have hypotensive and smooth muscle contractile properties (Von Euler et al., 1931). In 1934 Gaddum and Schild called this new agent Substance P, with P referring to the powder obtained after the extraction procedure.

The pioneering work of Lembeck in 1953 led to the proposal that substance P was a neuronal sensory transmitter associated with pain transmission, due to high concentrations of this agent located in dorsal root of the spinal cord.
Further evidence for its role came later with studies by Otsuka and Konishi in 1976 that showed that Substance P immunoreactivity increased after electrical stimuli applicated to rats.
In 1983 Erspamer et al., introduced Substance P as part of the tachykinin family.
Mammalian substance P derives from the preprotachykinin-A (PPT-A) gene, which originates from a common ancestral gene by duplication (De Regoli et al., 1994; Carter et al., 1990; Nakanishi 1997).
Expression of substance P and its mRNA are widely abundant in both the central nervous system (CNS) and the peripheral nervous system (PNS) (Kotani et al., 1986).
Substance P immunoreactivity has been demonstrated in the rhinencephalon, telencephalon, basal ganglia, hippocampus, amygdala, septal areas, diencephalon, hypothalamus, mesencephalon, metencephalon, pons, myelencephalon and spinal cord (Shults et al., 1984).
A number of techniques, such a polyclonal antisense, in situ hybridization and Northern blot analysis, have demonstrated the expression of PPT-A mRNA in nodose (Hamid et al., 1991), trigeminal (Lee et al., 1995; Kiyama et al., 1988), dorsal root ganglia (Gibbins et al., 1997; Sternini et al., 1991) and intrinsic neurons of the gut (Sternini et al., 1995).
The synthesis of Substance P takes place in the ribosomes (Harmar et al., 1982 and 1980) and is confined to the perikaryon. Substance P is then packed into storage vesicles (Plenderleith et al., 1990; Merighi et al., 1988), and axonally transported to terminal endings for final enzymatic processing (Brimijoin et al., 1980).
Biochemical (Takahashi et al., 1975) and immunohistochemical (Harmar et al., 1982) studies demonstrate that Substance P is transported to both the central and peripheral branches of primary sensory neurons.
The bulk of substance P is produced in the sensory ganglion cells and exported towards the terminal regions of the peripheral branches by a mechanism of axonal transport (Harrison et al., 2001).
The biological actions of substance P are mediated by tachykinin (neurokinin, NK) receptors, which belong to rhodopsin-like membrane structure. Substance P activates the NK1, NK2 and NK3 receptors in a great number of tissues (D. Regoli et al., 1994).
Nowadays it is fully recognized that substance P is released from both the central and peripheral endings of primary afferent neurons and acts like a pain neurotransmitter (Otsuka et al., 1993).

After more than 50 years from its discovery, substance P is starting to find its place as a marker of pain in human physiology and pathophysiology and in the last few years also in Veterinary Medicine (Harrison et al., 2001).

Peripheral Roles of Substance P: Neurogenic Inflammation

The effects produced by substance P and other tachykinins released from peripheral endings of capsaicin-sensitive primary sensory neurons, are collectively referred to as "neurogenic inflammation" (Marlier et al., 1991;Schaible et al., 1990).

Responses produced at the peripheral level by sensory neuropeptides are particularly prominent on the vasculature where they cause vasodilatation of arterioles, plasma protein extravasation in post-capillary venules, and leukocyte adhesion to endothelial cells of venules.

Additional tissue-specific responses produced by neurogenic inflammatory mechanisms are smooth muscle relaxation/contraction in the urinary bladder, ureter and iris, inotropic and chronotropic effect on the heart, bronchoconstriction in the airways. Peptide-containing primary sensory neurons are characterized by their unique sensitivity to capsaicin, the pungent principle contained in the plants of the genus *Capsicum* (Gibbins et al., 1987; Szallasi et al., 1999).

Subsets of primary sensory neurons are selectively stimulated by capsaicin that causes the release of sensory neuropeptides, thus promoting neurogenic inflammation. At higher concentrations capsaicin kills neurons, thus blocking the genesis of subsequent neurogenic inflammatory responses (Szallasi et al., 1999). The specific excitatory/desensitizing effect of capsaicin on these neurons is the reason why they have been defined as "capsaicin-sensitive" (Szolcsanyi et al., 1984).

Coetzee et al.'s Research

In 2008 Coetzee et al. made the first research about substance P on cattle, aware of the doubtful significance of the plasma concentration of cortisol during a painful procedure.

They compared the plasma concentration of substance P with cortisol in 10 calves undergoing true and simulated castration.

Calves were acclimated for 5 days before the experiment, than approximately 48 hours before study commencement, a jugular catheter was inserted in each calf.

The day of the experiment calves were castrated or sham-castrated. In the meanwhile behavioral observations were made and blood samples were collected immediately after and 10, 20, 30, and 45 minutes and 1, 1.5, 2, 2.5, 3, and 4 hours after castration or simulated castration.

Results demonstrated that mean ± SEM cortisol concentration in castrated calves (78.88 ± 10.07 nmol/L) was similar to that in uncastrated control calves (73.01 ± 10.07 nmol/L). However, mean SP concentration in castrated calves (506.43 ± 38.11 pg/mL) was significantly higher than the concentration in control calves (386.42 ± 40.09 pg/mL). This significant increase in plasma concentrations of SP after castration suggested a likely association with nociception.

Behavior

Behavior observation is of course a valid element to establish a diagnosis of pain. Champions of behavioral indicators of stress argue that behavioral responses are often correlated with physiological or immune responses, and so they can be used to predict the effect of pain on the biological functioning of the animal. Sometimes the interpretation of behavior is problematic. This problem of interpretation is the main limit for Veterinarians (Rushen et al., 2000).

Behavior in cattle has been studied after dehorning procedures (Stilwell et al., 2010,; Stafford et al., 2005; Vickers et al., 2005; Grøndahl-Nielsen et al., 1999; Morisse et al., 1995), but it is a very subjective evaluation.

In their recent research about dehorning Stilwell et al. evaluated some aspects of calves behavior after dehorning:

- head shake
- ear flick
- hind-limb scratching head
- head rubbing against objects
- quick transition from standing to lying and back to standing
- vocalization (Watts et al., 1999, 2000, 2001; Manteuffel et al., 2004).

Standardize the evaluation and make it the less subjective as possible can be, in the future, a very good method to make a diagnosis of animal suffering and to quantify pain (Rushen et al., 2000).

Pain Management in Cattle

Animal Welfare has recently hit the headlines. Nowadays, whether and how to alleviate pain are vital parts of the discussion about Animal Welfare (Broom et al., 1986, 1988, 1991, 2000, 2001, 2004, 2007; Gonyou et al., 1994; Sparrey et al., 1994; Jacobsen et al., 1996; Taschke et al., 1997; Carstens et al., 2000; Gentle et al., 2001; Berridge et al., 2003; Fraser et al., 2003, 1998; Sandoe et al., 2003; Bekoff et al., 2006; Weary et al., 2006; Martin et al., 2007; Virginia et al., 2011). Among Veterinarians, clinical practitioners in particular, Animal Welfare has always been an important concept and there is plenty of studies demonstrating that poorly treated animals are less productive (Ting et al., 2003; Whay et al., 2005; Van Reenen et al., 2005; Rust et al., 2007; Van Borell et al., 2007; Gonzalez et al., 2008; Marcillac-Embertson et al., 2009; Millman et al., 2009).
Italian Veterinarians read an Oath stating that the Veterinarian will use his/her knowledge and skills for the relief of animal suffering (Italian Veterinary Oath, 2009) and he/she is aware of the obligations of the Legislative Decree No. 146/2001 about Animal Welfare and, consequently, pain management.
Many advances in drug therapy are being made. Food animal Veterinarians are responsible for the preservation of a safe food product. To that end, Veterinarians must be cautious with cross-species applications of drugs and therapies (Anderson et al., 2005).
A key component of an adequate animal treatment is the recognition and control of pain, whether it is the result of disease, injury, or procedures such as castration or dehorning (Virginia et al., 2011).
In the last few years many surveys have been carried out by Veterinarians in the United States and Canada that provide useful insights about the current status of analgesic drug administration in Veterinary Medicine (Watts et al., 2000; Fitzpatrick et al., 2004; Huxley et al., 2006, 2008; Hewson et al., 2007; Misch et al., 2008) and demonstrate a growing awareness among Veterinarians about the estimation of pain caused by surgical procedures. Moreover, the use of analgesic drugs for pain treatment has substantially increased (Virginia et al., 2011).
The growing concern about the welfare of food animals has inspired research studies on the assessment of pain associated with various common procedures and medical conditions in cattle, such as castration, dehorning, and lameness (Lay et al., 1992; Molony et al., 1993, 1997; Lay et al., 1994; Hemsworth et al.,1995; Morisse et al., 1995; Schwartzkopf-Genswein 1997, 1998; Sylvester et al., 1998, 2004; Kent et al., 2000; Price et al., 2001; Schreiner et al., 2002; Stafford et al., 2002, 2003, 2005; Sutherland et al., 2002; Ting et al., 2003; Zulaf et al., 2003; Milligan et al., 2004; Stilwell et al., 2004; Vickers et al., 2005; Pang et al., 2006; Rushen et al., 2007; Thüer et al., 2007) and the development of validated

analgesic regimens for the treatment of pain associated with these conditions and procedures (Flower et al., 2008; Earley et al., 2002).
Drug therapy for pain or distress must be considered in the context of overall case management to optimize the patient's quality of life, restore function, and minimize adverse events (Hunt et al., 2001). In ruminants, this must be done in consideration of drug residues and in accordance with the Animal Medical Drug Use (Sundlof, 1998, D.lgs No. 193/2006).

Surgical Pain
The easiest type of pain to treat is induced pain. The magnitude of surgical pain is influenced by the procedure, the methods used, and the experience and skills of the practitioner (Anderson et al., 2005).
There are strategies to minimize pain before and after surgery.
The preemptive method includes local anesthesia, general anesthesia, sedation, and tranquilization.
Based on the available literature, the most important tool available in modern Veterinary Medicine is preemptive analgesia. Veterinarians must take the "opportunity" to prevent the onset of pain, avoid noxious stimuli or their perception, and limit the pain-stress-distress cascade that results in altered behavior and deviation from normal physiologic state. Rational treatment of pain requires an evaluation of its consequences, a fundamental understanding of the mechanisms responsible for its production, and a practical appreciation of the analgesic drugs currently available. The goal of pain treatment should be to restore normal (physiologic) pain responses and to eliminate pathologic pain processes.
Particular attention should be given to the physiologic processes induced by tissue injury that may lead to "pathologic" pain after surgery. Sedatives, tranquilizers, narcotics, and anesthetics inhibit pain detection or intensity by interfering with pain pathways, but these drugs do not act upon processes (for example, inflammation) to stop continued noxious stimuli (Anderson et al., 2005).
Many of the drug combinations used for field anesthesia in ruminants are also used in chemical restraint. Drug doses are typically lower when used in chemical restraint techniques, but the difference between these two applications is sometimes modest. The level of analgesia produced by chemical restraint varies with the technique and doses administered. The ideal dose can be difficult to predict, especially when recumbency is not desired. It sounds easy, making the wrong decision and it may result in the clinicians working on their knees or getting kicked in them (Abrahmsen 2008).
General anesthesia may be considered the "gold standard" for pain-free surgery. In studies comparing various methods of castration, however, general anesthesia

consistently stimulated the most severe rise in serum cortisol. These studies have suggested that general anesthesia may be intensely distressful for a patient despite the absence of a surgical pain stimulus.
In ruminant surgery, economic pressures and limitations of field surgery have caused selection against general anesthesia. Anyway, the behavior and demeanor of cattle favor the use of sedatives, local or regional anesthesia, or epidural anesthesia (Anderson et al., 2005).

Local Anesthetics: Lidocaine 2%
Local anesthetics are the most commonly used preemptive drugs in food animal practice (Muir et al., 1995; Locatelli et al., 2011). These drugs, especially lidocaine 2% HCl, are used to prevent incisional pain during surgery. Lidocaine 2% can be used in a variety of techniques, including surface active, local block, ring block, selected peripheral nerve block, and regional blocks (eg, paravertebral, epidural).
In Italy lidocaine 2% is the only anesthetic used in routine surgery in adult cattle and sometimes it is also used in calves. In a recent study (Locatelli et al., in press) a questionnaire was given to 5 Veterinarians taking care of cattle. It was asked them to write the anesthetic drugs they use during their interventions on adult cattle.
In total they made about 500 surgeries in 2010 and they always used lidocaine 2% for every animal and for every kind of surgery. Only in 3 clinical cases lidocaine 2% was associated to xylazine because of the temper of the cattle.
In Italy Lidocaine 2% is not registered for cattle and so it is used extra-label (Legislative Decree No. 193/2006 ch.11).
This study shows that lidocaine 2% is used also for surgery on calves, of course less than for adult cattle. Only 10% of calves received lidocaine 2% associated to general anaesthesia.
The use of lidocaine 2% in Italy is conspicuous, but despite this, this drug has never been searched during the National Residue Plan (Locatelli et al., 2009; Legislative Decree No. 158/2006).
This drug acts locally or regionally but has no systemic or behavioral effect.
Local anesthetics block nerve fibers (B fibers > C fibers > A fibers) (Muir et al., 1995). These nerve fiber types represent motor/touch (B fibers), nonmyelinated pain and temperature sensation (C fibers), and motor and proprioceptive (A fibers). Local anesthetic drugs act by inhibiting sodium channels to block nerve conduction by preventing depolarization of the nerve fiber. Lidocaine 2% must disassociate in an alkaline environment. In infected tissues, quality of local anesthesia is often poor because the relatively more acidic environment prevents disassociation of lidocaine 2% (Anderson et al., 2005).

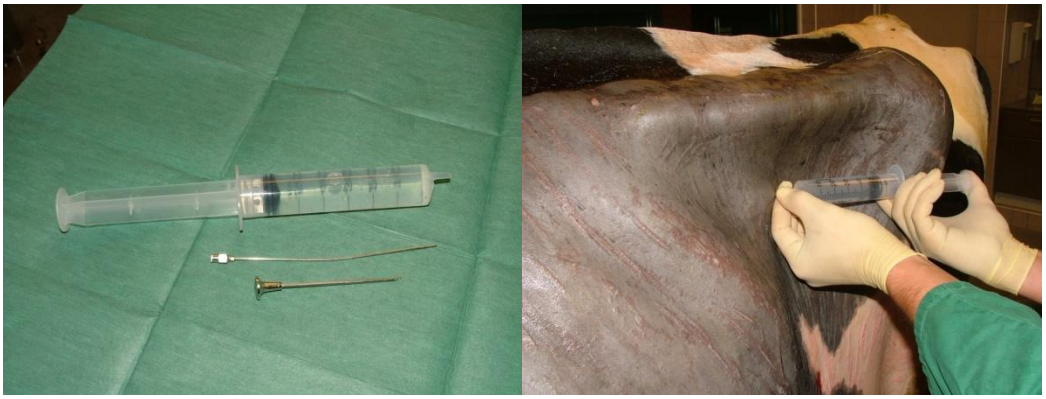


Figure 2 Regional anesthesia with lidocaine 2% before a rumen laparotomy for the presence of a foreign body.

There also exist lidocaine sprays and patches, successfully used in human medicine. They give a poor result in Veterinary Medicine; as a matter of fact bovine skin, especially dorsal skin, is too thick or resistant to anesthetic absorption to induce anesthesia. The patches were evaluated in cows after cruciate ligament surgery, septic arthritis surgery, and incisional pain, but most cows showed minimal detectable response (Anderson et al., 2005; Doherty et al., 2007; Edmonson et al., 2008).

Epidural Anesthesia

There are many drugs used for epidural anesthesia in cattle. Epidural anesthesia can be useful especially when we can keep the patient standing, minimizing motor nerve interference and optimizing analgesia. For this purpose Veterinarians use a2-agonists. (Anderson et al., 2005) Caudal epidural anesthesia is applied easily in cattle, but there are still many research projects in cattle in an attempt to minimize pain of surgery by using this technique (Lin et al., 1998; Prado et al., 1999; Fierheller et al., 2004; Lee et al., 2003; De Rossi et al., 2003; Caron et al., 1989; St-Jean et al., 1990).

NSAID

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX. COX acts on arachidonic acids to release prostaglandins and other mediators of inflammation. COX inhibitors prevent the production of these factors.

Nonspecific COX inhibitors include flunixin, ketoprofen, and phenylbutazone. Selective COX-2 inhibitors are rapidly evolving and include etodolac and carprofen.

NSAIDs have differential activity because of the presence of variable receptors and variable drug effects. Clinical observations suggest that flunixin provides excellent visceral analgesia but has less potent effects on many musculoskeletal

injuries. Alternatively, phenylbutazone seems to provide excellent musculoskeletal pain relief, but offers little benefit for the treatment of visceral pain (Balmer et al., 1997; Bath et al., 1998; Armstrong et al., 1999, 2002; Al-Gizawiy et al., 2004; Bunsberg et al., 2008)

Much of the research in the field of pain has shown tremendous benefits of preemptive analgesia. There is a consistently less impressive effect of administration of analgesic medication after the establishment of noxious stimulus.
In 2003, Zulauf et al. showed that if NSAIDs were administered before castration with Burdizzo, lower serum cortisol, greater feed intake, and less scrotal swelling occurred during the first 72 hours.
In 2002, Stafford et al. compared various surgical castration techniques with or without local anesthesia or NSAIDs. Local anesthesia alone did not prevent cortisol increase, but local anesthesia plus NSAID obtunded cortisol response. In the same year, Mellor et al. showed that there was a marked increase in cortisol and noradrenaline after castration, dehorning, and tailing amputation and that local anesthesia attenuated this response, but only so long as the local anesthesia was present.
In 2000, Faulkner and Weary showed that dehorning 4-to-8-week old calves under sedation and local anesthesia was beneficial only when combined with an NSAID. Calves that were administered NSAIDs for castration had less head shaking, head rubbing, and ear flicking and gained more weight compared with calves that received sedation and local anesthesia alone.
In 1999, Grondahl-Nielsen et al. compared cornual nerve block versus sedation (xylazine plus butorphanol) and found that the cornual nerve block significantly decreased pain responses as evidenced by lower serum cortisol and lower heart rate.
In 1998, McMeekan et al. used long-acting local anesthesia (bupivicaine, 3–4 hours' duration) for scoop dehorning. The cortisol response was obtunded only when period local anesthesia was active.

Alpha$_2$-adrenergic Agonists
Xylazine is the most frequently used drug for sedation in large animals. The initial behavior of the patient greatly influences the sedation produced by a given dose of an alpha$_2$-agonist.
The alpha$_2$-agonist can be administered intravenously (IV), intramuscularly (IM), or subcutaneously (SQ) and produce a dose-dependent degree of sedation, muscle relaxation, and analgesia. Intravenous administration of alpha$_2$-agonists provides a faster onset and more intense level of chemical restraint and analgesia.

Intramuscular administration results in a more gradual onset and provides a longer duration of less intense chemical restraint and analgesia. This route of administration is often used when patient cooperation does not allow intravenous administration or when extended duration is desired. The IM dose is traditionally twice the IV dose.
Subcutaneous administration results in the most gradual onset, longest duration, and mildest peak effect. Administering the intravenous dose IM or SQ is a method used to produce a degree of sedation with limited risk of recumbency (Anderson et al., 2005).

Ketamine, Opioids, Propofol, Benzodiazepines and Barbiturates
These anesthetics can be used extra label in cattle because they are not registered. (Legislative Decree No. 193/2006, Ch. 11).
Ketamine is a dissociative anesthetic commonly used in veterinary medicine. Ketamine possesses potent analgesic effects when administered at subanesthetic doses and it can be used in association with xylazine for example to amplify the analgesic power of the anesthesia (Abrahamsen et al., 2008).
An opioid (butorphanol, morphine) can be administered to increase the level of systemic analgesia in ruminants sedated, for example, with an $alpha_2$-agonist (Abrahamsen et al., 2008).
As regards benzodiazepines, they offer anxiolytic effect and muscle relaxation. They are also anticonvulsant (Fonda 2009, Corletto 2008, Doherty et al., 2006; Greene 2002; Van der Klejin et al., 1991).
Propofol and barbiturates are rarely used in cattle. The lack of experience, the costs and the need to associate them with other anesthetics make their use impractical, especially in farm everyday activity. (Corletto 2008, Singh et al., 2003; Genccelep 2005).

Dehorning and disbudding cattle

Dehorning and disbudding are routine painful procedures carried out on cattle to facilitate management (Vowles, 1976; Marshall, 1977; Stafford et al., 2005 and 2003; Laine et al., 2007; Duffield 2008; Gottardo et al., 2011).

There are two differences between dehorning and disbudding: the size of the horns and the age of the cattle, features which are often associated. As a matter of fact, disbudding is carried out when horn buds are 5–10 mm long and easily palpable. In this case, a heated disbudding iron can be used alone usually on calves up to around 8 weeks of age or a caustic paste on very young calves, aged less than one week (Kent 1999, Laine et al., 2007). Instead, when the horns become longer and a disbudding iron is not effective, horns have to be removed by amputation (Weaver, 1986).

The age at which horn buds become palpable varies between breeds as does the age at which disbudding becomes impossible and dehorning is necessary (Stafford et al., 2005).

Nowadays European, American, Canadian and Australian cattle farmers and Veterinarians are conscious that dehorning facilitates farm management and brings a lot of benefits (Groendahl-Nielsen et al., 1999; Breuer et al., 2003 and 2000; Stafford et al., 2005; Dockes et al., 2006; Hoe et al., 2006; Weary et al., 2006; Laine et al., 2007; Duffield 2008; Fulwider et al., 2008; Stilwell et al., 2010; Gottardo et al., 2011). Thus, cattle dehorning has become a very common procedure, especially in modern dairy production systems and it is considered necessary by most dairy farmers (Duffield et al., 2008; Gottardo et al., 2011).

A recent Italian research shows that, in Italy, dehorning is carried out in 80% of the farms (Gottardo et al., 2011). Farmers in favor of keeping horned cows are few.

Disbudding and dehorning will therefore probably be necessary until all cattle are polled. The breeding of polled cattle eliminates the need for dehorning and because horns are inherited as an autosomal recessive with polledness being dominant (Long and Gregory, 1978).

Although disbudding and dehorning are painful and stressful for cattle, the long-term consequences of not having horns are more beneficial than having them (Stafford et al., 2005).

The most important benefit dehorning can give is human safety. Dehorned cattle are safer to handle and dehorning reduces injury risk to the handlers during routine management practices, like milking, hoof trimming and calving (Stafford et al., 2005; Laine et al., 2007; Gottardo et al., 2011).

The second benefit dehorning can give is cattle safety. Horn damage causes bruising, especially during transport and lairage (Vowles 1976; Marshall 1977). Bruise trim from carcasses of horned cattle is about twice that from hornless

cattle (Meischke et al., 1974). Moreover, horned animals can cause injury to herdmates during aggressive interactions and competition at the feeding gate (Gottardo et al., 2011; AVMA, 2010; NFACC, 2009). Damage from horns can also result in complications associated with open wounds such as infection or fly strike (Faulkner et al., 2000).
Another benefit is that hornless cattle take up less trough space and need less room (Stookey and Goonewardene, 1996). Horned cattle require three times more space at a feed trough and during transport (McMeekan et al., 1999), and may also suffer financial penalties on sale (Faulkner et al., 2000).
It is also important to consider that dehorned cattle show no differences in weight gain, calf survival or fertility in comparison with non-dehorned cattle and they exhibit the same behavior during restraint (Goonewardene et al., 1999; Frisch et al., 1980).

Council Directive 98/58/EC of 20 July 1998 concerning the protection of animals kept for farming purposes and consequent Italian Legislative Decree No. 146/2001 about Animal Welfare in farms.
In the light of the increased attention towards the welfare of farm animals, several Member States in Europe introduced restrictive legislation on livestock mutilation.
Today, the practice of dehorning is regulated by the European Council Directive 98/58/EC, which lays down the minimum standards for the protection of farm animals.
According to this regulation, dehorning can be performed without anesthesia exclusively by means of thermal or chemical cauterization maximum at the third week of the calf's life and, in any case, under Veterinarinan supervision.
If cattle are more than 3 weeks old or if the Veterinarian believes it necessary, cattle must be anesthetized or sedated. For every animal undergoing dehorning, it is also important to consider whether it is appropriate to give an analgesic drug.
This European Directive is not so clear about the right protocol to keep during dehorning, but it is very clear about the role of Veterinarians. They are the people in charge of the dehorning procedure, which is of course a medical act.
Indeed, only Veterinarians are able to determine the health and welfare of farm animals and they have a double moral responsibility: a responsibility towards the consumer and a responsibility towards animals.
In Italy the Directive 98/53/EC was implemented in 2001 with the Legislative Decree No. 146.
Dehorning is treated in the Annex to this Legislative Decree, in Chapter 19 'mutilations'.

This chapter deals with the most different types of mutilation, on different animal species.

Below the entire Chapter 19:

Mutilation and other practices
19. It is prohibited burning and cutting tendons to the birds' wings and tails for cattle for therapeutic purposes unless certified. The corneal cauterization of the sketch is allowed under the three weeks of life. The cutting of the burner must be made in the first days of life with only the use of equipment to minimize animal suffering. Castration is allowed to keep the quality of products and traditional production practices provided that such operations are carried out before reaching sexual maturity by qualified personnel, while minimizing any suffering to the animals. From 1 January 2004 is not allowed the use of force-feeding ducks and geese, and the plucking of live birds. The practices described in this paragraph shall be under the control of the company's veterinary.

Once more, even in the Italian Legislation, it is confirmed that Veterinarians are actually responsible for animal protection and welfare. Only the Veterinarians, with their knowledge, can be guardians of that.
All procedures listed above, even if considered normal routine procedures in the agricultural field, must at least be supervised by Veterinarians who must always be present at the time of the mutilation.
The presence of a Veterinarian will ensure the application of the correct procedure, which, though simple, is always a medical act and cannot be delegated to other people.
In addition, Veterinarians are the guarantors of Animal Welfare safeguard and of Veterinary Public Health. No one outside Veterinarians can vouch for these concepts.
Veterinarians should evaluate each individual animal as a single clinical case and, therefore, decide the best way to protect its Welfare.
Legislation leaves the freedom of dehorning calves without the use of drugs if they are below 3 weeks of life. However, Veterinarians may consider it appropriate to use medicines for pain therapy after dehorning or for a pre-procedure sedation. This is a decision only Veterinarians can take based on their constant presence in farms and their knowledge of every clinical case, and is intended to protect Animal Welfare (Legislative Decree No. 146/2001).

Different Dehorning or Disbudding Methods and Drugs Used to Relieve Pain

Since 1977 many studies have been carried out to investigate pain caused by dehorning or disbudding. As a matter of fact behavioral and physiological research has determined that dehorning is a painful and unpleasant experience for animals, regardless of the method used (Graf et al., 1999; Grondahl-Nielson et al., 1999; Strafford et al., 2003; Laine et al., 2007).
The pain-induced distress caused by different methods of dehorning and disbudding has been evaluated using physiological, behavioral and production responses before, during and after the procedure with or without local anesthesia or systemic analgesia. These responses are interpreted to estimate the pain-induced distress caused by different techniques and to give an indirect indication of how is cattle experience of disbudding or dehorning (Mellor et al., 2000, 2005; Stafford et al., 2005).
During the last 10 years the pain-induced distress caused by amputation dehorning and cautery disbudding, and different strategies to alleviate pain, have been investigated extensively (Stafford et al., 2005), especially after the introduction of the recent Animal Welfare legislation and the growing feeling that has emerged in the field of animal suffering (Laine et al., 2007; Gottardo et al., 2011).

Methods to Evaluate and Quantify Pain Caused by Dehorning or Disbudding
Changes in plasma cortisol concentrations over time have been used more frequently than any other single parameter to measure the pain-induced distress caused by dehorning or disbudding (Boandl et al., 1989; Taschke and Folsch, 1993; Wohlt et al., 1994; Cooper et al., 1995; Morisse et al., 1995; Petrie et al., 1996; Sylvester et al., 1998; McMeekan et al., 1997, 1998; Graf and Senn, 1999; Grondahl-Nielsen et al., 1999; Sutherland et al., 2002).
There has been debate about the validity of using cortisol responses during disbudding in cattle. The strengths and weaknesses of this approach have been explored (Stafford and Mellor 1993; Mellor and Stafford 1997; Mellor et al., 2000; Stilwell et al., 2010). Stilwell et al. in 2010 confirmed that cortisol is absolutely a poor indicator of pain, but until now there have been few effective alternatives.
In 2003, Stafford said that it is not always appropriate to use plasma cortisol response as an indicator of distress, but if animals have an unpleasant experience which results in a significant elevation of plasma cortisol concentration, then it may be used as a guide in assessing the comparative intensity of that experience.
Stafford justified the use of plasma cortisol concentration as an indicator of pain during dehorning, emphasizing the importance of the function of the

hypotalamic-pituitary-adrenal (HPA) axis, which response probably reflects the severity of the pain-induced distress. As a matter of fact, an animal suffering pain-induced distress produces more or less cortisol proportionately to the intensity of the pain agent. This hypothesis is supported by the fact that the marked increase in plasma cortisol concentration observed immediately after dehorning is virtually abolished by local anesthesia (Petrie et al., 1996; MC Meekan et al., 1997 and 1998; Sylvester et al 1998).
Animals do not produce cortisol infinitely. Molony et al. in 2002 and 2005 tested the "ceiling effect": the increase in plasma cortisol concentration is limited and ends with a threshold. Different negative experiences may stimulate a maximum cortisol response which then cannot be used in a comparative sense.
Another parameter used to indicate pain-induced distress was the change in plasma catecholamine concentrations, but, of course, it can be used for comparing the experience of cattle in the minutes immediately after dehorning as described by Mellor et al. in 2002.
Other physiological parameters, such as heart rate (Grondahl-Nielsen et al., 1999), plasma beta-endorphin concentration (Cooper et al., 1995), plasma progesterone concentration (Cooper et al., 1995), or productive parameters, like weight gain (Goonewardene and Hand 1991) were used in previous studies as indicators of pain-induced distress, but these parameters were not considered entirely reliable by the Authors and they were not used anymore (Stafford, 1997; Mellor et al., 2000).
Behavior is commonly used to recognize and assess pain and distress in animals (Sandford et al., 1986) and the behavior of calves during and following dehorning and disbudding has been monitored (Taschke and Folsch, 1993; Graf and Senn, 1999, Grondahl-Nielsen et al., 1999; McMeekan et al., 1999; Stafford et al., 2000).
Pain-related behaviors, as for example tail shaking, head shaking and ear flicking can be good indices of the duration and the different phases of a painful experience (Stafford et al., 2005). In adult cattle it is also appropriate to evaluate if they graze and ruminate less (Stafford et al., 2000) after dehorning.

Drugs Used during Dehorning or Disbudding
Three recent studies evaluate the pain-induced distress in cattle associated with the dehorning or disbudding method (drugs) used. The first two were both conducted by Stafford in New Zealand, in 2003 and 2005. New Zealand is very sensible to this argument and in 2005 its Governament recommended to use drugs to relieve pain during dehorning.
The third one was conducted by Stilwell in Portugal in 2010.

First Study

In the first study, in 2003, Stafford et al., wanted to evaluate the effect of xylazine antagonized by tolazoline, with and without lignocaine, on the cortisol response of 3-month-old calves following amputation dehorning. The second objective was to assess the effect of ketoprofen and local anesthesia on the cortisol response of calves to amputation dehorning.

Thus, plasma cortisol concentrations were measured in 100 dehorned or non-dehorned 3-month-old calves over an 8-h period following five different sedative/analgesic or control treatments. Sedative/analgesic treatments were:

1. control (no anaesthesia);
2. local anaesthesia and ketoprofen;
3. local anaesthesia and xylazine;
4. local anaesthesia, xylazine and tolazoline;
5. xylazine only.

Within each sedative/analgesic treatment group, half of the calves (n=10 per group) were dehorned by amputation and half were not dehorned.

The result is that the change in plasma cortisol concentrations in calves dehorned after being given ketoprofen and local anesthesia did not differ significantly from that of non-dehorned control calves for at least 8 h. In contrast, the cortisol response of dehorned calves not given analgesic drugs peaked 30 minutes after dehorning and lasted >4 h. Xylazine injected before dehorning significantly reduced but did not eliminate the peak of the cortisol response. When both xylazine and local anesthesia were administered before dehorning the peak in the cortisol response was virtually eliminated. In the dehorned calves that received xylazine with or without local anesthesia, cortisol concentration increased significantly 3 h after dehorning and did not return to baseline values until at least 5 h later. When tolazoline was administered shortly after xylazine, it caused a marked cortisol response, higher than the response to any other treatment.

It is clear that combining ketoprofen and local anesthesia minimized the cortisol response, and by inference the pain-induced distress, following amputation dehorning in calves. Xylazine reduced the initial cortisol response to dehorning but not as much as when local anesthesia was also given. The increase in cortisol concentration from 3-8 h after dehorning in calves given xylazine alone or in combination with local anesthesia suggests that calves experienced pain-induced distress during this time and that xylazine had no long-term analgesic effect. Tolazoline, used to reverse the sedative effects of xylazine, caused a marked cortisol response.

Second Study

In this study, conducted only 2 years after the first one, Stafford et al. wanted to evaluate the pain-induced distress caused by dehorning and disbudding and to study the efficacy of different ways of alleviating that distress.

They were also concerned about giving Veterinarians and farmers practical advices to minimize animal suffering.

They found that the cortisol response to cautery disbudding is significantly smaller than that to amputation dehorning which infers that the latter is more painful.

Amputation dehorning stimulates a defined cortisol response with a rapid rise to a peak value within 30 min followed by a decline to a plateau which then declines to pre-treatment values after about 8 h.

A cornual nerve blockade using lidocaine 2% virtually eliminates the escape behavior seen during disbudding and dehorning and reduces the plasma cortisol response to dehorning for about 2 h. Thereafter there is an increase in the plasma cortisol concentration, a delayed response, which lasts for about 6 h. A cornual nerve blockade, using lignocaine combined with cauterizing the wound caused by amputation dehorning, virtually eliminates the cortisol response as does combining a lignocaine blockade with the non-steroidal anti-inflammatory drug (NSAID) ketoprofen.

When xylazine is combined with a cornual nerve blockade using lignocaine before dehorning, the cortisol response is virtually eliminated for about 3 h. When this regime is used before cautery disbudding and includes a NSAID given before and after disbudding the behavior of calves so treated suggests that pain may be alleviated for 24 h.

They concluded by saying that cautery disbudding is preferable to amputation dehorning, but for optimal pain relief xylazine sedation, local anesthesia and a NSAID should be used with both procedures.

Third Study

Cortisol and behavior have been investigated by Stilwell for the first hour after hot-iron disbudding of calves aged 37 ± 4 days. Calves were divided into four groups:

1. disbudding after IM xylazine (n = 10);
2. disbudding after IM xylazine and lidocaine (n = 10);
3. sham-disbudding after xylazine and lidocaine (n = 11);
4. sham-disbudding after IM saline and lidocaine (n = 10).

Xylazine-treated groups had higher cortisol than saline-treated animals and showed no differences among them at any time. Sham-disbudded calves with xylazine had lower cortisol at 60 min compared with all other times. Xylazine-alone disbudded calves struggled more during the procedure than all other

groups. Xylazine-alone disbudded calves showed more ear flicks at 10, 25 and 40 min and head shakes at 40 min than all other groups.
So, Stilwell et al. concluded that cortisol should not be used as an indicator of pain in disbudded calves while under the sedative effect of xylazine and that some behaviors (ear flicks, vocalizations, transitions, head rubs, head shakes) during and after the procedure are useful in showing that xylazine alone does not control hot-iron disbudding pain.

The lack of difference in cortisol with time in non-sedated animals shows that restraining and handling had no distress effect on these calves or that this hormone was not a good indicator of any such effects.
A significant increase in plasma cortisol in the xylazine-alone disbudded group was to be expected as xylazine does not have an anesthetic effect (Flecknell, 2000). Thus, for surgical procedures in cattle, it should always be supplemented with a local anesthesia (Greene, 2003). Although this lack of efficacy was expected, a xylazine-alone control group was needed to validate the study's cortisol and behavior results.
However, in Stilwell's study, this cortisol increase was temporary and showed no difference compared with the other xylazine-treated groups that did not suffer any pain. This may be explained by the already high baseline levels of all xylazine-treated animals and by the "ceiling effect" that occurs when very high levels of cortisol are attained (Mellor et al., 2005).
The high cortisol in all groups given xylazine is an interesting finding and highlights the disadvantages of using this measure to distinguish severe degrees of pain or when other factors cause a hormonal increase, as may be the case with xylazine although this effect has not been described.
Even if some studies have shown a decrease in cortisol in stressed cattle given xylazine (Brearley et al., 1990), Stafford et al. in 2003, studying amputation dehorning, showed that plasma cortisol concentration increases in xylazine-sedated calves even before any procedure is carried out. Several physiological or psychological factors may explain this effect. Alpha-adrenergic agonists reduce the tonic activity of the baroreflex, decreasing blood pressure and causing bradycardia (Campbell et al., 1979; Brest, 1980), and diminish tissue oxygenation (Hodgson et al., 2002). This may be a cause of distress to animals. But xylazine also causes muscle relaxation limiting the ability of the animal to react to human proximity and contact. This could mean that stress was induced when sedated calves in the study could not avoid human approach for blood collection. Although it was impossible to determine whether it was a physiological or psychological factor that contributed the most to the cortisol response, what these results show is that the HPA axis was activated in calves that were

xylazine-sedated and recumbent even if no painful procedure was performed, indicating that it is not a pain-related response.
In view of these results, the plasma cortisol values seem to be inadequate for the assessment of pain during the first 40–60 min after xylazine is given. In the Stafford et al. study, cortisol was considered useful in identifying animals in pain because cortisol levels were only compared between treatment groups 50 min after xylazine was given and because amputation dehorning is probably more painful.

Stilwell's one is the first study to look at behaviors of disbudded calves during the first hour after xylazine-sedation. Faulkner and Weary in 2000 measured the effect of disbudding on the behavior of calves given xylazine but only studied the effect from three to 24 h after the procedure. Grøndahl-Nielsen et al. in 1999 did not consider looking at behavior for the first four hours in xylazine-sedated calves probably because these animals received xylazine plus butorphanol.
In Stilwell's study, researchers looked at behavior for the first hour after disbudding and found that some signs are significantly related to pain and can be used in evaluating early pain in calves submitted to hot-iron dehorning after xylazine or other drug injection.

The Best Method to Dehorn Currently Available
Disbudding is of course less painful than dehorning. So, farmers should choose to do it in young animals aged less than 3 weeks (Gottardo et al., 2011; Parsons et al., 2006).
Recent studies confirm Stafford's theories. NSAID such as meloxicam, carprofen and ketoprofen, in association with local anesthesia, have proven to be effective in controlling post-disbudding pain-induced distress (Steward et al, 2009; Heinrich et al, 2010; Stillwell et al; 2011) and treated calves tend to have a higher weight intake compared with controls (Faulkner et al., 2000; Duffield et al., 2010; Heinrich et al., 2010).

Italian Current Situation about Dehorning and Disbudding
A very recent study thoroughly and precisely describes the Italian situation about cattle dehorning and disbudding. It has been conducted by Gottardo et al. in the Eastern Po Valley in 2011 on almost 650 farms through an anonymous questionnaire.
In Italy the practice of dehorning is carried out in 80.5% of the dairy farms. Farms that do not dehorn their animals are generally small, with less than 60 animals. The reasons against dehorning are aesthetic motivations and waste of time. These farmers declare no difficulties in managing horned adult animals.

Disbudding is the practice reported by all the interviewed dairy farmers who dehorn their replacement calves. Dehorning, which needs intensive preoperative (restraint), operative and postoperative care, is very uncommon in Europe (SANCO, 2009).
Mean age at disbudding is 32 days. It is very important to underline that only 24.5% of the surveyed farmers dehorn their calves within the third week of life and this data are in line with other European recent studies (Fulwieder et al., 2008). Sometimes 21-day old calves (as required by the Legislative Decree n.146/2001) have no horn buds yet.
Anyway, age at disbudding is a critical factor to limit the pain related to this practice because the horn bud is free-floating in the skin layer above the skull up until about 2 months of age. As the calf gets older, the horn bud attaches to the periosteum of the frontal bone and a small horn starts to grow (Parsons and Jensen, 2006). At this stage, the horn is best removed by amputation, which requires pain management, restraint, hemostasis and antiseptics. (Rebhun 1995).
Most of the farmers (90.6%) use hot-iron cauterization as disbudding method, whereas the remaining 9% use caustic paste.
Hot-iron cauterization is a simple method that does not require hemostasis and has minimal postoperative complications (Rebhum et al., 1995).
In the Unites States, the American Veterinary Medical Association recommends the use of local anesthesia and of an NSAID (AVMA, 2009).
The dehorner should be routinely checked and preheated to the correct temperature (600 °C) before use, to avoid the repetition of the procedure.
Caustic substances are corrosive compounds that cause liquefactive necrosis of the horning producing tissue. The incorrect application of these sticks or paste is frequent and may cause serious damage to the animals (Stafford and Mellor, 2005) and also to the operators (Gottardo et al., 2011). These caustics are not so practical to use, because they should be associated to sedations (Vickers et al., 2005) or, better, to the combined use of local anesthesia and NSAID (Stilwell et al., 2009).
The majority of farmers (75%) indicate that calf disbudding is performed by farm personnel. However, only a small percentage of the stockpersons in charge of the procedure receive specific training. This is in line with a recent European research (Misch et al., 2007). A direct involvement of Veterinarians is more frequent in very small farms, with less than 30 cattle.
The use of analgesia or of anesthesia is recommended by many Veterinarians and Governments worldwide. (New Zealand Government 2005; AVA, 2009; AVMA, 2010).
In Italy 10% of the farmers reported that their calves receive local anesthesia before disbudding. This is in line with other European studies (Hoe et al., 2006; Fulwider et al., 2008). Sedation is reported to be used by only 4% of farmers.

The limited use of preoperative treatments is justifiable because it is not required by law if cautery disbudding is performed within the third week of life of the calf.
Another obstacle is the absence of Veterinarians in farms so that farmers rarely use drugs. As a matter of fact, only Veterinarians can keep and administer local anesthetic and other drugs (Legislative Decree No. 193/2006). Farmers, however, mostly repot the use of local antibiotics (32%) that have no direct pain relief effect, maybe because they are easy to manage. The administration of analgesic is declared by only 5% of the respondents. This could be due to the perceived low painfulness of the procedures or, most probably, farmers do not know the beneficial effect of postoperative analgesia. Many of the interviewed farmers believe that pain after dehorning persist only a few minutes. This evaluation is done by observing calves behavior: farmers note that after dehorning calves shake head, flick ears, swish tail, rub and scratch the dehorned area. On these basis farmers consider dehorning-induced distress a moderate pain.
Another obstacle to the use of drugs is money. About half of the Italian farmers are reluctant to pay for sedation, anesthesia or analgesic treatment. This reluctance might arise from the fact that no detrimental effect of disbudding on calf growth performance has been documented so far (Groendahl-Nielsen et al, 1999) and it is also evident in everyday life.
However, recent studies show that calves treated with NSAID after disbudding eat more feed than controls (Duffield et al., 2010; Heinrich et al., 2010). It is also important to consider that the long-term effects of early painful or fearful experience on the productive performance of dairy heifers are likely underestimated. Another factor to consider is an ethological aspect: negative handling of young animals give a negative effect on milk yield and quality in commercial dairy herds (Breuer et al., 2000, 2003).

World Current Situation about Dehorning and Disbudding
In 2004 Canadian researchers made the first survey to investigate dehorning practices in Ontario. It provided new information on dehorning methods and attitudes about the practice.
Two hundred and seven producers and 65 Veterinarians completed a survey on dehorning practices.
Seventy-eight percent of dairy producers dehorn their own calves; 22% use local anesthetics. Veterinarians dehorn calves for 31% of dairy clients; 92% use local anesthetics. Pain management is the most common reason for use of local anesthetics for both groups, while time (Veterinarians) and time and cost (producers) are the most common reasons for lack of use.

The results of this survey indicated that most Veterinarians follow the Canadian Code of Practice by using a local anesthetic for dehorning, while only 22% of producers did. Almost half of the producers feel that pain management is not necessary for dehorning or are unaware that medications could be used.
Veterinary involvement in the producer dehorning decision-making is the main factor influencing producer medication use for dehorning.
In America (AVMA 2010), Australia and New Zealand (Stafford et al 2005) the situation of calf dehorning and disbudding is comparable to that of Canada.
It is clear that in every country producers who use local anesthetics are likely to involve Veterinarians in their dehorning decisions.
It is important to underline that attendance of a Veterinarian is compulsory for the Legislation of every country, but all over the world it is not respected. This aspect undoubtedly affects Animal Welfare.

Xylazine and Dexmedetomidine

Anesthesia is required in cattle in a lot of circumstances: surgery, restraint, diagnostic procedures, dehorning, etc.
In Italy the only anesthetic drugs also having analgesic power, registered for cattle are xylazine and detomidine. The use of any other anesthetic drug, which is not registered, is granted in derogation (Legislative Decree No. 193/2006).
Currently, although several studies have shown the effectiveness of pain treatment in cattle, it is still not valued as it should (Fajt et al., 2011; Hewson et al., 2007). This gap is particularly evident because of inadequate training of Veterinarians and because of a lack of analgesic registered drugs for the bovine species. To demonstrate this last aspect a study by Hewson et al. in 2007 showed a quite limited qualitative and quantitative use of analgesic drugs, even during painful and stressful procedures such as dehorning, castration and abdominal hernia surgery (Stafford et al., 2004).
So, the most used general anesthetics for cattle, especially in calves, is xylazine, an alpha$_2$-agonist. (Hewson et al., 2007; Stafford et al., 2005).

Alpha$_2$-agonists

Alpha$_2$-agonists constitute an important class of anesthetic drugs used for their analgesic, sedative and muscle relaxant properties (Robertson et al., 2004). Alpha$_2$-agonists have been applied in human medicine as antihypertensive drugs, then they have been introduced into anesthetic protocols for their sedative power.
The first studies on the sedative effects of agonists in Veterinary Medicine began in 1969 (Clarke et al.). Since then, these drugs have revolutionized the sedation and anesthesia of small and large animals. The availability of a specific antagonist has definitely contributed to the success enjoyed by these anesthetics.

Mechanism of Action
The alpha$_2$-agonists act by binding to alpha$_2$-adrenergic receptors, thus stimulating their activity and competing with the endogenous agonist. Alpha$_2$-receptors are mainly localized at pre-synaptic level and represent a mechanism to control the release of endogenous catecholamines.
Alpha$_2$-agonists promote a negative feedback for the release of norepinephrine (Cormack et al., 2005) resulting in a lower transmission of the synaptic cleft.
Alpha$_2$-agonists may also stimulate alpha$_1$-adrenergic receptors. Obviously, if less selective drugs are used, effects are more dose-dependent. The highly selective alpha$_2$-agonists are more reliable and more powerful in inducing alpha$_2$-mediated effects, but the precise dosage can be difficult to calculate (Corletto, 2006).

Pharmacokinetics

Alpha$_2$-agonists are drugs known to have a very good lipophily. This characteristic allows them to rapidly distribute among tissues and to quickly exert their effects. Intravenous administration is clearly the route that allows obtaining the quickest increase in plasma concentrations and the fastest onset of effects. By intramuscular administration, drug absorption can be delayed and plasma peak is reached more slowly. At low doses, alpha$_2$-agonists effects are dose-dependent, whereas at higher doses a maximal response can be easily reached. In fact, a characteristic of alpha$_2$-agonists is that they reach a threshold effect above which further drug additions do not increase neither the desired effects nor the adverse effects, but only the duration of drug action (Corletto, 2006). Alpha$_2$-agonists show a predominantly hepatic metabolism and a renal excretion which take place at variable rates based on dosage and route of administration. Regarding the metabolism timing, it is important to remember that alpha$_2$-agonists-induced hemodynamic changes can alter drug distribution volume and their subsequent elimination.

Clinical Effects

Due to the widespread diffusion of alpha$_2$-adrenergic receptors, the clinical effects of these drugs are not targeted to specific organs and structures. The sedative effect typical of these drugs, similar to physiological sleep, follows the inactivation of *locus coeruleus* (Doze et al., 1989), the cerebral structure with the highest alpha$_2$-adrenergic receptors concentration and responsible of the cortical inactivation caused by various stimuli. The analgesic effect also derives, at central level, from the stimulation of *locus coeruleus*. Namely, by inactivating this nucleus the release of norepinephrine at spinal level takes place accompanied by the consequent antinociception mediated by descendent noradrenergic neurons (Corletto, 2008). The muscle relaxant effect is not due to direct actions on the neuromuscular junction, but is probably caused by the inhibition of synaptic transmission at medullary level. Presynaptic alpha$_2$-receptor activation determines a decrease in norepinephrine release in the synaptic cleft. The relative increase in vagal tone that follows the reduction in the sympathetic activity involves the greatest systemic effects of alpha$_2$-agonists at cardiac level: vagus-mediated bradycardia, inotropism reduction and susceptibility to senoatrial and atrioventricular (AV) blocks. The inhibition of sympathetic nervous system, together with the presence of vasal alpha$_2$ receptors, also causes the main effects of alpha$_2$-agonists on blood pressure. In general, they follow a biphasic pattern involving an initial increase in blood pressure, followed by a gradual reduction to values lower than those at baseline (Campbell et al., 1979; Bloor et al., 1992; Murrel et al., 2005; Braz et al., 2008). Slow intravenous or intramuscular administration allows a gradual adaptation of cardiocirculatory system and

biphasic effect can damp. All cardiovascular effects do not seem, however, to be dose-dependent (Monteiro et al., 2009). In contrast to hemodynamic parameters, respiratory parameters are not subject to particular alterations unless recommended doses are exceeded. Nevertheless tachypnea and hemoglobin desaturation can occur in ruminants probably due to intrapulmonary alterations (Nolan et al., 1985). All alpha$_2$-agonists determine an increase in diuresis due to the inhibitory effect on antidiuretic hormone release at central level. Other effects associated with the stimulation of alpha$_2$-receptors are somatotropin inhibition, slowing of gastrointestinal motility and blood glucose increase by antagonization of insulin release from pancreatic beta cells.

Adverse Effects

If administered too fast, doses used to obtain an adequate sedation can cause significant cardiocirculatory effects that can negatively act upon tissue perfusion. After administration of an alpha$_2$-agonist, the heart shows a reduction in the contraction frequency and ability, whereas at vascular level hypotension of venous system and vasal alpha$_2$-receptors-mediated arteriolar vasoconstriction occur. Consequent preload reduction and afterload increase are badly compensated by heart inability to contract and can result in a dramatic reduction of cardiac output and tissue perfusion. Alpha$_2$ adrenergic receptor presence in the uterine smooth muscle can trigger uterus contractions in a bovine at full term pregnancy with the risk of inducing delivery, at least in this species. This effect is less marked if detomidine is used. A side effect of alpha$_2$-agonists, dangerous above all in newborn, is hypothermia caused by the inactivation of thermoregulatory hypothalamus center which has become insensitive to environment changes compensation (Corletto, 2008).

Xylazine

Xylazine was the first alpha$_2$-agonist used as a sedative and analgesic by Veterinarians. It was synthetized in Germany in 1962 to be used as an antihypertensive drug in human medicine. In the early 1970s xylazine appeared in American and European veterinary literature. It was found to have potent and profound sedative–analgesic effects and muscle relaxant action in cattle and other ruminants (Lumb and Jones, 1996).

Xylazine acts upon the CNS (spinal cord and brain) by simulating the effect of noradrenaline released by inhibitory descending pathways (Sullivan et al., 1987; Pertovaara, 2006).

Like every other alpha$_2$-agonist, xylazine exerts its sedative effects at alpha$_2$-adrenergic postsynaptic receptors localized in the cell bodies of the *locus coeruleus* (Hsu, 1981).

The muscle relaxant properties are related to inhibition of the interneural transmission of impulses in the central nervous system (Gross and Tranquilli, 2001).
Cattle are apparently one of the most sensitive species to the sedative and immobilizing actions of xylazine and therefore require a small dose. Clinical observations suggest that cattle are approximately 5 to 10 times more sensitive than horses to a given dose of xylazine. Sheep and goats are apparently slightly more sensitive than cattle (Green et al., 1988).
In cattle, the degree of sensitivity varies among breeds. Brahmans evidently are the most sensitive breed, followed by Herefords, Jerseys, Holsteins, and Angus (Lumb and Jones, 1996).
In ruminants, xylazine is a desirable adjunct when it is administered in conjunction with ketamine, telazol, guaifenesin ketamine, or a thiobarbiturate for inducing a short period of surgical anesthesia or when anesthesia is to be extended with an inhalant.
Other described effects of xylazine in cattle are: reduction in heart rate, cardiac output and arterial blood pressure; slowing of the respiratory rate (Gross and Tranquilli, 2001); increase in urine volume; transient hypoinsulinemia, due to its direct effect on alpha$_2$-adrenergic receptors of pancreatic islet beta cells resulting in an inhibition of insulin release (Hsu and Hummel, 1981), hyperglycemia and glicosuria, that is detected after 15 to 30 minutes and peaks at 2 hours (Raptopoulos and Weaver, 1988; Lima et al., 2001); reduction in plasma epinephrine; increase in body temperature (+1.9 °C) with the dose of 0.2 mg/kg, but a decrease when 0.4 mg/kg was used (Gross and Tranquilli, 2001); reduction in reticular rumen activity that can lead to bloat (Ruckebusch and Toutain, 1984; Ruckebusch and Allal, 2008); transient reduction in hematocrit values and hemoglobin concentration; and increase of uterine tone in late gestation (Abrahamsen, 2008) that can lead to abortion.
Several studies have investigated the cortisol responses of different species after they had been with xylazine (Brearley et al., 1990; Brearley et al., 1992). Most studies on cattle (Brearley et al., 1990; Brearley et al., 1992) found a lower cortisol level in sedated animals exposed to stress (e.g. transport, general anesthesia).
In 2010 Stilwell et al. demonstrated that calves disbudded after the administration of xylazine had higher cortisol than saline-treated animals. This may be explained by the already high baseline levels of all xylazine-treated animals and by the "ceiling effect' that occurs when very high levels of cortisol are reached (Mellor et al., 2005). The high cortisol in animals given xylazine is an interesting finding and highlights the disadvantages of using this measure to distinguish severe degrees of pain or when other factors cause a hormonal

increase, as may be the case with xylazine although this effect has not been described.
In their study on amputation dehorning (2003), Stafford et al. showed that plasma cortisol concentration increases in xylazine-sedated calves even before any procedure was carried out. Several physiological or psychological factors may explain this effect. Alpha-adrenergic agonists reduce the tonic activity of the baroreflex, decreasing blood pressure and causing bradycardia and diminish tissue oxygenation (Hodgson et al., 2002). This may be a cause of distress to animals. But xylazine also causes muscle relaxation limiting the ability of the animal to react to human proximity and contact. This could mean stress (Stilwell et al., 2010).
After IV injection cattle tend to lie down immediately (depending on the dose) but the effect is short. After IM injection the absorption and distribution is rapid (although incomplete) but the half-life is short (36 minutes in cattle). The IM injection of xylazine (0.2 mg/kg) in calves caused deep sedation, recumbency, useful analgesia that is evident at 5 minutes and maximum at 10 minutes. Analgesia usually last for 30-40 minutes (George, 2003).
It should be remembered that xylazine is not an anesthetic drug, that its analgesic effect is dose-dependent and that analgesia is not present except in deeply sedated animals (Nolan, 2000; Gross and Tranqulli, 2001). Also the sedation produced by alpha$_2$-agonists can be countered by elevated sympathetic tone in anxious or unruly patients (Abrahamsen, 2008) and by other unknown factors. This means that it is difficult for the practitioner to predict the effect of a certain dose in an individual animal. Usually xylazine at the dose of 0.05 mg/kg IV to 0.1 mg/kg IM results in recumbency in 50% of treatable cattle and 0.2 mg/kg IM cause recumbency in most cattle (Abrahamsen, 2008).
In cattle practice xylazine is used alone for restraining, physical examination of aggressive cattle, transport and minor surgeries (Stilwell, 2010). It is also used in association with regional or local anesthesia in major surgeries. Faulkner et al. (1992) showed a beneficial effect on performance and health of castrated bulls when butorphanol and xylazine were administered. Sometimes it is the only drug used for castrations and this suggests that its use is more frequently related to safety reasons than to its analgesic effect. This is evident when looking at the answers of a survey in which practitioners admitted using xylazine more often than lidocaine when castrating calves (Hewson et al., 2007). Xylazine is also used for hot-iron disbudding (Vickers et al., 2005; Faulkner and Weary, 2000; Mish et al., 2008) because its sedative effect facilitates handling and reduces activity after the procedure, giving the idea that distress is low. With the administration of xylazine the hot-iron disbudding may be performed by one person only.
The use of caudal epidural xylazine has been studied in heifers and the results show that there is less intraoperative distress during abdominal surgery (less

reaction to lidocaine injection, more sedation and ataxia) compared with controls, although no differences were found in signs of pain after the surgery (Chevalier et al., 2004).

Dexmedetomidine

Dexmedetomidine is a highly selective alpha$_2$-agonist that has been shown to have both sedative and analgesic effects (Venn et al., 1999; Kauppila et al., 1991). It is classified as a sedative–anxiolytic (Kamibayashi et al., 2000).

The hypnotic effect of dexmedetomidine is mediated by the hyperpolarization of noradrenergic neurons in the *locus ceruleus*.
When the alpha$_2$-adrenergic receptor is activated, it inhibits adenylyl cyclase. The latter enzyme catalyzes the formation of cyclic AMP (cAMP), a crucial second messenger molecule that acts in many catabolic cell processes. By reducing the amount of cAMP in the cell, dexmedetomidine favors anabolic over catabolic pathways. Simultaneously, there is an efflux of potassium through calcium-activated potassium channels and an inhibition of calcium influx into calcium channels in nerve terminals (Khan et al., 1999).
The change in membrane ion conductance leads to membrane hyperpolarization, which suppresses neuronal firing in the *locus ceruleus* as well as the activity in the ascending noradrenergic pathway (Kamibayash et al., 2000). When a hypnotic dose of dexmedetomidine was administered to laboratory animals, norepinephrine release from the *locus ceruleus* was inhibited.
The absence of inhibitory control over the ventrolateral preoptic nucleus (VLPO) resulted in the release of gamma-aminobutyric acid (GABA) and galanin, which further inhibited the *locus ceruleus* and tuberomamillary nucleus (TMN). This inhibitory response also caused a decrease in the release of histamine which resulted in a hypnotic response, similar to that found in normal sleep (Carollo et al., 2008).

Dexmedetomidine is widely used in human medicine where it is emerging as an effective therapeutic agent in the management of a wide range of clinical conditions with an effective and safe profile (Carollo et al., 2008).
As a matter of fact, dexmedetomidine has being investigated in human medicine for use in ICU (intensive care unit), where it proved to be a very effective agent for the management of sedation and analgesia after cardiac, general, orthopedic, head and neck, oncological and vascular critical surgery. It is also very appreciated in pediatrics (Venn et al., 1999).
Dexmedetomidine has several properties that may additionally benefit those critically ill patients who require sedation.

Cardiovascular stability was demonstrated, with significant reductions in rate–pressure product during sedation and over the extubation period.
Dexmedetomidine reduces the hemodynamic response to intubation and extubation (Jaakola et al., 1992; Aho et al., 1991), and attenuates the stress response to surgery (Aantaa et al., 1991), as a result of the alpha$_2$-mediated reduction in sympathetic tone. It should be possible to continue sedation with dexmedetomidine over the stressful extubation period without concerns about respiratory depression, while ensuring the preservation of hemodynamic stability. The short half-life of dexmedetomidine makes it an ideal drug for intravenous administration.
When dexmedetomidine is administered as a continuous infusion, it is associated with a predictable and stable hemodynamic response (Frangoulidou et al., 1998; Aantaa et al., 1993). However, care should be taken when administered to patients who are volume-depleted, vasoconstricted, or have severe heart block (Hassan 2000), as dexmedetomidine can cause hypotension and bradycardia.
Dexmedetomidine has other many advantages in comparison with more commonly used hypnotics. Although it produces sedative, analgesic, and anxiolytic effects (Aantaa et al., 1993), unlike other sedatives, it provides respiratory stability, in that it does not cause ventilatory depression (Frangoulidou et al., 1998). In spontaneously breathing volunteers, intravenous dexmedetomidine caused marked sedation with only mild reductions in resting ventilation at higher doses (Belleville et al., 1992).
As dexmedetomidine has the ability to potentiate opioids and other sedatives, this attribute suggests that these drugs can be administered in smaller doses (Carollo et al., 2008).
Dexmedetomidine also reduces the shivering in postoperative patients (Kamibayashi et al., 2000).
For all these reasons dexmedetomidine is now considered an effective therapeutic agent for many clinical and also critical conditions. (Carollo et al., 2008; Richard et al., 2000).
The side effect of dexmedetomidine is dry mouth, which is an advantage during fiber-optic intubation (Carollo et al., 2008).

In Veterinary Medicine, dexmedetomidine is the newest alpha$_2$-agonist introduced. Like in human medicine, also in Veterinary Medicine it has been appreciated for its minimal side effects on the respiratory tract, for its remarkable sedative and anxiolytic properties, for the possibility to be administered by continuous infusion (Tobias et al., 2007), and especially for the availability of a specific antagonist: atipamezole.
In Italy, dexmedetomidine is registered only for its utilization on cats and dogs.
Currently available studies have been conducted on these two species.

After muscle injection, a rapid attainment of the peak of plasma concentration and a half-life of elimination of 45-60 minutes are observed in these two species.
Metabolism, like other alpha$_2$-agonists, is mainly hepatic whereas elimination is mainly renal.
The high selectivity for the alpha-receptors confers to this drug a high therapeutic potency, at the expense of handling. In fact, a precise dosing is difficult to obtain and the drug dosage may vary depending on the temperament of the animal, its clinical condition and the desired result (Rioja et al., 2006).
Atipamezole is the specific antagonist of dexmedetomidine and has an affinity for the alpha$_2$ adrenergic five times higher than that of dexmedetomidine.
By displacing dexmedetomidine from the alpha$_2$-agonist receptors, atipamezole promotes the release and use of norepinephrine in central and peripheral synapses.
This predisposes to a rapid normalization of vital parameters and wakes up the animal in few minutes from the administration.
Atipamezole is generally given in doses 5 to 10 times higher than those of dexmedetomidine (Monography Antisedan).
Until now the use of dexmedetomidine in cattle has never been investigated.

CHAPTER 2

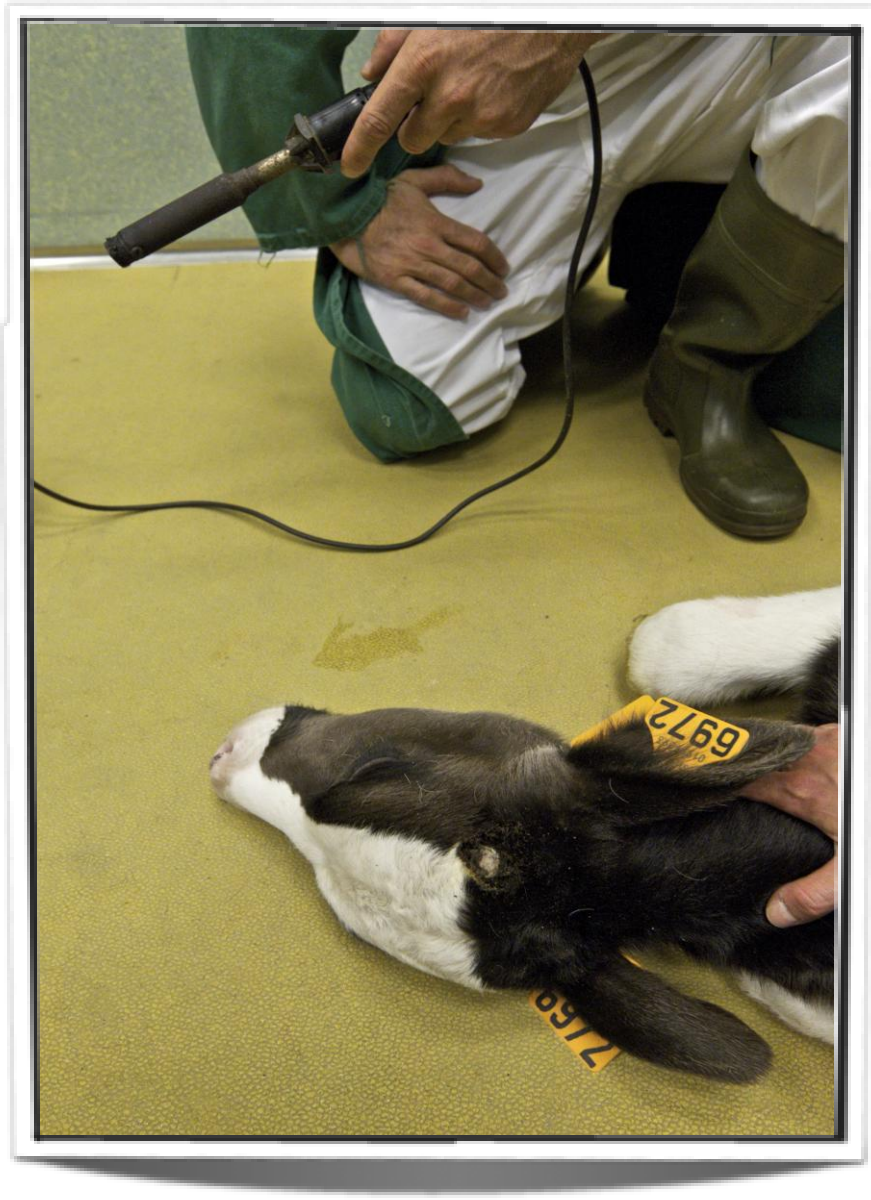
Objectives

Aim of the study

Disbudding is a routine painful procedures carried out on cattle to facilitate management (Gottardo et al. 2011, Duffield 2008, Laine et al. 2007, Stafford et al. 2005 e 2003, Marshall, 1977; Vowles, 1976).
To measure the pain-induced distress caused by dehorning or disbudding plasma concentration of cortisol have been used more frequently than any other parameter (Boandl et al., 1989; Taschke and Folsch, 1993; Wohlt et al., 1994; Cooper et al., 1995; Morisse et al., 1995; Petrie et al., 1996; Sylvester et al., 1998; McMeekan et al., 1997, 1998; Graf and Senn, 1999; Grondahl-Nielsen et al., 1999; Sutherland et al., 2002), but Stilwell et al. in 2010 confirm that cortisol is not a good indicator of pain.
Substance P is a neurotransmitter of pain used for the first time in cattle in 2008 by Coetzee et al. who found that substance P is a better indicator of pain than cortisol in calves after castration or simulated castration.
On these basis we hypothesized that SP could potentially be a more specific measure of pain in cattle undergoing disbudding than would the plasma cortisol response. We studied the effects of true and simulated disbudding on plasma concentration of cortisol and substance P in calves undergoing 2 different alpha2-agonists: xylazine and dexmedetomidine. In Veterinary Medicine dexmedetomidine is registered only for its utilization on cats and dogs and it is appreciated for its minimal side effects on the respiratory tract, for its remarkable sedative and anxiolytic properties and for the possibility it can be administered by continuous infusion. The utilization of dexmedetomidine in cattle has never been researched right now.

The aim of this study was:
-to compare plasma concentrations of cortisol and substance P as the best indicator of pain in 3-week-old dairy calves undergoing true and simulated disbudding.
-to compare dexmedetomidine and xylazine as the best alpha-2 agonist during a painful procedure such as disbudding, by using behavioural and physiological indexes.
-to evaluate the differences of plasma concentration of cortisol after the administration of dexmedetomidine and xylazine in calves undergoing simulated disbudding.

CHAPTER 3



Materials And Methods

Animals

Sixty Italian Friesian male (n. 17) and female (n. 43) calves, belonging to 3 different herds in Lombardy (Italy) were used in the study. The calves were 3 to 6 weeks old (mean 4.1 ± 1 week) and weighed approximately 50 kg (mean 49.5 ± 11.5 kg). On examination, the animals were clinically healthy, and they had not received any type of treatment in the previous 2 weeks.
The study protocol was approved by the Institutional Ethical Committee for Animal Care of the University of Milan (protocol No. 28/2011)[1].

Housing and Husbandry

On arrival at our facility, the calves were weighed and housed separately in 1.8 × 1.2 m (2.16 m^2) single boxes in an indoor stall with controlled temperature of 20 °C. Boxes were separated by solid walls and had straw litter. Calves were acclimated for 7 days prior to study commencement. During the acclimation period each calf had unlimited access to water, grass hay and pellets, and was fed 3 times/day with 2 L milk replacer (at 7.00 a.m., 1.00 p.m. and 7 p.m.).

Group Assignment

Calves were assigned to 6 treatment groups using a computer-generated randomized list (n = 10 calves/group). Treatment groups were:
Group 1 (disbudding – placebo): calves undergoing disbudding without sedative/analgesic treatment (administration of placebo consisting in 5 ml 0.9% NaCl saline solution).
Group 2 (simulated disbudding – placebo): calves undergoing simulated disbudding without sedative/analgesic treatment (administration of placebo consisting in 5 ml 0.9% NaCl saline solution).
Group 3 (disbudding – xylazine): calves undergoing disbudding after IV administration of 0.2 mg/kg b.w. xylazine diluted in 0.9% NaCl saline solution to a volume of 5 mL.
Group 4 (simulated disbudding – xylazine): calves undergoing simulated disbudding after IV administration of 0.2 mg/kg b.w. xylazine diluted in 0.9% NaCl saline solution to a volume of 5 mL.
Group 5 (disbudding – dexmedetomidine): calves undergoing disbudding after IV administration of 5 μg/kg b.w. dexmedetomidine diluted in 0.9% NaCl saline solution to a volume of 5 mL.

[1] http://www.unimi.it/cataloghi/comitato_etico/CE_18.10.2011_Verbale.pdf)

Group 6 (simulated disbudding – dexmedetomidine): calves undergoing simulated disbudding after IV administration of 5 μg/kg b.w. dexmedetomidine diluted in 0.9% NaCl saline solution to a volume of 5 mL.

Figure 1. Administration of dexmedetomidine through a jugular catheter to a calf of group 5

Jugular Catheterization

Approximately 48 hours before study commencement, the left jugular vein of each calf was catheterized. A 10 × 10 cm area over the left jugular vein was shaved and disinfected with 70% isopropyl alcohol and povidone iodine. A 14-gauge × 80-mm polypropylene catheter was introduced in the jugular vein through the skin. A 30 cm luer lock extension tube was connected to the catheter and both devices were sutured to the skin with 0 Supramid suture. Catheter patency was maintained by use of a 5 mL heparinized saline flush solution (25 U of heparin sodium/mL of 0.9% NaCl saline solution) administered 3 times/day.

Disbudding and Simulated Disbudding Procedures

The study was performed using 6-8 animals each time. Disbudding and simulated disbudding were always performed approximately at 10.00 a.m. by a single experienced veterinarian (DP) to minimize variations.

Disbudding was performed using a thermo dehorning device that was heated up for approximately 10 minutes. An animal holder was used to fix the calf so that it could not move its head. Each horn bud was burned out by turning the burner for 10 seconds while applying pressure. Finally, the inner parts of the burnt ring were peeled out with the tip of the instrument.
Simulated disbudding procedure was performed with a cold device applied for the same time with the same pressure on each horn bud.

Figure 2: true disbudding in a calf undergoing the administration of placebo (group 1)

Figure 3. Simulated disbudding in a calf undergoing the administration of xylazine (group 4)

Collection of Blood Samples

The samples for baseline cortisol and Substance P determination were collected 30 minutes prior to disbudding or simulated disbudding via the jugular catheter, at 9.30 a.m. (corresponding to plasma cortisol concentration zenith for cattle). Additional samples were collected immediately after disbudding or simulated disbudding (within 5 minutes from the procedure – Time 0), 20 minutes (Time 1) and 1, 2, 3, and 4 hours after the procedure (Times 2, 3, 4 and 5, respectively). Blood samples were collected in 10 mL vacuum tubes that contained potassium EDTA and lithium heparin and immediately centrifuged for 15 minutes at 1,500 rpm. Plasma was then harvested, placed in 1 mL tubes, and frozen at −80 °C until analysis.

Figure 4. Blood collection through a jugular catheter in a calf of group 2

Behavioral Scoring

Behavioral changes in response to disbudding or simulated disbudding were assessed by assigning a score for vocalization and behavioral changes during the procedure. Vocalization was scored on a scale of 0 to 3 (0, no vocalization; 1 snorting or grunting; 2, momentary vocalization; and 3, continuous vocalization during and immediately after head manipulation). Behavioral changes were scored on a scale of 0 to 3 (0, unchanged from pre-manipulation behavior; 1, head shaking, kicking; 2, momentary escape behavior [e.g., lunging against the head gate], head shaking, and kicking; and 3, violent escape behavior [e.g., repeated lunging against the head gate, head shaking, and kicking] throughout head manipulation). After disbudding we observed calf behavior for the entire period of blood sampling (4h). During observations we recorded the frequency (min) of three behaviors previously associated with pain after dehorning: head shaking, ear flicking (twitching of both ears when no flies present), head rubbing (with hind leg or against the sides of the pen).

Cortisol Analysis

Plasma cortisol concentrations were determined in duplicate by use of a competitive solid phase radioimmunoassay (RIA) validated for cattle. To perform the calibration curve, we labeled 2 uncoated 12x75 mm polypropylene tubes T (total counts) in duplicate. Because the NBS is characteristically low, the NSB tubes were omitted, as suggested by the procedure supplied with the kit. Than we labeled 12 Cortisol Ab-Coated tubes A (maximum binding) and B through F in duplicate. At the end of that, we pipetted 100 μL of the zero calibrator A into the A tube, and 75 μL of the zero calibrator A into the remaining calibrator tubes B through F and into the control tubes. We pipetted 25 μL of each control into the correspondingly labeled tubes. So, each calibrator, control and sample tube contain 100 μL.
After defreezing and gentle swirling of the samples, 100 μL of plasma were placed into coated tubes, added with 1 mL of ^{125}I-cortisol and than vortexed. The tubes were incubated for 90 minutes at 37 °C using a water bath. Afterwards the supernatant was aspirated and the tubes analyzed in a gamma-counter.

Figure 5. Aspiration of the supernatant from a tube before analysis of cortisol in the gamma-counter

Substance P Analysis

Plasma Substance P concentrations were determined by use of a commercial competitive immunoassay kit. Samples were subjected to solid-phase extraction with C-18 cartridges. (Waters. Spe-Pak Vac 3cc 500 mg C18 Cartridges) This immunoassay used a polyclonal antibody against Substance P that competitively bound to Substance P in the test sample or to an alkaline phosphatase molecule that was covalently attached to a Substance P molecule. The concentration of Substance P in the sample was inversely proportional to color intensity generated after incubation, as determined at 405 nm on a microplate reader. The analytic range of the assay reported by the manufacturer was 9.75 to 10,000 pg/mL, and sensitivity was 8.04 pg/mL.

The Substance P immunoassay has not yet been validated for bovine plasma. In human medicine it is done by use of calibration samples fortified with a stock solution of Substance P dissolved in assay buffer. Plasma was spiked with 3 concentrations (low, middle, and high) that spanned the expected analytic range of the assay. Each spiked sample was then analyzed in triplicate in accordance with manufacturer instructions. Briefly, 1 mL of sample was transferred into separate 13 × 100 mm glass culture tubes and acidified by addition of an equivalent volume of 1.0% aqueous TFA followed by vortexing at medium

intensity. Acidified samples were centrifuged at 3,000 × *g* for 10 minutes to separate precipitate from plasma. Concurrently, a 24-port vacuum manifold was used to prepare solid-phase extraction cartridges for use. Equilibration of the solid phase extraction cartridges was achieved by successive washings with 1 mL of high-performance liquid chromatography–grade methanols containing 0.1% TFA (1 mL) and distilled water containing 0.1% TFA (1 mL). After cartridges were loaded with the acidified samples, each was washed 6 times with 3 mL aliquots of distilled water containing 0.1% TFA. The Substance P-containing fractions were then collected during elution by use of 3 mL of methanol containing 0.1% TFA. Eluents were evaporated to dryness by use of a vacuum centrifuge under nitrogen gas at 37 °C. Dry samples were stored at –20 °C prior to competitive immunoassay, which was performed within 12 to 16 hours after extraction.

The competitive immunoassay was conducted by adding 50 µL of assay buffer to the nonspecific-binding and zero-standard wells, in duplicate. Remaining wells on each plate were filled with 50 µL of appropriately diluted sample. Thereafter, 50 µL of assay buffer was added to the nonspecific-binding wells, whereas the remaining wells received 50 µL of conjugate followed by 50 µL of antibody. Plates were then incubated at 22 °C on a plate shaker for 2 hours at approximately 500 rpm. Following incubation, contents of each plate were discarded, and wells were washed 3 times (400 µL of wash solution per wash).

After washing, wells were emptied, and plates were tapped on a paper towel to remove remaining wash buffer. Then, *p-nitrophenyl* phosphate substrate solution (200 µL) was added to each well, followed by incubation for 1 hour without shaking. Finally, 50 µL of stop solution were added to each well. Immediately after the stop solution addition, results for each plate were determined by use of a plate reader.

The results for each test well were compared with those for blank wells, and the optical density was then measured at 405 nm with correction at 570 nm. Computer software was used to process the data by converting the net optical density based on the following equation: net optical density of samples/net optical density of maximum binding wells. Resulting values were plotted versus the concentration of Substance P standards to create a standard curve.

The standard curve for Substance P was obtained by use of a 4-parameter logistic curve for concentrations from 9.76 to 10,000 pg/mL (R2, 0.98). A validation curve derived from the spiked bovine plasma samples was constructed by plotting net optical density versus corresponding concentrations of SP. The variation coefficient among triplicate bovine samples at each fortified Substance P concentration ranged from 6% to 22%. The linear regression line for the 3 points at each of the 3 concentrations had a correlation coefficient of 0.99.

CHAPTER 4

Results and discussion

Behavioural scoring

During disbudding procedure

Vocalization: animals true disbudded (groups 1, 3, 5) vocalize more than simulated disbudded ones (groups 2, 4, 6) during the procedure.
Calves that vocalize more belong to group 3 (true disbudded with xylazine). Mean result for this group is level 1: snorting or grunting vocalization during and immediately after the procedure. Animals disbudded with placebo and animals disbudded with dexmedetomidine reach level 0.5, a midway between snorting or grunting and no vocalization.
Animals sham-disbudded belonging to groups 2 and 4 reach level 0.2. In general they do not vocalize. Calves belonging to group 6 (simulated disbudding with dexmedetomidine) reach the similar level of animals belonging to group 1: 0.5.

Attitude
During the disbudding procedure animals struggling more belong to group 1 (true disbudded with placebo). On average they head shake, kick and have a momentary escape behavior. In group control (group 2) animals' attitude reaction is similar but less marked.
Animals belonging to group 3 (true disbudding with xylazine) do not show intense reactions due to pain. They reach level 0,2. In control group (group 4) calves do not present any reaction, they do not move during the procedure.
Animals belonging to group 5 (true disbudded with dexmedetomidine) show a similar reaction to animals of group 2 (simulated disbudding and placebo).

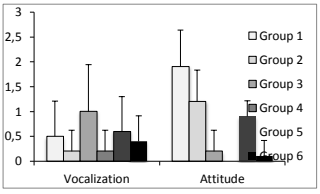


Table 1: Vocalization and attitude during disbudding procedure. Vocalization score: 0, no vocalization; 1 snorting or grunting; 2, momentary vocalization; and 3, continuous vocalization during and immediately after head manipulation. Attitude score: 0, unchanged from premanipulation behavior; 1, head shaking, kicking; 2, momentary escape behavior (e.g., lunging against the head gate), head shaking, and kicking; and 3, violent escape behavior (e.g., repeated lunging against the head gate, head shaking, and kicking) throughout head manipulation.

The intensity and the duration of vocalization and the attitude of calves during the disbudding procedure are thought to be behavioral indicators of pain (Groendhal-Nielsen et al. 1999). In our study animals vocalizing more belongs to groups undergoing true disbudding (group 1, 3 and 5). On average animals of group 3 reach the highest level in the intensity and in the duration of vocalization (level 1). Stillwell et al. 2010 demonstrate that vocalization is characteristic of animals sedated with xylazine, so should not be used as a sign of pain in animals treated with alpha2-agonists. Despite that, in our study, animals belonging to group 4 vocalize few, exactly like animals of group 2. Animals belonging to group 6 reach an higher level in intensity and duration of vocalization than group 2 and 4.

As regards calves attitude during the disbudding procedure, animals of group 1 and 2 show the more pronounced reaction to the procedure. Animals of group 1 meanly show a momentary escape behaviour and animals of group 2 kick and head shake. This is due to the pain caused by the procedure and the possibility to react, because they are not sedated. Of course the reaction is less intense for animals undergoing simulated disbudding.
Animals sedated with xylazine (group 3 and 4) show the lowest reactions to the procedure. For animals of group 4 the behaviour is unchanged from the premanipulation period. Xylazine causes a marked muscle relaxation limiting the ability of the animal to react to human proximity and contact (Stilwell et al. 2010), so it can be a very good drug for restraint.
Animals of group 5 show reactions similar to group 2 and animals of group 6 have similar attitude to group 3.
Dexmedetomidine could cause a less marked muscle relaxation than xylazine, so animals can react. Moreover xylazine could have more analgesic power than dexmedetomidine.

After disbudding procedure
Immediately after the disbudding procedure, calves were observed for 4 hours to evaluate head shaking and rubbing and ear flicking without the presence of flies.
Animals of group 1 are the most subjected to head shaking and head rubbing, followed by animals of group 2. Head shaking and rubbing in other groups do not give statistically significant results. In general calves treated with alpha2-agonists show more less head shaking and head rubbing than animals not treated.

Clincal effects

Heart rate

At the baseline mean heart rate varies from 66 bpm (group 1) to 83 bpm (group 6).

Group 1 shows a prominent increase of the heart rate five minutes after disbudding (87 bpm). Then 10, 15 and 20 minutes later the heart rate decreases progressively to become 77 bpm.

The heart rate in calves belonging to group 2 remains more or less constant: 72 bpm at the baseline and 68 bpm 20 minutes after disbudding.

The trend is very different after the administration of alpha-2agonists: calves belonging to groups 3, 4, 5 and 6 show a marked decrease of heart rate 5 minutes after the administration of the drug, both the disbudded ones than the simulated disbudded ones. For example the mean heart rate for group 6 at the baseline was 84 bpm; 5 minutes after the administration of an alpha-2 agonist it was 52 bpm. From there onwards the heart rate increases, but after 20 minutes it is still lower than the baseline in all 4 groups which assumed xylazine or dexmedetomidine .

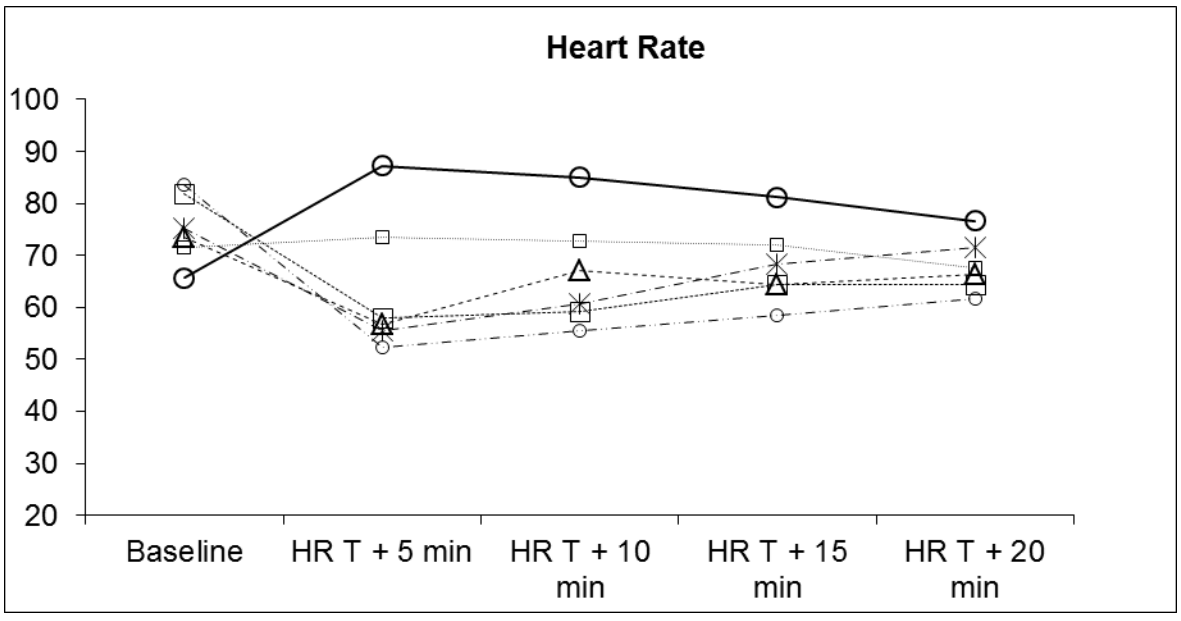


Table 4: mean heart rate at the baseline, 5 minutes, 10 minutes, 15 minutes, 20 minutes after the administration of alpha-2agonists or placebo.

——○—— group 1 (true disbudded-placebo), ……□… group 2 (simulated disbudded-placebo), — — □ — — group 3 (true disbudded-xylazine), -- Δ -- group 4 (simulated disbudded-xylazine), — · — *·— ·— group 5 (true disbudded-dexmedetomidine), — · · — · · ○ — · · — · · group 6 (simulated disbudded-dexmedetomidine).

Painful and stressful procedures physiologically cause an increase in heart rate (Jensen et al. 2008). Animals of group 1 show the highest peak in heart rate, followed by animals of group 2. Disbudding or simulated disbudding without preemptive analgesia cause pain and manipulation cause stress and consequently an increase in heart rate of these animals. Heart rate decrease by 20 minutes.
Animals treated with alpha2 show an initial bradycardia (Campbell et al., 1979; Brest, 1980), but after that, the pain induced distress cause an increase in heart rate, more marked in animals belonging to group 4.

Respiratory rate

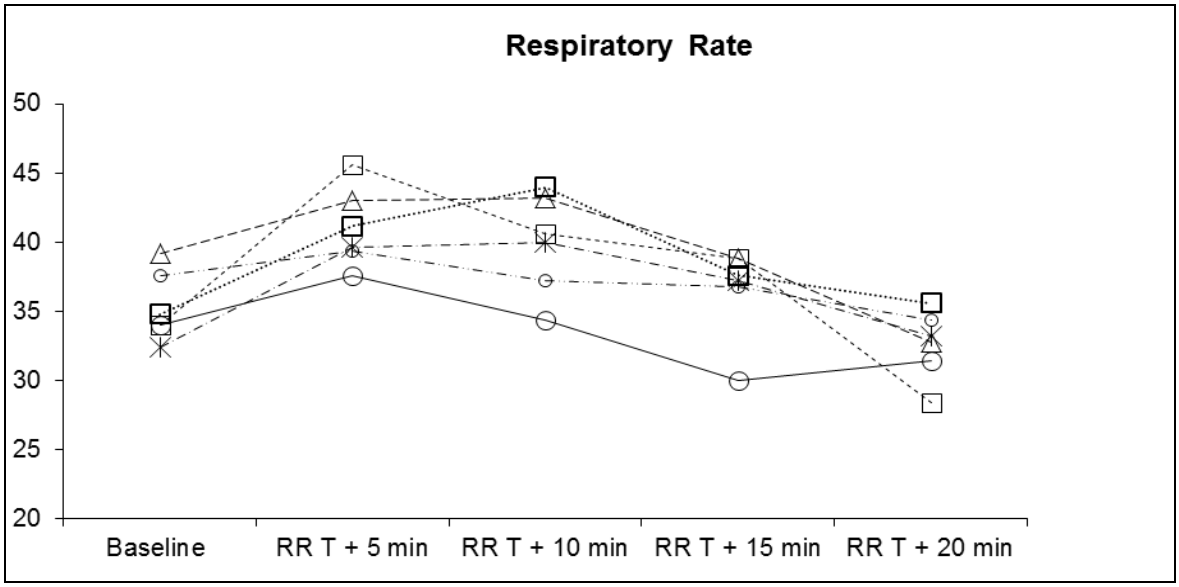


Table 4. mean respiratory rate at the baseline, 5 minutes, 10 minutes, 15 minutes, 20 minutes after the administration of alpha-2agonists or placebo.
——○—— group 1 (true disbudded-placebo), ……□… group 2 (simulated disbudded-placebo), — — □ — — group 3 (true disbudded-xylazine), -- **Δ** -- group 4 (simulated disbudded-xylazine), — · — *—· ·— group 5 (true disbudded-dexmedetomidine), — · · — · · ○ — · · — · · group 6 (simulated disbudded-dexmedetomidine).

The mean respiratory rate of animals belonging to group 1 at the baseline is 34 apm. It increases 5 minutes after disbudding (38 apm), than it decreases, and 15 minutes after is 31 apm.
The mean respiratory rate of animals of group 2 increases 10 minutes after the administration of placebo and it returns at the baseline after 20 minutes from the administration. In these cases the respiratory rate is influenced by the disbudding.
Animals which receive xylazine show the highest peak of the respiratory rate. The mean baseline respiratory rate of animals in group 3 is 34 apm. The

frequency rises sharply 5 minutes after from the administration (46 apm), then gradually decreases to get to 28 apm.
Calves subjected to simulated disbudding show a peak in respiratory rate 5 and 10 minutes after the administration of xylazine (43 apm). The frequency tends to decrease progressively and it returns to baseline values 20 minutes after the administration.
The animals receiving dexmedetomidine (group 5 and group 6) maintain a respiratory rate almost constant.

Plasma concentration of cortisol

Group 1 and 2 (disbudding and simulated disbudding-placebo)
Mean plasma cortisol concentration 30 minutes before disbudding (Baseline) is 1,7 ± 1,2 nM/L for calve of group 1 and 1,1 ± 0,22 nM/L for group 2.
Immediately after disbudding (Time 0) mean plasma cortisol concentration increases to 7,7 ± 8,1 nM/L for calves of group 1 and 2,5 ± 3,5 nM/L in group 2.
At Time 1, 20 minutes after disbudding or simulated disbudding, there is the peak of plasma cortisol concentration both for the true disbudded calves than for simulated disbudded ones, but for the group 2 it is lower than for the true disbudded calves: 45,4 ± 12,8 nM/L for group 1 and 12,6 ± 10,9 nM/L for group 2.
For group 1, thereafter, plasma cortisol concentration decreases by 2 hours (Time 2: 14,44 ± 12,20 nM/L; Time 3: 5,7 ± 4,44 nM/L) and rose up only slightly at Time 4 and 5 (Time 4: 9,81 ± 8,85 nM/L; Time 5: 12,93 ± 7,13 nM/L).
It occurs the same for the group 2 with similar values.
Mean plasma cortisol concentration from Time 0 to Time 5 reach mean baseline values anymore.

Group 3 and 4 (disbudding and simulated disbudding-xylazine)
Mean plasma cortisol concentration at the baseline is 4,89 ± 11 nM/L for calves of group 3 and 1,75 ± 1,52 nM/L for group 2.
Immediately after disbudding (Time 0) plasma concentration of cortisol increase substantially both in group 3 than in group 4, respectively 32,42 ± 18,7 nM/L and 18,15 ± 20,91 nM/L. This should lead to the belief that after the administration of xylazine, plasma concentration of cortisol increase, even without stress or pain.
Twenty minutes after disbudding (T1), plasma concentration of cortisol reaches its peak of 42,26 nM/L for calves of group 3 and 28,66 for group 4.
Then, from Time 1 to Time 5, the trend is very similar to groups 1 and 2.

Group 5 and 6 (disbudding and simulated disbudding-dexmedetomidine)
Mean plasma cortisol concentration at the baseline is 1,30 ± 0,6 for calves of group 5 and 5,57 ± 6,86 nM/L for group 6.
Immediately after disbudding (Time 0) mean plasma cortisol concentration increases and it becomes 9,96 ± 11,42 nM/L for calves of group 5 and 12,49 ± 9,24 nM/L in group 6.
At Time 1, 20 minutes after disbudding or simulated disbudding, there is the peak of cortisol concentration both for calves of group 5 than for group 6, but for group 6 it is lower than for group 5: 37,03 ± 44,46 nM/L for group 5 and 18,69 ± 10,34 nM/L for group 6. This trend is very similar to groups 1 and 2.
Then, from Time 1 to Time 5, the values are very similar to groups 1 and 2.

	Baseline	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**
Group 1	1,7 ± 1,2	7,7 ± 8,1	45,4 ± 12,8	14,4 ± 12,2	5,7 ± 4,4	9,8 ± 8,8	12,9 ± 7,1
Group 2	1,1 ± 0,2	2,5 ± 3,5	12,6 ± 10,9	2,3 ± 3,7	5,1 ± 8,1	10,0 ± 6,1	16,2 ± 9,9
Group 3	4,8 ± 11,0	32,4 ± 18,7	42,2 ± 25,3	10,6 ± 9,6	2,0 ± 1,5	9,4 ± 8,6	7,5 ± 5,8
Group 4	1,7 ± 1,5	18,1 ± 20,9	28,6 ± 21,9	14,2 ± 9,0	12,8 ± 12,9	6,6 ± 5,0	11,8 ± 7,4
Group 5	1,3 ± 0,6	9,9 ± 11,4	37,0 ± 44,4	13,4 ± 17,6	12,1 ± 7,0	12,7 ± 10,5	9,5 ± 5,7
Group 6	5,5 ± 6,8	12,4 ± 9,2	18,6 ± 10,3	8,7 ± 6,5	11,4 ± 5,8	6,0 ± 5,9	15,3 ± 8,1

Table 5. mean ± SD of cortisol plasma concentration in the 6 different groups at the baseline (30 minutes before disbudding), at T0 (immediately after disbudding), at T1 (20 minutes after disbudding), at T2 (one hour after disbudding), T3 (2 hours after disbudding), T4 (3 hours after disbudding), T5 (4 hours after disbudding).

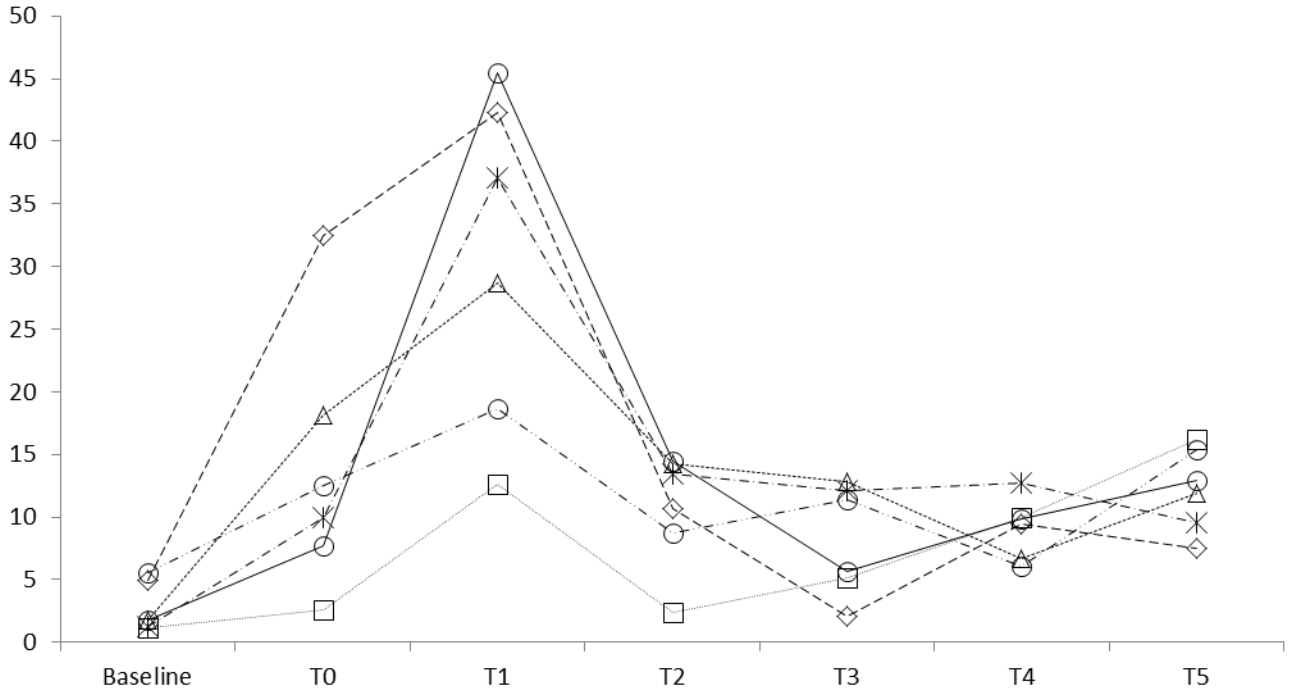


Table 6. Mean plasma cortisol concentration: ——○—— group 1 (true disbudded-placebo), ……□… group 2 (simulated disbudded-placebo), — — □ — — group 3 (true disbudded-xylazine), -- Δ -- group 4 (simulated disbudded-xylazine), — · — *·— · — group 5 (true disbudded-dexmedetomidine), — · · — · · ○ — · · — · · group 6 (simulated disbudded-dexmedetomidine).
Plasma cortisol concentration increases significantly immediately after disbudding or simulated disbudding (T0) in calves of group 3 and 4. At T1, 20 minutes after disbudding, every group reaches its peak in plasma cortisol concentration. It is higher in group 1. After that plasma cortisol concentration decreases by 2 hours in every group and rose up slightly at T4 and T5. Mean plasma cortisol concentration from Time 0 to Time 5 reach mean baseline values anymore in no group.

To measure the pain-induced distress caused by castration and disbudding plasma concentration of cortisol have been used more frequently than any other parameter (Boandl et al., 1989; Taschke and Folsch, 1993; Wohlt et al., 1994; Cooper et al., 1995; Morisse et al., 1995; Petrie et al., 1996; Sylvester et al., 1998; McMeekan et al., 1997, 1998; Graf and Senn, 1999; Grondahl-Nielsen et al., 1999; Sutherland et al., 2002).
Group 1 shows the higher peak of cortisol at T1 (mean 45,4 ng/L). This is due to the pain induced distress of the procedure without any kind of analgesic drug. In group 2 the peak of plasma cortisol concentration is the lowest one. Of course animals belonging to this group can feel stressed by the manipulation during the procedure, that is the reason of the peak, but they do not feel pain.
The high cortisol levels found in animals belonging to groups treated with xylazine (groups 3 and 4) can be induced by xylazine administration. Although some studies have found a decrease in cortisol in stressed cattle treated with

xylazine (Brearley et al., 1990), Stafford et al. (2003), studying amputation dehorning, showed that plasma cortisol concentration increases in calves xylazine-sedated even before any procedure is carried out, exactly like in this study. Animals belonging to group 3 and 4 show a sensible increase in plasma cortisol concentration also at T0, immediately after the administration of the drug, than they reach the peak in plasma cortisol concentration 20 minutes after disbudding. Several physiological or ethological factors may explain this effect. Alpha-adrenergic agonists reduce the tonic activity of the baroreflex, decreasing arterial pressure and causing bradycardia (Campbell et al., 1979; Brest, 1980) and reduce tissue oxygenation (Hodgson et al., 2002). This may be a cause of distress to animals. But xylazine also causes muscle relaxation limiting the ability of the animal to react to human proximity and contact. For this reasons it is difficult to distinguish between the real increase in plasma cortisol concentration due to the pain induced distress procedure and the increase due to the effects of xylazine. (Stilwell et al. 2010). In this case plasma cortisol concentration seems to be not a good indicator of pain.

Animals belonging to group 5 show an initial increase in plasma cortisol concentration immediately after disbudding and reach the peak of plasma cortisol concentration 20 minutes after disbudding (37,7 nM/L). The initial increase in plasma cortisol concentration, even if lower than group 3, and the high peak at T1 can be due to the effects of this alpha2-agonist to the baroreflex, inducing bradycardia and reducing tissue oxygenation (Campbell et al., 1979; Brest, 1980, Hodgson et al., 2002). Animals belonging to group 6 have a similar trend of group 2, but with values slightly higher respect group 2. This can be also due to pharmacological properties of dexmedetomidine and to an ethological aspect: animals feel stressed if they feel handled without the possibility to react, as observed by Stilwell et al. as regards xylazine.

Statistical analysis

The statistical analysis shows that the baseline, T4 and T5 have no significant differences among groups.

There are significant differences if we compare T0 among groups. Plasma cortisol concentration in group 3 is statistically significant if compared to groups 1, 2, 5 and 6. As a matter of fact at time 0, group 3 show an increase in plasma cortisol concentration due to the features of xylazine, that causes stress. Animals of group 4 have no statistically significant differences with group 3 because these animals, too, show an increase in plasma cortisol concentration, even if less marked than group 3.

At T0 there is another important difference between group 2 and 6. In group 6 plasma cortisol concentration increase already immediately after injection. Animals of this group are more stressed than animals of group 2, even if they are treated with dexmedetomidine. This can explain that the administration itself of

dexmedetomidine cause an increase in plasma cortisol concentration, due to the features of alpha2-agonists. Of course these effects are less marked than for xylazine.
At T1 it is important to evaluate that plasma cortisol concentration increases in a statistically significant way in group 1 respect group 2 and 6. This confirm that true disbudding is much more painful than simulated disbudding. The peak in plasma cortisol concentration of animals of group 2 is only due to stress induced by manipulation and in animals of group 6 is due to manipulation stress and features of dexmedetomidine. At T1 there are no statistically significant differences between animals of group 1 and animals treated with xylazine (group 3 and 4), because this drug causes a marked increase in plasma cortisol concentration for its effect on heart, breath and muscle.
At T2 there are no significant differences among groups except that for group 2 compared to group 4. Despite the calves belonging to these groups undergo simulated disbudding, group 4 show a higher level of plasma cortisol concentration, always due to xylazine properties.
At time 3 the level of plasma cortisol concentration of group 3 is statistically low if compared to group 4 and 5. The increase in heart rate and blood pressure due to pain could help to eliminate xylazine from tissue and consequently its effects on the increase in plasma cortisol concentration. At this time animals belonging to group 5 show still a high level of serum plasma cortisol. This means that dexmedetomidine has poor analgesic power because pain induced distress is kept for long time after disbudding.
Statistically there are some significant differences inside groups.
Group 1. In group 1 T1 has significant differences if compared to every other time. This is the peak in cortisol plasma concentration and it appears 20 minutes after the disbudding, which is of course a painful and stressful procedure.
Immediately after disbudding (T0) and at T3, plasma cortisol concentration is similar to baseline. At T4 and T5 values of plasma cortisol concentration have not yet been normalized.
Group 2. In this group cortisol plasma concentration reaches its peak at T1 because of the procedure, but the values are high at T4 and T5 if compared to the baseline. This can be due to stress induced by handling for blood collection.
Group 3. In this group it is important to consider that at T0 and T1 plasma cortisol concentration reaches high significant levels if compared to baseline and to T2, T3, T4 and T5. This confirm that xylazine increases plasma cortisol concentration immediately after the administration, but one hour later plasma cortisol concentration has already normalized.
Group 4. In this group baseline values show significant difference with every other time. It means that the only administration of xylazine increases plasma cortisol concentration. Cortisol keeps high even after 4 hours from the

administration. Simulated disbudding does not increase blood pressure and heart rate, so that it is difficult to eliminate xylazine and its effects.
Group 5. In group 5 baseline values show significant differences with every other time. This trend confirm that dexmedetomidine has poor analgesic features and that cortisol plasma concentration is always too high.
Group 6. In group 6 plasma cortisol concentration shows no significant differences among times. In general, plasma cortisol concentration is higher if compared with animals belonging to group 2, that could mean that the administration of dexmedetomidine increase cortisol in blood.

Plasma concentration of substance P

The Substance P immunoassay has not yet been validated for bovine plasma. Consequently the first objective of our work was to perform a standardization of the method.
We prepared samples for a 96-well plate (Cayman Chemical Company - Substance P EIA Kit). Some samples were purified by a cold spike procedure, other samples were used without being purified.

Purification procedure. 0,8 mL of plasma sample was transferred into separate 13 × 100 mm glass culture tubes and acidified by addition of an equivalent volume of 1.0% aqueous TFA followed by vortexing at medium intensity. Acidified samples were centrifuged at 3,000 × *g* for 10 minutes to separate precipitate from plasma. Concurrently, a 24-port vacuum manifold was used to prepare solid-phase extraction cartridges for use. Equilibration of the solid phase extraction cartridges was achieved by successive washings with 1 mL of high-performance liquid chromatography–grade methanols containing 0.1% TFA (1 mL) and distilled water containing 0.1% TFA (1 mL). After cartridges were loaded with the acidified samples, each was washed 6 times with 3 mL aliquots of distilled water containing 0.1% TFA. The Substance P-containing fractions were then collected during elution by use of 3 mL of methanol containing 0.1% TFA. Eluents were evaporated to dryness by use of a vacuum centrifuge under nitrogen gas at 37 °C. Dry samples were stored at –20 °C prior to competitive immunoassay, which was performed within 12 to 16 hours after extraction.

Standardization trial

We analyzed a not-purified plasma of a healthy calf. It was compared to the same plasma after the purification procedure, carried out in duplicate. The 2 purified samples gave the same result, so we found that purification procedure gave repeatable results.

The first parameter to perform was the volume of the eluent (methanol containing 0.1% TFA) necessary to obtain the Substance P-containing fractions during the purification procedure. We purified 2 samples by using 3 mL of methanol, than the same samples were eluted again by using 3 mL more and other 3 mL collected in different tubes. The result we obtained is that 3 mL of methanol containing 0.1% TFA is enough to get all the substance P into the tube. As a matter of fact the second and the third elutions did not residual substance P.
The cold spike purification permits to find the more reliable concentration of substance P. Without purification, values are much lower than after purification and results are not repeatable.
Then we tested some plasma samples diluted with the EIA Buffer. The same samples were purified and not-purified. The first proportion was 50% plasma and 50% EIA Buffer, than 25% plasma and 75% EIA buffer. Purified samples gave more reliable values than not purified ones, and proportions were maintained.
Other samples were obtained by adding known concentrations of substance P to the plasma of the healthy calf without purification and after purification. Concentrations were 250 pg of substance P, 125 pg and 62,5 pg.
Then we prepared samples with EIA buffer and known concentrations of substance P: 250 pg, 125 pg and 62,5 pg. These values were not reliable maybe for a manufacturing defect of the substance P EIA standard we added.
We prepared a 96-well plate (Cayman Chemical Company – Substance P EIA Kit) adding 50 nL of each sample per well in duplicate The plate contained 2 blanks, 2 non-specific binding wells, 2 maximum binding wells and an eight point standard curve run in duplicate. We followed the entire procedures. After the overnight period, we emptied the wells and rinsed 5 times with Wash Buffer. We added 200 mL of Ellman's Reagent to each well, and we added 5 nL of tracer to the Total Activity wells. We covered the plate with plastic film and placed it in the dark room. The Substance P EIA Kit instruction provided to wait from 90 to 120 minutes before reading the plate, so we read the plate both after 90 minutes and after 120 minutes.
The results were extremely different. In general, reading the plate after 90 minutes, the plasma concentration of substance P resulted double than after 120 minutes. The gap of time (30 minutes) to read the plate and indicated as safety in the instructions of the EIA kit, in practice give totally different results. In 30 minutes the concentration of substance P it decreases by half. This resulted to be a manufacturing defect of the plate.
During the standardization of the method we found a defect in in the performance of the standard curve. We followed the procedure for the preparation of the essay, but the standard curve was wrong.

We reconstituted the substance P EIA standard with 2 mL of EIA Buffer. The concentration of this solution was 5 ng/mL (the bulk standard). We labeled 8 clean test tubes from 1 to 8. We pipetted 900 μL of EIA Buffer to tube 1 and 500 μL of EIA Buffer to tubes 2 from 8. We transferred 100 μL of the bulk standard (5 ng/ml) to tube 1 and vortex. We serially diluted the standard by removing 500 μL from tube 2 and placed it into tube 3 and vortex. We repeated this process for tubes from 4 to 8 and we used the diluted standards immediately.
Despite that the standard curve was wrong. It is probably due to a manufacturing defect of the substance P EIA standard of the EIA kit.
Coetzee et al., in 2008, have been the first researchers to use substance P as an indicator of pain in cattle undergoing castration and simulated castration. They found that on average the baseline value of substance P in cattle was 500 pg/mL with a peak of almost 1000 pg/mL.
In human medicine plasma levels of substance P determined by EIA, after C-18 SPE purification, ranges between 5-115 pg/mL with a mean of 38 pg/mL (Fehder et al. 1998). These values are similar to those obtained by RIA analysis of substance P in plasma of healthy adults (Pernow et al. 1983).
EIA kits, in general, work in a range of 3.9 to 500 pg/mL. This range of values is similar to those we found.
In conclusion we can affirm that, right now, the purification method is standardized, with reliable and repeatable results, while the analysis of plasma concentration of substance P on plasma has to be performed.

Discussion

Plasma concentration of cortisol can give important information about pain and distress. Probably plasma concentration of substance P is a better indicator of pain if compared to cortisol concentration because it is more specific for pain and it is not influenced by stress or drugs administration. Despite that the methods to measure substance P, in Veterinary Medicine, have still to be performed. In this research we evaluated important findings as regards the cold spike purification procedure, that is necessary and it is the base to perform the Elisa Immunocompetitive Assay. The substance P EIA kits nowadays available show some problems and are to be performed.

In the future our purpose is to improve the EIA methods to measure substance P in cattle. Substance P can be a very good indicator of pain and consequently of Animal Welfare. As a matter of facts we suppose that these results can be very useful also for the new analgesic drugs that will be produced. The suppression of the production or of the perception of substance P can be the purpose to reach for many analgesic treatments.

Plasma cortisol concentration has not been an useful method to compare the analgesic features of the 2 alpha 2agonists xylazine and dexmedetomidine. In fact they both act on the plasma concentration of cortisol, increasing it because of their effects on baroreflex and on muscles.

Animals belonging to groups undergoing the administration of xylazine show a sensible increase in plasma cortisol concentration immediately after the administration of the drug. Several physiological or ethological factors may explain this effect. Alpha-adrenergic agonists reduce the tonic activity of the baroreflex, decreasing arterial pressure and causing bradycardia (Campbell et al., 1979; Brest, 1980) and reduce tissue oxygenation (Hodgson et al., 2002). This may be a cause of distress to animals. But xylazine also causes muscle relaxation limiting the ability of the animal to react to human proximity and contact. For this reasons, after the administration of xylazine, plasma cortisol concentration increases not only for pain induced distress but also for the characteristic of the drug.

Dexmedetomidine increases plasma concentration of cortisol, but less than xylazine. Dexmedetomidine has less effects on baroreflex, so the increase in plasma cortisol concentration is limited.

Dexmedetomidine, of course, has less negative effects than xylazine on respiratory and cardiocircular system, but has poor analgesic and sedative effects.

It is hopeful to complete as soon as possible the method to measure substance P because it can guarantee a more reliable evaluation of pain in cattle, even after the administration of alpha2-agonists.

Summary

Disbudding is a routine painful procedures carried out on cattle to facilitate management (Gottardo et al. 2011, Duffield 2008, Laine et al. 2007, Stafford et al. 2005 e 2003, Marshall, 1977; Vowles, 1976).
To measure the pain-induced distress caused by dehorning or disbudding plasma concentration of cortisol have been used more frequently than any other parameter (Boandl et al., 1989; Taschke and Folsch, 1993; Wohlt et al., 1994; Cooper et al., 1995; Morisse et al., 1995; Petrie et al., 1996; Sylvester et al., 1998; McMeekan et al., 1997, 1998; Graf and Senn, 1999; Grondahl-Nielsen et al., 1999; Sutherland et al., 2002), but Stilwell et al. in 2010 confirm that cortisol is not a good indicator of pain.
Substance P is a neurotransmitter of pain used for the first time in cattle in 2008 by Coetzee et al. who found that substance P is a better indicator of pain than cortisol in calves after castration or simulated castration.
On these basis we hypothesized that SP could potentially be a more specific measure of pain in cattle undergoing disbudding than would the plasma cortisol response, but the methods to measure its concentration in cattle plasma is still to set up, so we are improving it.
We studied the effects of true and simulated disbudding on plasma concentration of cortisol in calves undergoing 2 different alpha2-agonists: xylazine and dexmedetomidine. Plasma cortisol concentration has not been an useful method to compare the analgesic features of the 2 alpha 2agonists xylazine and dexmedetomidine. In fact they both act on the plasma concentration of cortisol, increasing it because of their effects on baroreflex and on muscles.
In Veterinary Medicine dexmedetomidine is registered only for its utilization on cats and dogs and it is appreciated for its minimal side effects on the respiratory tract, for its remarkable sedative and anxiolytic properties and for the possibility it can be administered by continuous infusion. The utilization of dexmedetomidine in cattle has never been researched right now.

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Ministero della Salute- PNR 2007 Relazione finale. 2008

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