Study of cow subclinical hypocalcemia and development of new tools for its diagnostic and prevention

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Resum

Les vaques de llet sofreixen pèrdues de calci (Ca) quan comença la lactació i poden veure's afectades per la hipocalcèmia clínica (sèrum Ca < 6mg/dL) o subclínica (Ca < 8.5mg/dL). Donat que la incidència clínica és del 5%, el problema més rellevant es troba en els casos subclínics (SCHC) perquè tenen una prevalença més elevada. Aquests casos no poden ser tractats degut a la falta d'eines de diagnosi. Per tal d'estudiar l'impacte, les conseqüències i la regulació de SCHC, i per desenvolupar estratègies de diagnòstic i de prevenció, es van dur a terme quatre estudis. Al primer estudi, es va avaluar l'associació de SCHC amb vàries malalties del peripart. El 75% de les vaques tenien SCHC. El desplaçament d'abomàs, la ketosi, la retenció de placenta i la metritis van ser més propenses a trobar-se en vaques amb SCHC. Les vaques afectades tenien una producció de llet més elevada. El zel apareix abans en vaques normocalcèmiques. A més, la gravetat de les diferents malalties està relacionada amb la intensitat de la SCHC. Per entendre els mecanismes involucrats, un segon estudi es va dur a terme per clarificar els papers de la calcitonina a l'inici de la malaltia i a la prevenció de la hipocalcèmia sota acidosi metabòlica. Una pujada de calcitonina en vaques amb SCHC greu després de parir perjudica la recuperació del Ca sanguini perquè la resposta del receptor de la PTH (PTHR) no és suficient com per activar la vitamina D i compensar l'efecte de la calcitonina. L'acidosi metabòlica prevé la hipocalcèmia perquè l'expressió de PTHR s'incrementa al ronyó. A més, l'activitat de la calcitonina es veu perjudicada a pHs baixos i això incrementa el rol hipercalcèmic de la PTH. Basat en aquest fet, al tercer estudi es va intentar una estratègia per prevenir la hipocalcèmia basada en immunització passiva contra calcitonina. Anticossos policlonals van neutralitzar la calcitonina *in vitro* i a un model de rata, augmentant la concentració de Ca en sang. Un mètode assequible per produir en massa va ser derivat d'una naïve phage library de ScFv. El ScFv B10 va reconèixer i neutralitzar la calcitonina *in vitro*, però no va afectar els nivells de Ca en sang quan es va administrar en vedells o vaques. Més estudis seran necessaris per entendre les dificultats de l'estratègia proposada. Un sistema de diagnòstic seria molt útil per identificar i tractar vaques hipocalcèmiques. En el quart i últim estudi, es va desenvolupar un sistema analític semiautomàtic i portable basat en transistors de camp ió-selectius amb membranes fotocurables selectives per ions de Ca^{2+}. Aquest sensor determina concentracions de Ca en sang de boví de forma ràpida i fiable i pot ser aplicat en mesures en vaques al camp.

Summary

Dairy cows suffer blood calcium (Ca) losses as lactation begins and might be affected by hypocalcemia in its clinical (serum Ca < 6mg/dL) or subclinical state (serum Ca < 8.5mg/dL). Since clinical incidence is only about 5%, the most relevant problem concerns subclinical cases (SCHC) because of a higher prevalence. These cases cannot be treated due to the lack of diagnostic tools. In order to study the impact, consequences and regulation of SCHC and to develop preventive and diagnostic strategies, four studies were conducted. In the first study, the association of SCHC with several periparturient diseases was evaluated. Seventy five percent of cows incurred SCHC. Displaced abomasum, ketosis, retained placenta, and metritis were more likely to happen in cows with SCHC. Affected cows had a greater milk production. Normocalcemic cows showed their first heat sooner. Also, the severity of SCHC is related to the severity of the different periparturient diseases. In order to understand the exact mechanisms involved, a second study was performed to clarify the potential roles of calcitonin in the onset of SCHC and in the prevention of hypocalcemia under metabolic acidosis. A calcitonin rise in severe SHCH cows after calving impairs the recovery of blood Ca because PTH receptor (PTHR) response is not sufficient to activate vitamin D and compensate the calcitonin effect. Metabolic acidosis prevents hypocalcemia because the expression of PTHR is up-regulated in kidney. Moreover, an impairment of calcitonin activity at low pH enhances the hypercalcemic role of PTH. Based on this calcitonin role, in the third study an approach to prevent hypocalcemia through passive immunization against calcitonin was tested. Polyclonal antibodies neutralized calcitonin *in vitro* and in a rat model, raising the blood Ca concentration. An affordable method of mass-production was designed from a naïve ScFv phage library. The ScFv B10 recognized and neutralized calcitonin *in vitro*, but it did not affect blood Ca levels when administered to cattle requiring further research to understand the main difficulties of the proposed strategy. A diagnostic system would be very useful to identify and treat hypocalcemic cows. In the fourth and last study we developed a portable semiautomatic analytical system based on ion-selective field effect transistors with Ca^{2+} ion selective photocurable membranes. This sensor determines bovine serum calcium concentration in a reliable and fast way and can be applied in the field in cow-side measurements.

List of abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D3
$25(OH)_2D$-1α	25-hydroxyvitamin D 1α-hydroxylase enzyme
AI	Artificial insemination
BSA	Bovine serum albumin
BW	Body Weight
Ca	Calcium
CALC	Calcitonin
CALCR	Calcitonin receptor
cAMP	Cyclic adenosine monophosphate
CI	Confidence interval
CP	Crude protein
DCAD	Dietary cation-anion difference
DHI	Di-(n-hexyl)-itaconate
DIM	Days in milk
DM	Dry matter
DOS	Dioctyl sebacate
EDTA	Ethylenediaminetetraacetic acid
EE	Ether Extract (crude fat)
EIA	Enzyme Immunoassay
Fab	Fragment antigen-binding
HFBuA	Hexafluorobutyl acrylate
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
ISFET	Ion-selective field effect transistor
K-TpClPB	Potassium tetrakis(p-chlorophenyl)borate
LDA	Left displaced abomasum
NDF	Neutral detergent fiber
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween

PMMA	Polymethylmethacrylate
PTH	Parathyroid hormone
PTHR	Parathyroid hormone receptor
qPCR	Quantitative real time PCR
RIA	Radioimmuno assay
SCC	Somatic cell count
SCHC	Subclinical hypocalcemia
SEM	Standard error mean
TBS	Tris buffered saline
TMB	Tetramethylbenzidine

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Chapter I – Literature review

Block I CALCIUM IN MAMMALS

1. History of the biological importance of calcium

Sir Humphry Davy discovered calcium as an element in 1808. Soon after, he recognized its presence and central role in mammalian bones and other mineralized tissues such as teeth (Thomas *et al.,* 2008). Almost 75 years later, the british physiologist Sidney Ringer was interested in the effects of cations on frog-heart muscle (Ringer, 1882). He suspended them in a saline medium using London tap water instead of distilled water and saw them contract. When he replaced tap water with distilled water, the beating of the hearts became weaker until it stopped. Ringer found that calcium ions, present in the tap water, distributed in millimolar concentrations, were necessary for muscle contraction and tissue survival (Ringer, 1883). Calcium went from a strict structural element to a messenger: it carried the signal that initiated heart contraction.

In the coming years, Ringer and Sainsbury (1894) discovered that calcium was involved in the fertilization of eggs and development of the tadpole. Locke (1894) and Overton (1904) found that Ca^{2+} was critical for impulse transmission between nerve and muscle. Heilbrunn and Wiercinski (1947) injected minimum amounts of calcium to the cut ends of frog muscle fibers. After seeing them contract, they concluded that calcium had diffused from the cut ends to the internal contractile elements. Bozler (1954) found that the removal of calcium by EDTA relaxed muscle fibers. Anne-Marie Weber (1959) discovered that Ca^{2+} ions activate actomyosin after binding to myofibrils. Actomyosin is a complex protein in skeletal muscle that is formed by actin and myosin which, when stimulated, shortens to cause muscle contraction (Figure 1).

Today it is widely recognized that Ca2+ ions are central to a complex intracellular messenger system that is mediating a wide range of biological processes: bone formation, muscle contraction, secretion, glycolysis and gluconeogenesis,

ion transport, cell division, growth, enzyme co-factor, stabilization of membrane potentials, blood coagulation and stimulus-response coupling (Forsén and Kördel, 1994). The binding of Ca2+ to proteins alters their structures and, thus, its functions.

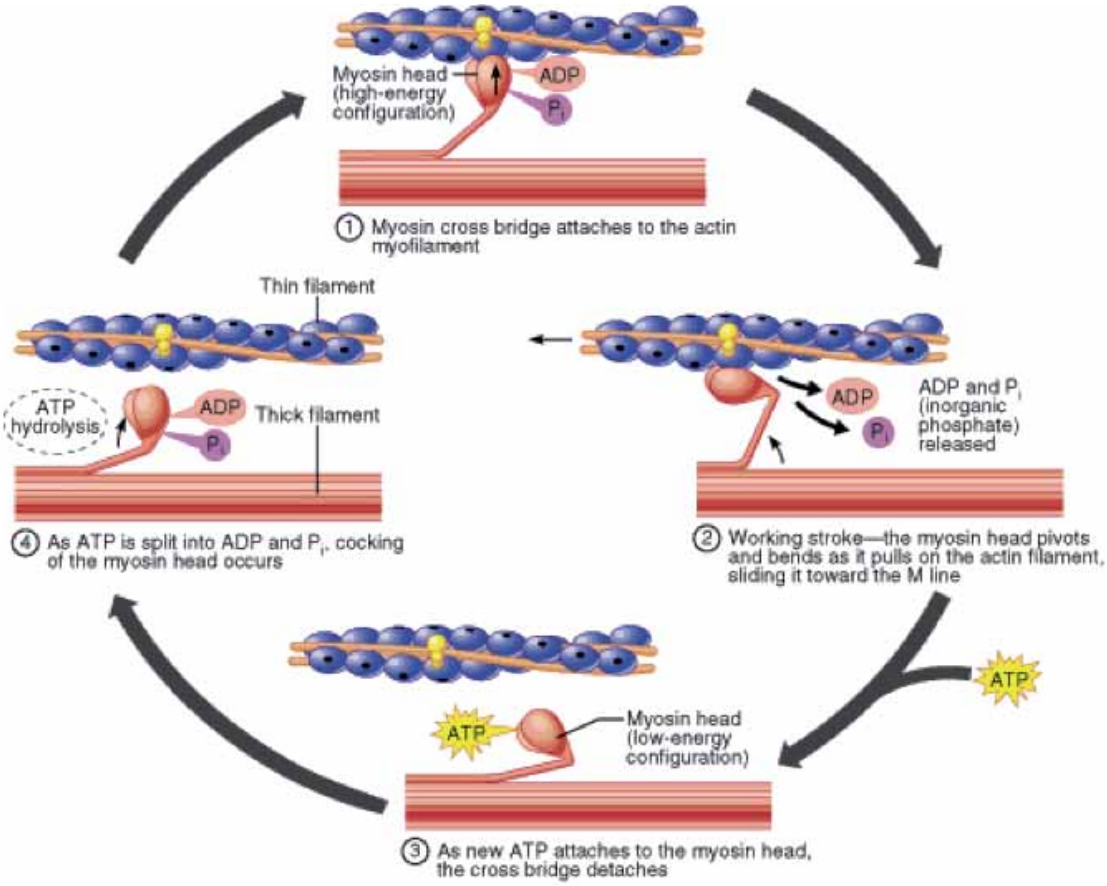

Figure 1. Myosin heads bind to actin, thus becoming actomyosin in the sliding filament model of skeletal muscle (adapted from Human Anatomy & Physiology, 2007).

Calcium is transferred to the newborn through the milk. At the onset of lactation, a large quantity of colostrum is produced. Colostrum is the pre-milk fluid produced by female mammals in the mammary glands just before they give birth, often called “first milk”. This first milk is crucial to the survival of the newborn. It contains a rich source of nutrients, immunoglobulins, vitamins and growth factors for the suckling. It also contains large amounts of calcium to rapidly calcificate the bones and teeth in the growing mammal neonate (Uruakpa *et al.,* 2002).

A 99% of the calcium in mammals resides in teeth and bone as hydroxy-apatite crystals. Of the remainder, approximately 1% is intracellular and a tiny fraction, less than 0.1%, is extracellular. It is this small extracellular fraction of Ca2+ that is homeostatically regulated by hormones and that determines calcium balance within the body (Nussey and Whitehead, 2001).

2. Calcium homeostasis

Three main calcium-regulating hormones regulate serum calcium levels: parathyroid hormone (PTH), vitamin D, and calcitonin. This regulation is done through their specific effects on the intestine, kidneys, and skeleton (Figure 2) (El-Samad *et al.,* 2002).

Parathyroid hormone (PTH) is the main hormone responsible for increasing blood calcium. It is released in response to low blood calcium (DeGaris and Lean, 2008) and secreted by the parathyroid gland. PTH receptors are located on osteoblasts and in kidney. Osteoblasts stimulate osteoclasts to resorb bone by releasing enzymes onto the surface causing a local reduction of pH at the surface of bone, helping for mineral dissolution and increasing blood calcium levels (Block, 1994; Horst *et al.,* 2003). PTH is also responsible for renal reabsorption of calcium by a larger number of PTH receptors being expressed in kidney (Horst *et al.,* 1994).

As a consequence of the PTH increase, 25-hydroxyvitamin D 1α-hydroxylase enzyme increases in kidney (Wasserman and Fullmer, 1995). This enzyme is responsible for the conversion of 25-hydroxycholecalciferol to its active form, 1,25 dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) in kidney (Goff, 2008). Active vitamin D is responsible for enhanced calcium absorption from the intestines (Horst, 1986), exerting its biological effect at the level of transcription by interacting with its intracellular receptor (VDR) (Pike, 1985). It also stimulates bone calcium resorption in concert with PTH (Horst *et al.,* 2005).

The calcitonin hormone, with an opposite effect to PTH and $1,25(OH)_2D_3$, inhibits the reabsorption of Ca from the bones and increases blood calcium losses by urinary

excretion (Murray *et al.,* 2008). Calcitonin secretion from the thyroid gland C-cells increases in response to hypercalcemia (Austin and Heath, 1981; Findlay and Sexton, 2004) and in response to food intake, which is one condition where calcium levels may increase (Roos *et al.,* 1980).

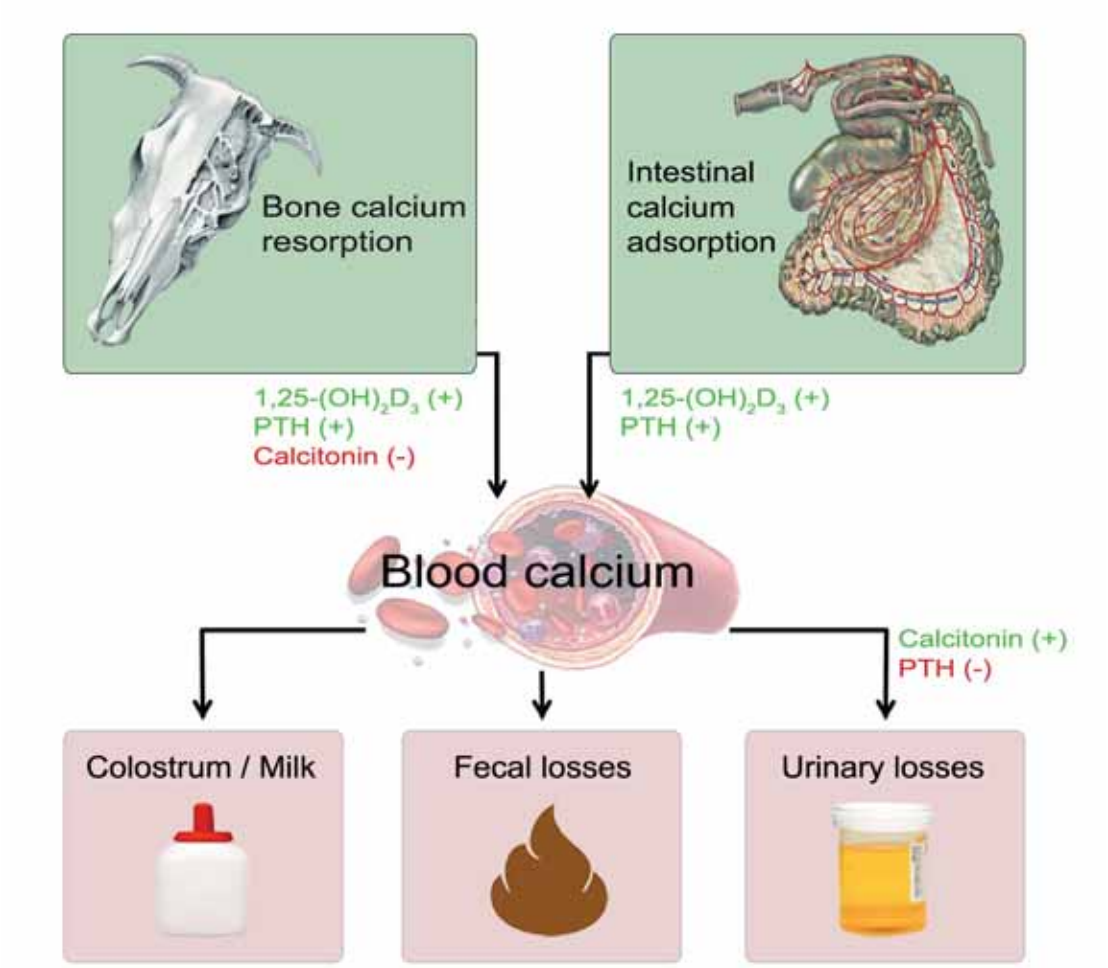

Figure 2. Calcium homeostasis in the postpartum cow. Calcitonin, PTH and $1,25(OH)_2D_3$ maintain blood calcium with calcium from the bone and intestine. Calcium losses in early lactation mainly occur in colostrum or milk. To a lesser extent, through fecal or urinary elimination, the latter being controlled by calcitonin and PTH. The symbols in parentheses indicate the biological effect exerted by hormones on the target tissues.

Block II BOVINE CLINICAL AND SUBCLINICAL HYPOCALCEMIA

1. Etiology

A regular dairy cow is bred during the 3rd month of lactation and gives birth about 8 weeks after their previous lactation ceases (Horst *et al.,* 2005). Unlike other mammals, the dairy cow lactates for 10 months. During the final two months of pregnancy, the cows are not milked anymore and they are forced to enter into a dry period to regenerate the senescent cells of mammary gland and improve the milk production at the following lactation. During the dry period, the calcium needs are low despite the calcium invested in the development of the fetus. The calcium losses going into the fetal skeleton are ~80mg/$kg^{3/4}$ at this stage. However at the onset of lactation, very high amounts of calcium are secreted into colostrum and the calcium needs go up to ~500mg/$kg^{3/4}$ (Horst *et al.,* 2005). A forced evolution towards a greater milk production through genetics and management has led the cows to huge calcium requirements at the onset of lactation (Capuco and Akers, 1999).

The colostrum of a lactating dairy cow has a calcium concentration of 62-75 mM, while milk has 25-30 mM. In contrast, blood calcium concentration is around 2.5 mM, representing a 10 and 30-fold increase compared to milk and colostrum, respectively. Having the cow a blood calcium pool of 2-4 grams, it has to be depleted several times per day to meet the demands of milk production (Horst *et al.,* 2005). The blood calcium needs could be fulfilled through absorption in the intestine and bone resorption, but the main problem is that these mechanisms dedicated to recover calcium levels are almost inactive at the start of lactation (Horst *et al.,* 2005). Most cows cannot replenish the blood calcium pool in time and the total blood calcium decreases. This situation dramatically affects the animal's homeostatic ability to maintain blood calcium levels (Goff, 2008). If the loss of calcium is not compensated properly, the cow is at risk of suffering from a metabolic disease called hypocalcemia or parturient paresis, with a high incidence in cattle. It can also be called milk fever, but fever is a misnomer, as the temperature of the body will usually go below normal.

The puerperal paresis increases significantly in animals starting their third or later lactations (Curtis *et al.,* 1984). This is because they have higher milk production and therefore a greater calcium demand. Also, calcium mobilization from bones is slower in older than younger cows due to fewer active osteoblasts, fewer receptors for 1,25 dihydroxy-vitamin D_3 and because of an increase in vitamin D inactivating enzymes such as C-24 hydroxylase (Horst *et al.,* 1994; Johnson *et al.,* 1995). Even with young calves, when lactation starts there is a high demand of calcium and the homeostatic mechanisms of the cow are challenged and can fail (Horst *et al.,* 1997).

Hypocalcemia can be a clinical (total blood calcium below 6mg/dL) or subclinical (below 8mg/dL) disease (Goff, 2008). It can have its clinical manifestation between 24 hours pre-natal until 72 hours after delivery (Shappell *et al.,* 1987). The early clinical signs of hypocalcemia are as follows: languidness, cold ears, dry nose and uncoordinated walking. Just prior to or after calving, the cow shows symptoms including loss of appetite, reduced body temperature and anxiety (Houe *et al.,* 2001). As the illness advances, the cow is unable to stand, followed by a progressive lack of awareness. The heartbeat becomes almost imperceptible and the heart rate increases from 60-70 to 120 beats per minute. Milk secretion fails, digestion processes are suppressed and the stomach bloats, there is urinary retention and the animal gets constipated. Breathing is slow and body temperature keeps on decreasing. If animals are not treated, they die in 2 or 3 days of prolonged coma or heart failure (Horst *et al.,* 2005; Murray *et al.,* 2008).

Subclinical hypocalcemia also occurs in the puerperal period and can be present for the first 40 days after a normal delivery in cows with high milk production (Kamgarpour *et al.,* 1999). In these cases, the alterations of blood calcium levels are lower, but also dramatically affect the health and welfare of animals. There are no clinical signs of the hypocalcemia itself, but food intake decreases and muscle contraction is reduced, affecting ruminal, intestinal and abomasal mobility (Goff, 2008; Clarck *et al.,* 2001).

Regarding milk production, there are different studies with different conclusions. Rajala-Schultz *et al.,* (1999) reported that cows with milk fever had a higher milk

yield in average than healthy cows, but later on the lactation, they had milk production losses between weeks 4 to 6. In contrast, Østergaard and Larsen (2000) found that a low concentration of calcium in blood was not affecting milk yield. This is a fact difficult to state, because cows with milk fever usually are high yielding cows. This fact plus being the onset of the disease at early lactation, it is difficult to show the milk reducing effect of the periparturient paresis. As for subclinical hypocalcemia, Jawor *et al.,* (2012) compared normocalcemic cows with subclinical hypocalcemic cows and found that milk yield was greater among third-lactation cows with subclinical hypocalcemia.

2. Relationship with other metabolic and infectious diseases

Clinical hypocalcemia is associated with an increased incidence of displaced abomasum (Chapinal *et al.,* 2012; Seifi *et al.,* 2011), uterine prolapse and retention of fetal membranes (Risco *et al.,* 1984), likely mediated by the effects of low blood Ca suppressing smooth muscle contractions (Hansen *et al.,* 2003). Cows with puerperal hypocalcemia have abnormally long intervals between calving and first postpartum ovulation, and a longer luteal phase in the next reproductive cycle (Murray *et al.,* 2008). Cows with parturient paresis also exhibit a greater decrease in dry matter intake than cows without milk fever (Goff and Horst, 1997a). Goff and Horst (1997b) speculated that the decline in intake also results in a less full rumen which floats above the floor of the abdomen and reduced depth of the rumen mat. The risk of ruminal dysfunction, displaced abomasum and ketosis triples (Massey *et al.,* 1993; DeGaris and Lean, 2008). Moreover, the proportion of neutrophils with phagocytic activity was lower in cows suffering from this disease (Ducusin *et al.,* 2003) and there was an impairing in the activity of mononuclear blood cells (Kimura *et al.,* 2006). Through this reduction in the immune cell functions, hypocalcemia has also been linked to periparturient diseases such as retained placenta (Melendez *et al.,* 2004) and mastitis (Curtis *et al.,* 1983).

In less severe cases of calcium deficiencies such as subclinical hypocalcemia, muscle contraction is also reduced, increasing the risk of abomasal displacement and ketosis

(Goff, 2008; Clarck *et al.,* 2001). Ribeiro *et al.* (2013) reported that early-lactation cows with low serum Ca in the first 7 DIM had an increased incidence of ketosis. The closure of the teat sphincters after milking is also affected; the cows suffer more infections and have an increased incidence of mastitis (Martinez *et al.,* 2012). It has also been shown to adversely affect cell immune response. In this context there is a link between low levels of calcium in blood and low levels of intracellular calcium in polymorphonuclear inflammatory cells, which decreases phagocytosis processes and increases the risk of mastitis and metritis of the exposed animals (Ducusin *et al.,* 2003). Martinez *et al.* (2012) also support this relationship between metritis and subclinical hypocalcemia. In contrast, Chapinal *et al.* (2012) noted that subclinical hypocalcemia in the first week postpartum was not associated with retained placenta or metritis, but was associated with an increased risk of developing a displaced abomasum. Massey *et al.* (1993) found that the risk of left abomasum displacement was almost 5 times higher among cows with subclinical hypocalcemia. However, LeBlanc *et al.* (2005) reported no relationship between serum calcium concentrations and the subsequent incidence of abomasum displacement. Also, the size of ovulatory follicles and the corpus luteum is reduced when the next reproductive cycle is resumed (Kamgarpour *et al.,* 1999). Jawor *et al.* (2012) found that dairy cattle with subclinical hypocalcemia (serum calcium ≤1.8mmol/L) during the 24-h period following calving did not exhibit production or behavioral changes associated with poor health. It also reduces intake and affects the energy metabolism in nonpregnant, nonlactating dairy cows (Martinez, 2014).

In general, in these animals with clinical and subclinical hypocalcemia, productivity may be reduced, the susceptibility to other metabolic or infectious diseases is increased and animal welfare is compromised (Goff, 2008; Murray *et al.,* 2008). Therefore, although there are treatments to prevent the death of the animal, subclinical cases are not detected, so it is much more important to invest in prevention instead of treatment.

3. Treatment

The treatment can be applied only when clinical hypocalcemia is detected. It is usually an intravenous injection of calcium (typically from 8 to 10 grams of calcium) to prevent the death of the animal until calcium homeostatic mechanisms adapt to the new situation (Horst *et al.,* 1997). The most preferred way to administrate this amount of calcium is an infusion of 23% calcium borogluconate (Figure 3). However, it is very important to avoid the injection of calcium in a short period of time, because very high blood calcium concentrations can be reached (>25mg/dL), and this can lead to cardiac arrest (Goff, 2008). Nevertheless, 25% of the animals relapse 24 hours after receiving the initial infusion of calcium and require additional treatments (Horst *et al.,* 2005).

Figure 3. Cow being administered calcium borogluconate solution warmed to body temperature by slow intravenous injection into the jugular vein.

Calcium salts may also be administered subcutaneously, but absorption is variable and often blood flow in the periphery is compromised. In addition, taking into account the concentrations of the preparations, the maximum amount that can be injected subcutaneously is 1-1.5 g and 0.5-1 g via intramuscular; implying the need to perform several injections (Goff, 2008). Although the applied treatment overcomes the poor condition of the animal, the consequences that remain are great, and productivity in the subsequent lactation is affected. The most frequent causes of treatment failure are overdosing or underdosing, inadequate management of the animal and lack of clinical care.

4. Economical implications

In the last 30 years there have been some field studies indicating the incidence of hypocalcemia. In its clinical form, it is affecting 3.45% of North American cows. It reaches 6.17% in Europe and 3.5% in Australia (DeGaris and Lean, 2008). Taking subclinical cases into account, surveys conducted in the US show that while about 4% of the cows develop puerperal paresis each year, the incidence of subclinical hypocalcemia is 50% in the older cows (Goff, 2008).

Manuals as Nutrient Requirements of Dairy Cattle (2001) consider that virtually all animals from a farm producing milk cattle suffer clinical or subclinical hypocalcemia the first days after delivery.

The average productive life of a cow can be reduced 3.4 years with hypocalcemia (Horst *et al.,* 1997). Also, animals with high rates of milk production suffer a greater risk of being affected by puerperal paresis and subclinical hypocalcemia (DeGaris and Lean, 2008). With these data it can be affirmed that farms have large economic losses due to hypocalcemia. Costs can easily exceed 400 dollars per cow as estimated in 1996 (Horst *et al.,* 1997). This value is based on the direct costs associated with treatment of clinical cases and the estimated production losses of clinical and subclinical cases. Even if the cows are recovered from puerperal paresis and subclinical hypocalcemia, they are more likely to develop other diseases. Given the specialization of this sector in milk production, hypocalcemia can compromise the profitability of the farm. Avoiding this illness is a key factor not only in the animal welfare but also in the economy of the dairy industry.

5. Preventive strategies

Prevention of milk fever has been the focus of much research over the last several decades because of the direct and indirect effects it can have on cow performance. Although treatment with calcium solutions prevents the death of the animal in clinical cases, more than half of the animals suffer subclinical hypocalcemia and the risk for other metabolic and infectious diseases increases. Therefore, it directly

affects the profits for the producer and the average life of the animal, also compromising animal welfare. The last years of study have focused on finding new prevention strategies, but to date, none meets the needs of the industry, increasing its efforts into trying to prevent this disease. So far there have been several strategies to prevent puerperal paresis but none has been truly effective.

One of the things that can influence effectiveness of calcium homeostatic mechanisms is the blood acid-base balance. Acid-base balance can be manipulated by adjusting the dietary cation-anion difference (DCAD) in the feed. Cows can suffer from metabolic alkalosis, and it may be caused due to a diet supplemented with more cations than anions. Diets that generally apply to ruminants cause metabolic alkalosis, because all forages are rich in K^+ due to the usual agronomic practices. One of the main theories correlating metabolic alkalosis with the incidence of puerperal paresis and subclinical hypocalcemia is that high pH lowers the tissue response to PTH (Abu *et al.,* 1994). On the contrary, inducing metabolic acidosis by adding anionic salts to the diet creates a negative calcium balance by mechanisms that are not entirely clear. Block (1984) found that significantly fewer cows developed milk fever when fed diets containing greater concentrations of anions (Cl^- and S^- in particular) and less Na+. This method of prevention has been later supported by other researchers (Oetzel, 1996; Goff *et al.,* 1991). The function of PTH (Horst *et al.,* 2005) is recovered and prevents hypocalcemia at calving (Goff, 2006). The problem of adding anions in the diet such as Cl^- and $SO42^-$ is their low palatability (Horst *et al.,* 1997). An alternative to the addition of anions is the removal of cations such as K^+ (Goff, 2006), but it is important not reduce the cation levels below the animal's nutritional requirements. However, this task is difficult because, as stated before, all forages are rich in K^+. Formulating pre-partum feed, for a particular group of animals, taking into account all these parameters greatly hinders the management of a farm. Also, anionic diets are not always fully effective (Romo *et al.,* 1991).

A lot of effort has been put into adapting the diet to prevent hypocalcemia. The feeding of the cows prior to parturition can be involved in the periparturient paresis and hypocalcemia incidence. Specially, feeding high amounts of calcium to the cow before calving may accentuate the risk of hypocalcemia because the mechanisms for

calcium upregulation are inactive (Lean *et al.,* 2006). One strategy is based on reducing the intake of calcium prior to delivery to maintain homeostatic mechanisms active and to respond effectively when the puerperal hypocalcemia appears. Following this hypothesis it has been shown that a diet low in calcium, not even meeting the calcium requirements of the animal, is effective in preventing parturient paresis and stimulating the secretion of PTH and $1,25(OH)_2D_3$ before calving (Horst *et al.,* 2005; Goff, 2008). After delivery, the animals were maintained on a diet high in calcium to maintain normocalcemia. Despite these results, this strategy is very difficult to implement in the field because most of ruminant feed containing calcium concentrations are incompatible with the formulation of diets low in calcium (<20g/day). Some studies have proposed the use of clays such as zeolite and free fatty acids of longer chains, such as calcium scavengers (Goff, 2006). These agents work well with diets with Ca^{2+} concentrations below 50g/day, but they don't specifically bind calcium and compromise other nutritional components (Thilsing - Hansen *et al.,* 2002).

High volumes of calcium salts have been orally administered to promote the passive diffusion of calcium to the blood during the puerperal period. Traditionally, $CaCl_2$ solutions and gels that provide a concentrated source of calcium (36%) have been used. Giving these sources of calcium to the cows a few days before labor or even after delivery reduces the incidence of puerperal paresis and displaced abomasum (Oetzel, 1996). The disadvantage of these solutions is that they are very caustic and can cause mouth and gastrointestinal ulcers (Horst *et al.,* 2005). An excess of $CaCl_2$ oral administration can induce severe metabolic acidosis that results in loss of appetite, and intake is very important in this (Goff and Horst, 1993). An alternative product, which does not have these disadvantages, is calcium propionate. However, this calcium compound can only be found at a concentration of 21.5%, making it very difficult to administer orally because of the large volumes needed. For a better control of hypocalcemia, a dose is given at calving and another one is given 24 hours later. However the effect is limited and an increase in these doses may be toxic (Goff et al 1996; Melendez *et al.,* 2002).

The prevention of puerperal paresis was also attempted by the administration of

calcitropic hormones like 1,25$(OH)_2D_3$ (vitamin D_3 derivative) and parathyroid hormone (PTH), with the aim of promoting the mobilization of calcium reserves. Treatments with Vitamin D3 or 1-alpha-hydroxy-Vitamin D3 metabolites prevent puerperal paresis but, 10-14 days postpartum, animals develop hypocalcemia again and have other clinical symptoms of the disease (Horst *et al.*, 2003). This is due to the withdrawal of the treatment: when the hormones are not administered, the animal is unable to produce 1,25$(OH)_2D_3$ due to the feedback in the enzyme 1-alpha-hydroxylase and the high concentrations of vitamin D_3 metabolites in blood. In the search for new metabolites and analogues with a more active and lasting effect, a subcutaneous implant of 24F-1,25$(OH)_2D_3$ was tested, avoiding high concentrations of the compound in plasma. Although the results are positive, this compound has never been used beyond experimental phases (Goff *et al.,* 1988). Treatment with 1,25-dihydroxyvitamin-D and its analogs or with PTH before delivery may be helpful, but their effective doses are close to the toxic dose. It is very difficult to find the optimal timing of administration and these treatments are really expensive, resulting in non-viable preventive treatments (Clarck *et al.,* 2001).

Until now the tested preventive measures are difficult to implement, have low efficiency and can have side effects. Puerperal paresis and subclinical hypocalcemia have a great health and economic importance, so it is crucial to develop more effective preventive tools, easy to implant in the actual management systems and with lower economic costs.

6. Diagnosis and the potential of biosensors

The diagnosis of hypocalcemia ultimately depends on the clinical observations (Larsen *et al.,* 2001). Rectal temperature, surface temperature, muscle shivering and ataxia are the physical checks most often monitored. The obvious advantage of clinical observations is that diagnoses can be generated immediately (i.e., on the spot), but the disadvantage is that subclinical hypocalcemia, affecting the major number of animals, cannot be detected. Therefore, a need for a better diagnosis exists in order to have a tighter control of the blood calcium levels of the animals.

The use of certain blood parameters as indicators of the physiological, nutritional, metabolic and clinical status of farm animals can be an important point to take into account in the control of puerperal paresis. However, variables such as breed, age and number of lactation can have an influence on many blood parameters.

Efforts in the development of new types of biosensors that are small, relatively inexpensive, easy for non-scientists to operate, rapid and accurate, have been made to perform cowside diagnostics for both individual and herd-level medicine. So far, the “gold standard” cow-side is test for the diagnosis of subclinical ketosis.

The ability to measure serum electrolytes would aid in the field diagnosis of hypocalcemia. Bioluminescence, complexing agents with calcium-dependent light absorption, fluorescence or NMR spectra can measure concentrations of uncomplexed or “free” calcium (Forsén and Kördel, 1994). Unfortunately, none of these methods can be used in the field and no simple cowside diagnostic tests exist at this time. Hence, such blood analysis has remained primarily “in-house” at veterinary clinics, although large farms could also justify the equipment to carry out such analysis. The more recent development of portable blood analysis equipment, such as the VetScan i-STAT 1 (Abaxis, Union City, CA) and the Quick Test II (Midland Bio Products, Boone, IA) is beginning to bring limited blood assays for electrolytes and other substances to the field for rapid diagnosis by veterinarians.

Research in the development of new cow-side biosensors specially focused at measuring ionic or total blood calcium will be really relevant in the control of dairy cow hypocalcemia and periparturient diseases.

Block III PASSIVE IMMUNIZATION: REGULATING A METABOLIC DISEASE

1. Definition and applications

Being the tested prevention strategies hard to apply on the field, new strategies have to be contemplated and the administration of antibodies to regulate metabolic and physiological disorders offers a new field to be explored. Passive immunization is the administration of exogenously produced antibodies (polyclonal and monoclonal) (Dunman and Nesin, 2003). Introduced in 1890 to treat diphtheria (Wesselhoeft, 1936), it became the primary mode to control pathogens and infectious diseases, soon followed by vaccination (active immunization) and the use of antimicrobial chemotherapy such as antibiotics (Kelly *et al.,* 2001).

But antibodies can also bind to other molecules instead of just pathogens and toxins. The greatest impact of passive immunization has been in the field of human research, for example in rheumatoid arthritis (Taylor *et al.,* 2001; Dalum *et al.,* 1999), in anaphylactic shock (Heusser and Jardieu, 1997) and in cancer therapy (Huls et al, 1999; Scott and Welt, 1997). But the application of passive immunization in the therapy of farm animals is also demonstrated and supported by a large number of studies aimed primarily to immunoneutralization of hormones involved in reproduction and growth (Reeves *et al.,* 1989). Passive immunization enables the neutralization of regulatory molecules by exerting its effect in a more controlled way than an active immunization. The dose and the number of treatments are fully controllable and the effects are immediate and reversible. These antibodies are removed from the body in a period of a few weeks. One of these applications was the one discovered by Fitzgerald *et al.* in 1985. They demonstrated that passive immunization against luteinizing hormone (LH) increased circulating concentrations of follicle stimulating hormone (FSH) and the duration of estrus cycle and the ovulation rate. Immunoneutralization of inhibin increased FSH secretion stimulating follicular growth and ovulation rate in goats (Medan *et al.,* 2004). The administration of anti-cholecystokinin antibodies in the cerebrospinal fluid increases lamb, sheep and pig's intake (Reeves *et al.,* 1989). Finally, passive immunization is a technique

successfully used for veterinary applications in the treatment and prevention of infectious diseases (Lopez *et al.,* 2007; Monger *et al.,* 2006).

Approaching the calcium homeostasis regulatory mechanisms, it has been found that in rats, the inhibition of calcitonin results in an increase in blood calcium levels (Kalu *et al.,* 1976, Roos *et al.,* 1980). Passive transfer of circulating antibodies against rat calcitonin inhibits its biological activity and consequently increases blood calcium levels to a similar level as the thyroparathyroidectomy (Roos *et al.* 1980). Moreover, in diseases such as postmenopausal osteoporosis (in which, unlike in parturient paresis, there is hypercalcemia and exogenous calcitonin is administered as treatment for reducing blood calcium levels), it has been shown that neutralizing antibodies are what cause the patient to have resistance to calcitonin and unresponsiveness to treatment (Grauer *et al.,* 1995). Therefore, a strategy based on the inhibition of blood calcitonin by passive immunization could help to prevent hypocalcemia.

So long, evidence for an association of calcitonin in hypocalcemia in the cow is inconsistent: whereas some studies point out that this hormone secretion is increased, others state that it is diminished or that it does not change at all. Shappell *et al.* (1987) gave low or high calcium diet to primiparous and multiparous cows. In the case of the multiparous cows, when the blood calcium drops below subclinical hypocalcemic levels (below 8mg/dL), there is an increase in the levels of calcitonin at day 1 postpartum. Mayer *et al.* (1975) observed that the levels of calcium dropped significantly from 1 day before to 1 day after parturition while parturient hypocalcemia and paresis were happening. Prior to parturition and even before the levels of calcium dropped below 8mg/dL, the levels of calcitonin diminished from day -3 from parturition until the calving day. Finally, Hollis *et al.* (1981) studied cows with milk fever and did not see significant changes in the levels of calcitonin around the calving day. So far, this strategy has not been studied but for sure it will be very interesting to be explored. In any case, the few studies that include an analysis of this hormone show that in cattle there are basal calcitonin levels in the bloodstream, so as in previous studies in rats, it could be immunoneutralized with passive antibody administration (Bandzaite *et al.,* 2005; Hollis *et al.,* 1981; Mayer *et al.,*

1975). In addition, since studies examining the profiles of calcitonin and PTH show that there is an inverse correlation between the levels of these two hormones (Kovacs and Kronenberg, 1997; Shappell *et al.,* 1987), calcitonin inhibition by circulating antibodies could have a positive effect on serum PTH hormone, also strengthening the prevention of hypocalcemia or subclinical parturient paresis.

2. Recombinant antibodies.

Genetic engineering techniques have provided the tools to produce recombinant antibodies in microbial systems, obtaining large amounts of antibody at a low cost. In this context, fragments of IgG can be produced in Escherichia coli or Pichia pastoris, completely retaining the specific recognition properties of the parent antibody (Ning *et al.,* 2003; Corisdeo and Wang, 2004, Kim *et al.,* 2001). Many of these antibody fragments are monovalent and bind to a single antigen (Fab, ScFv, heavy and light chain variable VL or VH) but they can also be bivalent and bind to two antigens (Fab'2, diabodies, minibodies, etc.) (Holliger and Hudson, 2005). The advantages of using smaller recombinant antibodies like single-chain Fv antibodies (ScFv) rely on its high penetrability while maintaining the same affinity and specificity as the full antibody. They are also easier and faster to produce (Holt *et al.,* 2003). Moreover, the affinity, specificity, and stability of can be improved by several mutagenic technologies (Pansri *et al.,* 2009). One of the most important advances in the production of recombinant antibodies has been the development of phage-display antibody technology (McCafferty *et al.,* 1990).

Phage display was first developed with M13, an *E.coli* specific bacteriophage. Its success has made possible the development of alternative display systems such as λ-phage, T4 phage and systems using eukaryotic viruses but M13-based phage display is still the best option (Sidhu, 2000). The M13 phage particle consists of a single-stranded DNA core surrounded by a coat composed of five different proteins (Figure 4). In early examples of phage display, polypeptides were fused to the amino-terminus of pIII (Scott and Smith, 1990; McCafferty *et al.,* 1990). These systems were severely limited because large polypeptides compromised coat protein function and

so could not be efficiently displayed. The development of phagemid display systems solved this problem because, in such systems, polypeptides were fused to an additional coat protein gene encoded by a phagemid vector. Subsequent infection with a helper phage produced particles with phagemid DNA encapsulated in a coat composed mainly of wild-type coat proteins from the helper phage but also containing some fusion coat proteins such as the antibody fragment. Phagemid systems permit the display of polypeptides that could not be displayed in simple phage systems, because the deleterious effects of the fusion protein are attentuated by the presence of wild-type coat proteins from the helper phage (Sidhu, 2000).

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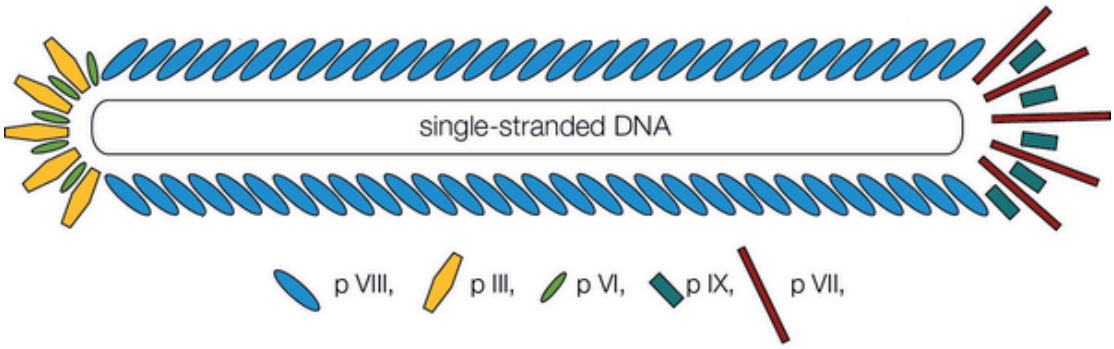

Figure 4. M13 phage with its 5 coat proteins depicted.

Another advantage of these recombinant antibodies is that can be obtained without a need for an animal immunization if using a naïve phage display library. A phage display library is a collection of phages expressing recombinant fusion proteins (containing antibody fragments) in the surface of the bacteriophages. Thus, each phage displays a single antibody fragment. In order to construct a library, antibody genes are fused to phage genes. Antibody genes can be isolated from B-lymphocytes of non-immunized donors, thus obtaining a naïve library. This library will be a great source of monoclonal antibody fragments against billions of antigens (Pansri *et al.,* 2009).

An ScFv is an antibody fragment that consists of the variable antigen binding domains of the heavy (V_H) and light (V_L) chain regions of a complete antibody. The

variable domains are connected by a flexible peptide linker and encoded by a single gene. For phage display, V_H and V_L genes are cloned and used to construct ScFv gene repertoires. With this technology it is possible to isolate monoclonal ScFv antibodies specific for proteins and small molecules. Therefore, they can be separated from nonbinding phage antibodies by standard immunological assays. Once selected, the antibody genes are subcloned in expression systems for recombinant microorganisms such as E.coli or Pichia pastoris (Sheets *et al.,* 1998).

Chapter II – Objectives

Hypocalcemia is an important disease in the dairy industry affecting the welfare of the animals and hampering its production. Subclinical hypocalcemia is known to have an incidence of 50% in dairy cows, but its consequences are not as studied as clinical hypocalcemia.

The main objective of the thesis was to study the mechanisms of action of subclinical hypocalcemia and to develop a preventive treatment. The specific objectives were:

1. To evaluate the incidence of subclinical hypocalcemia and its consequences on the cow's health, fertility and production.

2. To describe the role of calcitonin in the homeostasis mechanisms when the animal suffers subclinical hypocalcemia.

3. To develop a new method for a quick and reliable blood calcium measurement applicable on the field.

4. To try a preventive approach based on metabolic acidosis by altering the diet of the animals.

5. To develop a new preventive strategy based on the passive immunization of the cows against calcitonin.

To achieve these objectives, four studies were conducted:

Study 1 "Effects of subclinical hypocalcemia on periparturient diseases and fertility of dairy cows"

Analysis of the different periparturient diseases, milk production changes and fertility affectation associated with subclinical hypocalcemia in the dairy cow. Evaluation of subclinical hypocalcemia severity.

Study 2 “Is calcitonin an active hormone in the onset and prevention of hypocalcemia in dairy cattle?

Clarification of the role of calcitonin, and its interactions with PTH and Vitamin D metabolites, in the onset of subclinical hypocalcemia and its association with the mechanisms underlying the prevention of hypocalcemia under metabolic acidosis.

Study 3 “Evaluation of calcitonin neutralization as a new strategy to prevent cow hypocalcemia”

Evaluation of a passive immunization method to prevent hypocalcemia. Neutralization of calcitonin *in vitro* and *in vivo*.

Study 4 “New flow-through analytical system based on ISFETs with optimized Ca selective photocurable membrane for bovine serum analysis”

Development of an analytical system based on ion-selective field effect transistors (ISFETs) with Ca^{2+} ion selective photocurable membranes to offer a semiautomatic, quick and portable analysis of serum calcium concentration of cows.

Chapter III – Study 1

Effects of subclinical hypocalcemia on periparturient diseases and fertility of dairy cows.

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1. INTRODUCTION

The dairy cows experience important physiological changes around parturition. Due to the onset of lactation, the levels of blood Ca decrease suddenly in the 2-3d around calving (Quiroz-Rocha *et al.,* 2009). The sudden increased demand for Ca can cause the failure of homeostatic mechanisms of the cow due to a lack of ionized Ca availability (Horst *et al.,* 1997). The animal is then affected by hypocalcemia or puerperal paresis, which can be a clinical disease, in about 5% of the cows (NAHMS, 2002), or a subclinical disease with an incidence of around 50% in adult cows (Horst *et al.,* 2003). Despite the fact that the severity of the disease is greater in the clinical cases, subclinical case are more important because 1) they are far more frequent, 2) they cannot be easily diagnosed, and 3) may impair the longevity and productivity of the cow (Goff *et al.,* 2008; Murray *et al.,* 2008). Since there is a good correlation between blood ionized and total Ca concentrations (Blum *et al.,* 1972; Kvart and Larsson, 1978; Kvart *et al.,* 1982), the latter can be used to determine the range of blood Ca concentration affected by clinical or subclinical hypocalcemia (SCHC). It is commonly assumed that dairy cows experience SCHC when total blood Ca is below 8mg/dL and clinical hypocalcemia when blood Ca leves are below 6mg/dL (Goff, 2008), but there are other studies that apply different ranges to determine the severity of hipocalcemia. For example, Chapinal *et al.* (2012) defined that a normal cow would have blood Ca levels above 8.8mg/dL, Goff (2008) widened this range from 8.5mg/dL to 10mg/dL, and Martinez *et al.* (2012) proposed 8.59mg/dL as the cutoff value.

Clinical hypocalcemia causes immunosuppression by depleting intracellular Ca stores in peripheral blood mononuclear cells (Kimura *et al.,* 2006), which may compromise immune function (Hammon *et al.* 2006; Silvestre *et al.,* 2011) through decreased neutrophil function (Martinez *et al.,* 2012; Ducusin *et al.,* 2003). Low Ca concentrations in blood affect contraction of smooth and skeletal muscles (Murray *et al.,* 2008), and thereby may compromise the teat sphincter and myometrium (Chamberlin *et al.,* 2013), which may be one of the reasons, along with impaired immune function, behind the increased risk of mastitis, metritis, and retained placenta (Goff and Horst, 1997; Curtis *et al.,* 1983). Furthermore, induced SCHC

decreases chewing activity and the rumen and abomasum muscle contractions that can predispose the cow to displacement of abomasum (Daniel, 1983; Hansen *et al.,* 2003).

Curtis *et al.* (1983) evaluated the association of milk fever (clinical hypocalcemia) with 8 periparturient disorders in cows: dystocia, retained fetal placenta, metritis, LDA, foot problems, ketosis, mastitis, and mastitis, and reported a significant association between hypocalcemia and dystocia, retained placenta, ketosis, mastitis, and a tendency for an association with LDA. Other studies have reported inconsistent associations between SCHC LDA, with some reporting SCHC as a risk factor for development of LDA (Massey *et al.,* 1993) but others reporting no association (Chamberlin *et al.,* 2013; LeBlanc *et al.,* 2005).

Moreover, clinical hypocalcemia hampers fertility, conception and pregnancy rates of dairy cows (Maizon *et al.,* 2004; Roche, 2006; Martinez *et al.,* 2012). In fact, retained placenta and metritis negatively affect fertility, and mastitis negatively affected reproductive efficiency (Maizon *et al.,* 2004). Metabolic disorders delay the first detected oestrus, predispose the cows to metritis, and reduce submission rate to AI (Roche, 2006). Martinez *et al.* (2012) found that the rate of pregnancy was reduced under SCHC, and the interval to pregnancy was extended by 15 d, although there were no differences in conception rate at first AI between groups. However, Chamberlin *et al.* (2013) did not find increases in the occurrence of metritis or other uterine related diseases, and no differences were found with regard to services per conception or days open.

Lastly, there are contradictory reports about the potential association between milk yield and SCHC, with some studies reporting no correlation (Shappell *et al.,* 1986; Østergaard and Larsen, 2000, Martinez *et al.,* 2012) and others describing greater milk production in SCHC than in normocalcemic cows (Curtis *et al.,* 1984; Jawor *et al.,* 2012).

Because of the lack of data about the possible health consequences of SCHC, the objectives of the current study were to provide more data about potential

associations between SCHC and milk yield, reproductive performance, and the most important post-partum afflictions of dairy cattle.

2. MATERIALS AND METHODS

2.1. Animals

A total of 764 Holstein cows from different lactations were analyzed in this study. The animals were from six different Spanish farms. All animals were managed under typical European production conditions

2.2. Blood sampling, analysis, and data collection

Blood was collected by venipuncture of the tail vein using evacuated tubes without additives between 24 and 48h post-parturition. Serum from blood samples was recovered after centrifugation at 2,000 x *g* for 15 min. Serum samples were stored at -20 ºC until further analysis. Serum Ca concentration was determined using atomic absorption spectrophotometry. Cows with analyzed serum Ca below 8.5mg/dL were considered to suffer SCHC. Length of the dry period was measured. Milk yield in the first 60d of lactation was recorded for each cow, and, within the first 30 DIM, milk samples were collected at different days to determine milk SCC.

The occurrence of postpartum disorders (ketosis, LDA, retained placenta, metritis, mastitis) were recorded from each herd using defined standard definitions for each affliction. These diseases were recorded as either present or absent. Ketosis was diagnosed based upon a color change on a Ketostix urine dipstick (Bayern Corporation, Elkhart, IN) on the first 30d post-calving. Left displaced abomasum was diagnosed when a veterinarian performed the corresponding surgical intervention. Retained placenta was diagnosed when the fetal membranes failed to be completely expelled from the birth canal within 12h of parturition. Metritis was defined as a cow with a fever and a discolored, foul-smelling uterine discharge diagnosed by farm personnel during the first 30d post-calving. Cases of clinical mastitis were recorded when a cow was treated with intra-mammary antibiotics on the first 30d post-

calving. Fertility measurements included number of AI per conception, DIM at first AI, and presence or absence of heat within the first 60 DIM.

2.3. Calculations and statistical analysis

Data were analyzed using a mixed-effects logistic regression model with SCHC as a fixed effect and herd as a random effect. Confidence interval was set at 95%. Continuous data (e.g., milk yield) were analyzed using a mixed-effects model with SCHC as a fixed effect and herd as a random effect.

A second analysis was performed categorizing serum Ca levels according to the quartiles of their distribution, thus each category represented 25% (191) of the total number of cows (764). Then, the incidence of several post-partum afflictions (LDA, metritis, mastitis, retained placenta) as well as SCC at first test, number of AI needed to conceive, milk production in the 60 DIM and length of the dry period were contrasted among the four quartiles using a mixed effects model accounting for the random effect of herd. Mean separation among the 4 quartiles was performed using a Tukey's test for continuous variables. Odds ratios among quartiles were analyzed for nominal data (e.g., presence of ketosis).

Values with $P<0.05$ were considered significant. Values with $P<0.10$ were considered a tendency to be significant.

3. RESULTS

The overall serum Ca average concentration from all cows was 7.87 ± 0.335mg/dL (mean ± SE). From the 764 cows of the study, 575 (75.26%) could be considered SCHC. The mean of the serum Ca of cows with SCHC (serum Ca between 6 and 8.5mg/dL) was 7.54 ± 0.333mg/dL. Normocalcemic cows had a serum Ca concentration of 8.90 ± 0.253mg/dL. The parity of SCHC cows (2.41 ± 0.062) was greater ($P<0.0001$) than that of normocalcemic (1.70 ± 0.082) cows (Table 1). Cows classified as SCHC produced more ($P=0.001$) milk in the first 60 DIM than normocalcemic cows (2,398.8 ± 26.27 vs. 2,218.2 ± 44.73 kg, respectively). Cows

suffering from SCHC were dry for almost 11 more days (P<0.002) than healthy cows (53.7 ± 1.53 vs. 42.8 ± 3.13 d, respectively). No associations were found between the SCC and presence or absence of hypocalcemia, but high SCC within the first 30 DIM was associated with the presence of mastitis (1.8 ± 0.28 vs. 3.0 ± 0.55 cells x 10^5/mL, respectively). Normocalcemic cows were more likely (P<0.001) to have their first heat within the first 60 DIM compared with cows suffering SCHC. There was no correlation between the presence of SCHC and the number of AI needed to conceive.

Variable	SCHC	Normocalcemic	P-value
Blood calcium, mg/dL	7.54 ± 0.333	8.90 ± 0.253	-
Parity, n	2.41 ± 0.062	1.70 ± 0.082	<0.0001
Milk, kg in first 60 DIM	2398.8 ± 26.27	2218.2 ± 44.73	0.001
Days dry, d	53.71 ± 1.531	42.79 ± 3.134	0.0018
SCC, cells x 10^5/mL	2.2 ± 0.29	1.3 ± 0.48	0.11
Zeal in first 60 DIM, %	29.4 ± 2.68	61.7 ± 5.15	<0.0001
AI to impregnate, n	3.4 ± 1.17	3.1 ± 0.20	0.31

Table 1. Standard least squares model with SCHC at 24-48h post-calving as the fixed effect. Number of parturitions, milk yield in the first 60 DIM, days dry, first measured SCC, apparition of zeal in the first 60 DIM and AIs needed to impregnate are evaluated. Values are expressed as mean ± SEM.

Variable	Odds ratio	95% CI	P-value
LDA			
No	Referent		
Yes	2.71	1.23-7.18	0.01
Mastitis			
No	Referent		
Yes	1.50	0.99-2.35	0.06
Ketosis			
No	Referent		
Yes	3.62	1.88-7.87	<.0001
Metritis			
No	Referent		
Yes	2.56	1.76-3.78	<.0001
Retained Placenta			
No	Referent		
Yes	3.42	1.78-7.44	<.0001

Table 2. Nominal logistic model including likelihood of LDA, Mastitis, Ketosis, Metritis and Retained placenta to happen in SCHC cows expressed in odds ratios.

Table 2 shows the odds ratios for different health complications between cows with or without SCHC. There was an association (P<0.01) between SCHC and LDA (2.71), ketosis (3.62), metritis (2.56), and retained placenta (3.42). Furthermore, SCHC cows tended (P=0.06) to have 1.50 greater chances of incurring mastitis than normocalcemic cows.

When data was treated by quartiles of the blood calcium levels (Table 3), parity was severely influenced by the quartiles of serum Ca levels. As parity increased, the hypocalcemia was more severe (P<0.0001). The same differences as with the first approach were found regarding LDA, cows with SCHC had a greater risk of suffering LDA than normocalcemic cows (P<0.05). No differences were found in mastitis incidence between quartiles. Severe SCHC cows (serum Ca < 7.399mg/dL) had a greater (P<0.01) SCC than cows with serum Ca above 7.896mg/dL. Cows with serum Ca below 8.496mg/dL suffered a higher incidence (P<0.0001) of metritis than normocalcemic cows (serum Ca > 8.496mg/dL). Retained placenta incidence was greater (P<0.001) in cows with serum Ca below 7.399mg/dL than in cows with serum Ca between 7.399 and 7.986mg/dL and normocalcemic cows. Cows with less than 7.399mg/dL of serum Ca had a greater (P<0.0001) incidence of ketosis than cows with serum Ca above 7.896mg/dL. Also, normocalcemic cows had a lower incidence of ketosis than SCHC cows (P<0.0001). Milk production during the first 60 DIM was affected by the quartiles of serum Ca levels (P<0.0001). The dry period was longer (P<0.0001) in cows with severe SCHC (serum Ca < 7.399mg/dL) than in normocalcemic cows. Normocalcemic cows were more likely (P<0.0001) to have their first heat within the first 60 DIM compared with cows suffering SCHC.

4. DISCUSSION

The incidence of SCHC found in this study was 75.26%. To our knowledge, this is one of the greatest figures of SCHC incidence described in the literature far from, for example, the 47% assigned to multiparous cows by Reinhardt *et al.* (2011). Our analysis considered 2 models. A model assessing risk of diseased based on absence

Table 3. Analysis based in the quartiles of the blood calcium distribution at 24-48h post-calving. Incidence of LDA, mastitis, ketosis, metritis and retained placenta, number of parturitions first measured SCC, total milk in the 60 days post-partum, days dry and apparition of zeal in the first 60 DIM are evaluated. Values are expressed as mean ± SEM. Levels not connected by same letter are significantly different.

Variables	Quartiles by blood calcium (mg/dL)				P value
	< 7.399	7.399 - 7.986	7.986 - 8.496	≥ 8.496	
Parity, n	3.0 ± 0.10^{a}	2.2 ± 0.10^{b}	2.1 ± 0.10^{b}	1.7 ± 0.10^{c}	<0.0001
LDA, %	7.9 ± 1.84^{a}	7.9 ± 1.84^{a}	8.9 ± 1.84^{a}	3.1 ± 1.84^{b}	<0.05
Mastitis, %	23.0 ± 2.96	22.0 ± 2.96	23.0 ± 2.96	16.8 ± 2.96	0.36
SCC, cells x 10^5/mL	339.1 ± 49.04^{a}	203.2 ± 52.04^{ab}	134.4 ± 47.72^{b}	133.8 ± 47.72^{b}	<0.01
Metritis, %	42.4 ± 3.45^{a}	42.9 ± 3.45^{a}	41.4 ± 3.45^{a}	22.5 ± 3.45^{b}	<0.0001
Retained placenta, %	18.8 ± 2.35^{a}	11.0 ± 2.35^{b}	13.6 ± 2.35^{ab}	5.2 ± 2.35^{c}	<0.001
Ketosis, %	20.4 ± 2.38^{a}	13.7 ± 2.39^{ab}	12.0 ± 2.38^{b}	4.7 ± 2.38^{c}	<0.0001
Milk, kg in first 60 DIM	2,600.8 ± 44.78^{a}	2,303.9 ± 44.8^{b}	2275.4 ± 43.9^{b}	2214.4 ± 43.4^{b}	<0.0001
Days dry, d	56.5 ± 2.50^{a}	53.2 ± 2.67^{ab}	50.7 ± 2.82^{ab}	42.8 ± 3.13^{b}	<0.0001
Zeal in first 60 DIM, %	26.0 ± 4.64^{a}	28.9 ± 4.71^{a}	33.0 ± 4.64^{a}	61.4 ± 5.09^{b}	<0.0001

or presence of SCHC (logistic regression) (Table 1 and 2) and another model comparing the incidence of disease according to the quartiles of the distribution of serum Ca concentrations. This second approach helped to evaluate the risks of different serum Ca levels more precisely.

As observed previously, the incidence of SCHC increases as the parity increases (Curtis *et al.,* 1984; Reindhart *et al.,* 2011). Primiparous cows have a lower risk of suffering from SCHC while multiparous cows have an increased risk (Table 1). Multiparous cows, especially after the third parturition (Table 3), have an increased risk of suffering from severe SCHC.

As stated in previous studies, milk production in SCHC cows was greater than normocalcemic cows (Jawor *et al.,* 2012), but it is also related to the length of the dry period. A short dry period leads to milk losses in the subsequent lactation and a regular dry period ensures a correct mammary involution (Pezeshki *et al.,* 2010). Cows that incurred SCHC were dried a mean of 11d later than normocalcemic cows, and had greater milk production during the first 60 DIM in the next lactation. The downside of this greater milk production was the amount of Ca excreted in the milk. When dissecting data into quartiles, cows with serum Ca concentrations <7.399mg/dL produced more milk than cows in the other quartiles, or in other words, only cows with a milk production of around 2,600 kg in the first 2 mo after parturition seem have greater incidence of SCHC. A similar outcome was observed with the days spent dried. Cows with serum Ca levels below 7.399mg/dL had a 13-d longer dry period than cows with more than 8.496mg/dL of serum Ca, and the production increase of those 13d of additional dry period may be provoking too much Ca excretion into the milk. The production of a full lactation has to be further evaluated to assess whether this shift in the first 2 mo of production is significant. As previously suggested (Hardeng *et al.,* 1995), high milk productions could imply a higher risk of post-partum complications and the selection of cows should include health traits that contribute to profitability, and not only productive traits (Uribe *et al.,* 1995).

Although there was no relationship between first measured SCC and the presence of SCHC, there is, as expected, an association between the SCC and the presence of mastitis. Contrarily, when analyzing by quartiles, there are significant differences in the first measured SCC. Cows suffering severe SCHC have an elevated SCC and consequently, a greater risk to be affected by mastitis.

Clinical hypocalcemia has been previously associated to an increased risk of ketosis (Curtis *et al.,* 1984). Both of our approaches evidence a strong relationship between SCHC and ketosis as well. The severity of ketosis is directly correlated with the severity of SCHC. Cows with ketosis have low tissue responsiveness to insulin (Sakai *et al.,* 1993) and ketoacidosis is one of the reasons for insulin resistance (Van Putten *et al.,* 1985; Holtenius, 1993; Steen *et al.,* 1997). Previous studies have reported low concentrations of insulin in hypocalcemic cows despite having high concentrations of glucose (Forslund *et al.,* 2010). Hypoglycemia of parturient paresis is associated with a defect in the insulin response to hyperglycemia (Littledike *et al.,* 1970), probably because insulin is secreted by a Ca-dependent exocytosis system (Hou *et al.,* 2009). Also, insulin decreases liver non-esterified fatty acid uptake through stimulating lipogenesis and inhibiting lipolysis in adipose tissue, enhancing peripheral tissue ketone utilization, and altering enzyme activities and availability of substrates involved in ketogenesis (Brockman, 1979). In agreement with our results, ketosis is associated with SCHC through a defect in the insulin secretion in response to hyperglycemia and, thus, a decreased ketone depletion. Severe SCHC hypocalcemia is also associated with an elevated milk yield. These facts also support previous associations of ketosis with an increased milk production, since a greater milk yield is associated with a greater risk of negative energy balance in early lactation, leading to the apparition of ketosis (Uribe *et al.,* 1995, Hardeng *et al.,* 2001).

Cows suffering clinical hypocalcemia tend to have a higher risk of suffering LDA as previously reported (Curtis *et al.,* 1984). Supporting previous findings (Massey *et al.,* 1993) and in contrast to others (Chamberlin *et al.,* 2013; LeBlanc *et al.,* 2005), in the present study SCHC cows had a significantly higher risk of being affected by LDA. The levels of calcium in blood are not as reduced as in clinical hypocalcemia but they are low enough to alter the contraction of the abomasum, probably caused by a reduced

chewing activity as seen when SCHC has been induced (Daniel, 1983, Hansen *et al.,* 2003).

SCHC is also related to metritis and retained placenta. Previous studies associate clinical hypocalcemia with these two periparturient diseases (Goff and Horst, 1997; Curtis *et al.,* 1983), and Martinez *et al.* (2012) uses the incidence of metritis as a tool to designate SCHC cows as animals with a serum Ca below 8.59mg/dL. There is a diminished immune function and, along with a diminished muscle contraction, metritis and retained placenta are more prone to happen in SCHC cows (Martinez *et al.,* 2012). Our results agree in the association of metritis with SCHC and relate it with retained placenta incidence as well. When the data was grouped into quartiles, the retained placenta incidence differed between the different severities of SCHC. Retained placenta in cows suffering severe SCHC was almost four times more likely to happen than in normocalcemic cows.

Given that metritis and retained placenta incidence is higher in SCHC, previous studies suggest a negative correlation between these afflictions and the fertility of dairy cows (Maizon *et al.,* 2004). The present data confirm that SCHC affects fertility but not as heavily as previous studies (Roche, 2006). Healthy cows are more likely to have its zeal sooner than SCHC cows although no other fertility traits were correlated with the illness in the present study. Contrarily to previous findings (Martinez *et al.,* 2012) and agreeing with Chamberlin *et al.* (2013), we found no correlation with the number of AI needed to impregnate the cows.

In addition to all of these results, and taking into account all the previous studies defining the range to determine if an animal has SCHC or not (Goff, 2008; Chapinal *et al.,* 2012; Martinez *et al.,* 2012), the quartile distribution helped us to characterize the implications of hypocalcemia more precisely. Most of the results obtained when data were dissected into quartiles could be divided into three groups: normocalcemic cows (serum Ca > 8.496mg/dL), mild SCHC cows (serum Ca from 7.399 to 8.496mg/dL) and severe SCHC cows (serum Ca < 7.399 md/dL). This distribution provides a better understanding of SCHC and its implications in the different afflictions and post-partum traits of the cows. Not every affliction is related

in the same way to hypocalcemia and mild or severe SCHC have to be distinguished. There are substantial differences if we differ in the calculation of the range of SCHC. The lower cutoff value of SCHC (serum Ca = 6mg/dL) should also be re-evaluated in further experiments.

5. CONCLUSION

In conclusion, SCHC is a very important periparturient disease with high incidence and strong consequences in the health and production of the cows. Subclinical hypocalcemia is associated with ketosis, LDA, mastitis, metritis and retained placenta. It also affects the fertility of the cows by delaying the zeal apparition and is strongly correlated with the milk production and the dry period. A differentiation between mild and severe SCHC should be considered.

6. ACKNOWLEDGEMENTS

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Chapter IV – Study 2

Is calcitonin an active hormone in the onset and prevention of hypocalcemia in dairy cattle?

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1. INTRODUCTION

Parturient paresis is an important disease following calving in dairy cattle resulting from an inability of homeostatic mechanisms to regulate calcemia during a high demand for Ca when the lactating period begins. Hypocalcemia appears not only in clinical cases but also, in about 45% of animals, as a subclinical process. In both clinical and subclinical cases, the longevity and productivity of the cow are impaired (Goff *et al.,* 2008; Murray *et al.,* 2008), resulting in important economical losses for the dairy cattle industry. Under physiological conditions, the actions of calcitonin (CALC), parathyroid hormone (PTH), and $1,25(OH)_2D_3$ control blood Ca concentrations. A decrease in blood Ca triggers the parathyroid gland to secrete PTH, which increases renal Ca reabsorption and induces the expression of 1-a-hydroxylase in the kidney, responsible for producing $1,25(OH)_2D_3$ (Wasserman and Fullmer, 1995). One of the most important effects of $1,25(OH)_2D_3$ is to stimulate Ca absorption through the active transport across intestinal epithelial cells (Perez *et al.,* 2008). In addition, $1,25(OH)_2D_3$ stimulates, in conjunction with PTH, osteoclastic resorption activity of bone Ca (Horst *et al.,* 2003). By contrast, CALC inhibits the resorption of bone Ca and increases blood Ca losses through urinary excretion (Murray *et al.,* 2008). Blood CALC concentrations increase in response to hypercalcemia (Austin and Heath, 1981; Findlay and Sexton, 2004) and in response (or even in advance) to feed intake (Roos *et al.,* 1980).

Until now, there is not a consensus about the triggering cause of the sudden drop of blood Ca at a hormonal level. It is known that PTH and $1,25(OH)_2D_3$ increase after a decrease in blood Ca around parturition (Wasserman and Fullmer, 1995). Because cow hypocalcemia is a disease related with low levels of Ca in blood, it could be argued that CALC may be involved in the onset of this affliction. However, evidence supporting a role of CALC in hypocalcemia in the cow is inconsistent. Capen and Young (1967) first proposed an etiological role of CALC in the incidence of parturient paresis. They found a diminished CALC content in thyroid glands of cows with parturient paresis and suggested that an abrupt release of CALC near parturition may be related to the development of hypocalcemia. Barlet (1967) induced hypocalcemia in young and mature cows by intravenous administration of porcine CALC and also

concluded that CALC release contributed to hypocalcemia or parturient paresis. In contrast, Mayer *et al.* (1975) suggested that the development of hypocalcemia at parturition may be associated with a diminished prepartal secretion of CALC. Similarly, Hollis *et al.* (1981) did not observe changes in blood CALC concentration along the periparturient period, but found that paretic animals had lower blood concentrations of CALC than normocalcemic animals. Lastly, Shappell *et al.* (1987) detected fluctuations in serum CALC between parturition and the 5th day postpartum in contrast to the response reported by Hollis *et al.* (1981). According to Mayer *et al.* (1975) and Hollis *et al.* (1981) studies, cows with normocalcemic or subclinical levels of blood Ca showed the highest peak of CALC at postpartum, whereas the lowest concentrations of serum CALC were found in clinical hypocalcemic cows.

On the other hand, the diet of the cows prior to parturition may be involved in the incidence of periparturient paresis and hypocalcemia. Most prepartal diets based on forages are high in cations such as Na, and especially K. These diets may induce metabolic alkalosis and promote hypocalcemia because renal production of $1,25(OH)_2D_3$ and osteoclastic bone resorption are decreased in contrast to anionic diets (Goff *et al.,* 1998). Hence, the induction of metabolic acidosis through the supplementation of anionic salts creates a negative metabolic balance of Ca and prevents hypocalcemia at calving (Goff, 1998; Goff, 2008). However, the mechanism by which addition of anions to counteract cations in the diet of a cow enhances Ca homeostasis is not well understood. Leclerc and Block (1989), Goff *et al.* (1991), and Phiilippo *et al.* (1994) provided indirect evidence that physiological functions stimulated by PTH, such as bone resorption and production of $1,25(OH)_2D_3$, were enhanced in cows fed diets containing added anions. Also, Goff *et al.* (2014) have recently demonstrated that metabolic alkalosis reduces tissue sensitivity to PTH injections in comparison to cows under metabolic acidosis. Previous studies have also reported that metabolic acidosis increases the expression of CALC receptor (CALCR) in mouse osteoclasts (Biskobing and Fan, 2000) and up-regulates the expression of PTHR along with PTH in rat osteoblast-like cells (Disthabanchong *et al.,* 2002). Nevertheless, to our knowledge no study has been conducted in cattle to assess the role of PTHR and CALCR in Ca homeostasis during metabolic acidosis.

Furthermore, to our knowledge no previous study has evaluated whether CALC activity is affected under metabolic acidosis. The objective of this study was to assess the role of CALC in the onset of subclinical hypocalcemia and in the physiological mechanisms participating in the prevention of bovine hypocalcemia under metabolic acidosis.

2. MATERIALS AND METHODS

2.1. Experiment 1

2.1.1. Animals and Sample Collection

Fifteen Holstein cows (4.01 ± 1.1 years old, average lactation number = 1.6 ± 0.7, lactation length = 362 ± 86.1, average milk production/year = 8,550 ± 2,503.5 kg, average BW of 674 ± 22.8 kg, and with a milk production peak in previous lactations above 30 kg/d) incurring in subclinical hypocalcemia during the first 5d postpartum were classified in 2 groups depending on the levels of blood Ca. Eight animals were categorized as undergoing a low subclinical hypocalcemia (LSH) with blood Ca levels ranging between 7.5 and 8.5mg/dL, and 7 animals were classified as high subclinical hypocalcemia (HSH) with blood Ca levels between 6 and 7.4mg/dL. Blood samples were taken daily from day -5 to day 5 relative to parturition to perform subsequent hormonal analyses.

2.1.2. Blood Calcium and Hormone Analysis

Plasma Ca was determined by atomic absorption spectrophotometry. Plasma PTH and $1,25(OH)_2D_3$ concentrations were analyzed using commercial ELISA kits from Immunotopics (San Clemente, CA, USA) and Immunodiagnostic systems (Boldon, UK), respectively. Plasma CALC was measured by a double-antibody RIA. Synthetic CALC was iodinated with [^{125}I]Iodine (PerkinElmer) following the chloramine T method (Greenwood *et al.,* 1963) and used as the tracer. The same synthetic CALC was used as the standard and to raise a polyclonal serum in rabbits. Two rabbits were inoculated subcutaneously with bovine CALC conjugated to ovalbumin in complete

Freund's adjuvant. At 3, 6, and 9 wk after the first inoculation they received a booster injection with the same preparation, except that incomplete Freund's adjuvant was used. Exsanguination was performed 2 wk after the last booster injection and polyclonal serum was collected.

Before determining CALC using RIA, plasma samples were mixed with 1.5 volumes of acetone (Sigma, Spain) and incubated on ice. After centrifugation, the liquid phase was dried up by a nitrogen stream, and the wet material reconstituted with assay buffer (bovine albumin 0.25%; Triton X-100 0.1%; EDTA 25 mM; sodium phosphate 50 mM; pH 7.4). All samples that were compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 5.6%.

2.1.3. Statistical Analysis

Data from Experiment 1 were analyzed using a mixed-effects model with repeated measures; the model included treatment, time, and their 2-way interaction as fixed effects, and animal as a random effect. Data were log-transformed or root-transformed when necessary to achieve normal distributions.

Data from Experiment 2 were analyzed with an ANOVA with treatment (control vs. metabolic acidosis) as main effect. Data were log-transformed or root-transformed when necessary to achieve normal distributions.

2.2. Experiment 2

2.2.1. Animals, Treatments and Sampling

Twenty-four Holstein bulls (497 ± 69 kg of BW and 342 ± 10.5d of age) were randomly distributed to 2 treatments: control or metabolic acidosis. Animals were randomly distributed in 6 identical covered pens (12 x 6 m) bedded with straw. In each pen, animals had access to a drinker and received *ad libitum* concentrate (14.2% CP, 6.8% EE, 20.4% NDF, 5.1% ash in DM basis) and straw. The animals assigned to the metabolic acidosis treatment were given an oral drenching of 133 g of NH_4Cl (Panreac, Castellar de Vallès, Spain) diluted in water twice a day (morning and evening) during 10d (2.5 mEq/d). At day 10 from the onset of the study, animals

were transported to the slaughterhouse. Samples of venous blood were obtained from the coccygeal vein before slaughter in vacuumed tubes containing sodium-heparin (Beckton Dickinson, Rutherford, NJ, USA). Blood pH was measured immediately after the extraction. Then, blood was centrifuged at 3,500 rpm for 15 min and plasma immediately frozen for subsequent hormonal analysis. Parathyroid hormone and $1,25(OH)_2D_3$ were quantified in plasma by ELISA (Immunotopics, San Clemente, CA) and 1,25-dihydroxy vitamin D using a EIA kit from Immunodiagnostic systems (Boldon, UK) following the manufacturer instructions. At the slaughterhouse (3h after blood sampling) urine was collected from the bladder immediately after animal sacrifice and urine pH measured. Also, tissue samples of kidney (glomerular cortex region), parathyroid gland, and thyroid gland were collected and immediately preserved in RNAlater™ (Invitrogen, Carlsbad, CA) for subsequent gene expression analysis.

2.2.2. Gene Expression Analyses

Tissue samples from the kidney, parathyroid gland, and thyroid gland were homogenized prior to RNA extraction using a homogenizer (VDI 12; VWR, Radnor, PA). Messenger RNA was isolated with the use of Trizol® (Invitrogen, Carlsbad, CA) and mRNA retrotranscribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Specific bovine primers for b-actin (reference gene), PTH, PTHR, CALC, CALCR, and human CALCR genes (Table 1) were designed using the Quantprime software (Arvidsson *et al.,* 2008). Quantitative PCR was performed using IQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) with the MyIQ™ Real-Time detection system from BioRad (Bio-Rad Laboratories, Hercules, CA). The PCR reactions were performed in triplicates and water was used as negative control. Gene expression levels were analyzed by the method of comparative critical threshold ΔΔCt (Livak and Schmittgen, 2001) using b-actin gene as a reference gene.

2.2.3. Calcitonin Activity Assay

Studies of CALC activity in acidosis were performed in cultures of T47D breast cancer cells purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine

serum (FBS; Invitrogen, Carlsbad, CA) and dexamethasone (Sigma-Aldrich, St. Louis, MO) 100nM using 100 ml/well at a density of 10^5 cells/mL in 96 well plates. Cells were incubated at different pH (7.1, 7.2, 7.3 and 7.4) for 48h at 37ºC in a 5% CO_2 atmosphere. The pH of the medium was adjusted using HCl or NaOH. After this period of time, cells were used in a CALC activity assay or lysed with Trizol® (Invitrogen, Carlsbad, CA, USA) to analyze the expression of CALCR by real-time PCR.

In the CALC activity assay, a final concentration of 40nM bovine CALC (synthesized in the Proteomics Department of the Universitat Pompeu Fabra, Barcelona, Spain) was added to the wells for 90 min. Then, cells were washed twice with PBS and lysed with 0.1M HCl for 15 min. The cell extracts were frozen and further analyzed for cAMP (Lamp *et al.,* 1981) using a commercial ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA).

Gene		Sequence (5'-3')	Annealing (ºC)	Amplicon (bp)
β-actin	Forward	CTGGACTTCGAGCAGGAGAT	53'5	75
	Reverse	CCCGTCAGGAAGCTCGTAG		
Parathyroid hormone	Forward	GTGCACAACTTTGTTGCCCTTGG	57'5	66
	Reverse	TCGAGGTCTCTGGGAACTACCATC		
Parathyroid hormone receptor	Forward	AAATCCACACTGGTGCTCATGCCGC	55'5	165
	Reverse	GCAGAAACAGTATATGATGGC		
Calcitonin	Forward	TGAGTACCTGTGTGCTGAGTGCT	58	84
	Reverse	GTGTTTCAGGCCCGAAGCCCA		
Calcitonin receptor	Forward	CCTGAATCGCCCGCTTCCGG	60	194
	Reverse	AGCCATCCCACGTGCGGTTG		
Human Calcitonin receptor	Forward	GTGAAGCGCCAATGGGCCCA	60	111
	Reverse	GATGTCGCCAGCCTCCGCAG		

Table 1. Sequence of primer sequence for different genes and optimized qPCR conditions.

3. RESULTS AND DISCUSSION

To assess the role of CALC in the onset of hypocalcemia, the hormonal profile of cows incurring in subclinical hypocalcemia were analyzed in the peripartum. It is considered that cows with blood Ca levels below the traditional threshold of 8.0mg/dL have subclinical hypocalcemia. Recent research has indicated that this threshold should be raised to 8.59mg/dL, the level at which negative health effects actually become apparent (Martinez *et al.,* 2012). In the present study, cows were classified within the range of 8.5-7.5mg/dL blood Ca as having LSH or as HSH when blood Ca was 7.4-6mg/dL. As expected, cows on HSH had lesser ($P < 0.0001$) Ca concentrations along the 5d postpartum than before calving, whereas LSH cows only showed a moderate reduction ($P < 0.0001$) of blood Ca at calving (d 0) or at d 4 post-calving (Figure 1). Blood PTH concentration tended to differ ($P = 0.09$) between HSH and LSH cows, and they were greater ($P < 0.0001$) at postpartum than before calving in both groups. The increase in blood PTH was maintained during 3d post-calving in HSH animals whereas it was sustained for at least 5d in LSH animals (Figure 1). Interestingly, LSH cows had greater ($P < 0.05$) blood $1,25(OH)_2D_3$ concentrations (39.34 ± 7.537 pmol/L) than cows on HSH (20.11 ± 6.98 pmol/L) throughout the peripartum (-5 to 5d from calving) and blood $1,25(OH)_2D_3$ concentrations increased ($P < 0.0001$) during the postpartum in both groups. Hence, although blood PTH concentrations tended to be greater in HSH than in LSH cows, blood levels of $1,25(OH)_2D_3$ were lower ($P < 0.05$) in HSH than in LSH cows (Figure 1). This finding supports that the production of $1,25(OH)_2D_3$ is not only controlled by PTH through the regulation of 1-alpha hydroxylation of $1,25(OH)_2D_3$, but also by the PTHR, as previously hypothesized by other authors (Phiilippo *et al.*1994; Goff *et al.* 2014). Most likely, PTH was not able to stimulate the enzyme 1-alpha-hydroxylase to produce $1,25(OH)_2D_3$ with the same efficiency in HSH than in LSH cows, probably due to the amount and/or sensitivity of the PTHR.

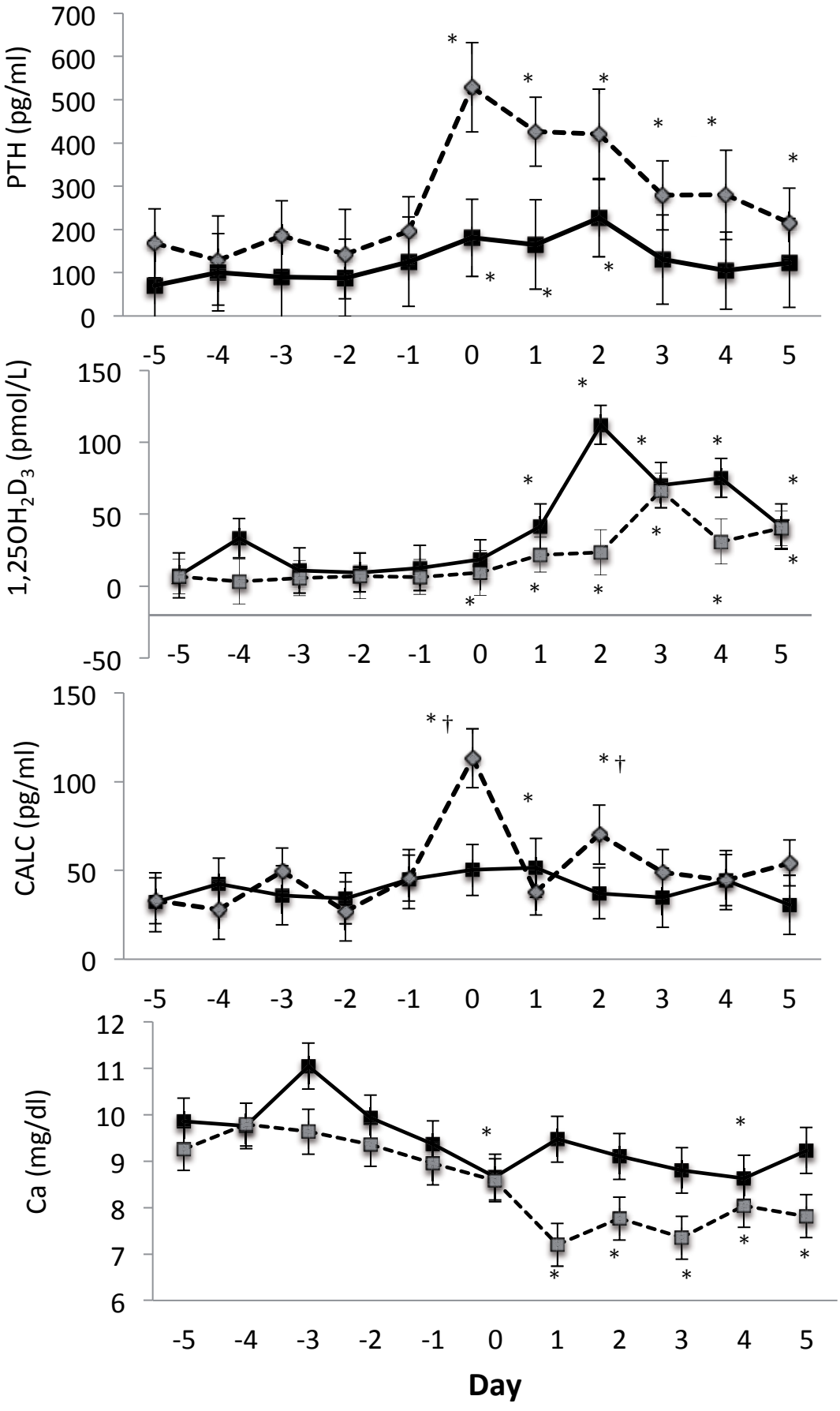
PTH (pg/ml)
700
600
500
400
300
200
100
0
-5 -4 -3 -2 -1 0 1 2 3 4 5
1,25OH2D3 (pmol/L)
150
100
50
0
-50
-5 -4 -3 -2 -1 0 1 2 3 4 5
CALC (pg/ml)
150
100
50
0
-5 -4 -3 -2 -1 0 1 2 3 4 5
Ca (mg/dl)
12
11
10
9
8
7
6
-5 -4 -3 -2 -1 0 1 2 3 4 5
Day

Figure 1. Profiles of blood parathyroid hormone (PTH), $1.25(OH)_2D_3$, calcitonin (CALC), and calcium along the periparturient period (-5 to 5d relative to calving). Solid black lines correspond to cows classified as having low subclinical hypocalcemia (LSH) and dashed black lines correspond to cows classified as having high subclinical hypocalcemia (HSH). Asterisks indicate differences ($P < 0.05$) regarding day -5 and crosses depict differences ($P < 0.05$) between LSH and HSH groups.

Overall, blood CALC concentration was not affected ($P = 0.29$) by the severity of subclinical hypocalcemia, but it was influenced by days from calving as it has been previously reported by Hollis *et al.* (1981) and Shappell *et al.* (1987), showing greater ($P < 0.0001$) concentrations at days 1, 2, 3 in HSH and at day 1 in LSH cows (Figure 1). However, there was an interaction ($P < 0.05$) between severity of subclinical hypocalcemia (HSH and LSH) and days since calving (Figure 2). At calving (day 0) and at day 2 postpartum, HSH cows had 2.25 and 1.89 folds more blood CALC than LSH, respectively. Accordingly to Shappell *et al.* (1987), peak concentration of blood CALC appeared to coincide with or follow peaks in circulating PTH concentration reflecting the feedback between the roles of theses 2 hormones: PTH increasing resorption of bone, and CALC decreasing resorption and increasing urinary Ca excretion. Interestingly, in the study of Shappell *et al.* (1987), the animals had subclinical hypocalcemia (values > 6mg/dL) and the results of hormone concentrations were similar than the ones presented herein. However, Hollis *et al.* (1981) studied hormonal differences between animals with clinical hypocalcemia (< 6mg/dL) and normocalcemic animals and did not detect neither the feedback between PTH and CALC, nor the CALC peak after calving, probably because the low concentration of Ca inhibited the synthesis of CALC. This fact should be taken into account in future studies, because it is possible that mechanisms involved in subclinical or clinical hypocalcemia are different and some preventive strategies that do not work for animals clinically affected could be effective in subclinical cases.

Overall, the results of this study suggest that after calving, CALC appears to exert a feedback on PTH release when cows are under subclinical hypocalcemia. The CALC rise causes a decrease in blood Ca concentration, which could make it even more difficult for the HSH cows to recover from hypocalcemia because the PTH-PTHR

response could be not sufficient to activate $1,25(OH)_2D_3$ and compensate the CALC feedback and subsequent decrease in blood Ca.

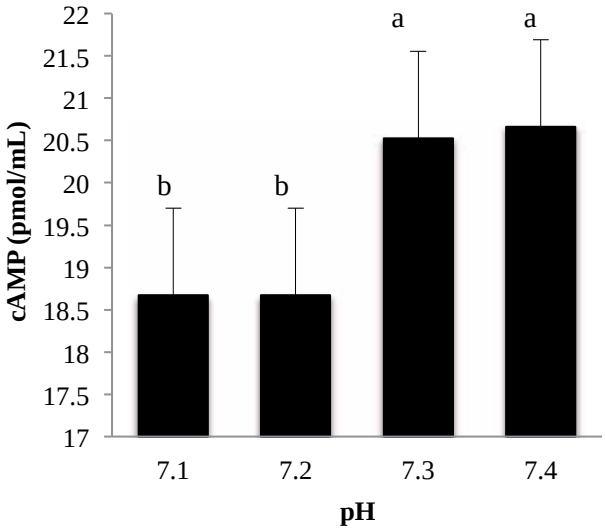

Figure 2. Activity of calcitonin (CALC) at different pH values assessed as cAMP production in T47D cells. Different letters depict differences ($P < 0.05$) between groups.

In the experiment 2, supplementing diets with ammonium chloride led to a reduction in blood ($P < 0.01$) and urine ($P < 0.001$) pH (Table 2) in bulls receiving the metabolic acidosis treatment (7.57 ± 0.015 and 5.95 ± 0.135, respectively) in comparison with the control group (7.64 ± 0.015 and 7.38 ± 0.135, respectively). These data indicate that the strategy adopted to induce metabolic acidosis was successful.

No differences were found in the expression of PTH and CALC genes in the parathyroid gland or the thyroid gland (Table 2). Moreover, PTH serum concentrations were not different between the two experimental groups (Table 2). Serum concentrations of $1,25(OH)_2D_3$, which depend mainly on PTH activity (DeGaris and Lean, 2008), did not differ between treatments either. The expression of PTHR in the parathyroid gland was similar between groups (Table 2). The expression of PTHR in the kidney was greater ($P < 0.05$) in the animals under metabolic acidosis than in the control ones (Table 2). These results indicate that metabolic acidosis enhances the activity of PTH in cattle through an increase of the expression of its receptors in the kidney, potentially leading to an increased coupling of the hormone with its receptor and, in consequence, a possible increased synthesis of $1,25(OH)_2D_3$, which would ultimately trigger an hypercalcemic response. These results are in agreement

with an *in vitro* study performed with rat osteoblast-like cells, where the number of PTH receptors increased under metabolic acidosis (Disthabanchong *et al.,* 2002).

	Treatment			
	Control	Anionic	SEM	*P*-value
pH				
Blood	7.64*	7.57*	0.015	0.003
Urine	7.38*	5.95*	0.135	<.0001
Gene expression, ΔΔCt				
Calcitonin (thyroid)	122.2	82.9	37.89	0.20
Calcitonin receptor (thyroid)	0.0062	0.0067	0.00257	0.48
Calcitonin receptor (kidney)	0.00073	0.00070	0.000328	0.77
Parathyroid hormone (parathyroid)	0.010	0.014	0.0064	0.64
Parathyroid hormone receptor (parathyroid)	0.00144	0.00147	0.000322	0.94
Parathyroid hormone receptor (kidney)	0.069*	0.115*	0.0139	0.03
Blood concentration				
Parathyroid hormone , pg/mL	207.7	213.5	27.36	0.88
1,25$(OH)_2$-vitamin D_3, pmol/L	455.1	603.9	68.73	0.14

Table 2. Blood and urine pH, gene expression, and blood concentration of different parameters as affected by the supplementation of anionic salts.

The role of CALC under metabolic acidosis has not been previously described and the results of hormonal expression of the present study (Table 2) only show a non-significant decline in CALC expression when metabolic acidosis was induced. To study deeply the effect of acidosis on CALC activity, the expression of CALCR was also analyzed in the thyroid gland and in the kidney. Metabolic acidosis did not affect the gene expression of CALCR, neither in the thyroid gland nor in the kidney. Moreover, to assess whether the activity of CALC could be somehow affected by blood pH, an *in vitro* experiment was conducted with the cell line T47D that expresses CALCR (Goldring *et al.,* 1997). Cells were incubated at different pH with bovine CALC and hormone activity was monitored through the synthesis of cAMP produced after the binding of CALC with CALCR. The activity of CALC was greater at physiological pH 7.4, and decreased significantly as pH decreased (Figure 2). The synthesis of CALCR was also analyzed in T47D cells, but it was not affected by pH (data not shown). These results support the findings of the *in vivo* experiment, where metabolic acidosis did not affect the expression of CALCR, but point out that acidosis impairs CALC activity,

which could be explained by conformational changes affecting CALC stability or its binding affinity for CALCR. The decrease of CALC activity under acidosis would have a synergistic effect with the upregulation of the expression of PTHR and the consequent increase of PTH activity, leading to a final raise in blood Ca levels.

4. CONCLUSIONS

In conclusion, this study demonstrates that CALC has an important role in the onset of hypocalcemia because despite that Ca demand at the beginning of the lactation is the main stimulus responsible for the decline in circulating Ca levels, there is also an increase of CALC that could exacerbate subclinical hypocalcemia. The clue of this negative effect of CALC resides in the delay between the PTH synthesis and the PTH effect that seems to be miss-controlled by unknown reasons in HSH cows. Moreover, the expression of PTHR is up-regulated in the kidney under metabolic acidosis increasing the effect of PTH and inducing increased $1,25(OH)_2D_3$ concentrations. An impairment of CALC activity, in combination with an increase in the number of PTHR, could foster a hypercalcemic status under metabolic acidosis.

5. ACKNOWLEDGEMENTS

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Chapter V – Study 3

Evaluation of calcitonin neutralization as a new strategy to prevent cow hypocalcemia

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1. INTRODUCTION

In dairy cows, at the onset of lactation there is a high demand of calcium. Many cows cannot fulfill the blood calcium demands because its blood calcium pool has to be depleted several times a day to meet the milk production needs (Horst *et al.,* 2005). A cow is healthy if its blood calcium levels are around 10mg/dL (Goff, 2000). If the calcium homeostasis of the cow cannot meet these calcium requirements, the cow suffers from subclinical (total blood calcium below 8mg/dL) or clinical hypocalcemia (below 6mg/dL) (Goff, 2008). The clinical hypocalcemia affects approximately 5% of dairy cows while subclinical hypocalcemia affects about 50% of all multiparous cows (Goff *et al.,* 2014).

In the clinical state of the illness, animals treated with an infusion of calcium borogluconate will relapse in 24h (Horst *et al.,* 2005). Also the cows can be treated with calcium salts administered subcutaneously but its effect is variable and several injections can be needed (Goff, 2008). However, although there are treatments to prevent the death of the animal in clinical cases, the productivity may be reduced, the susceptibility to other metabolic or infectious diseases is increased and animal welfare is compromised in both clinical and subclinical cases (Goff, 2008; Murray *et al.,* 2008). Subclinical hypocalcemia is a risk factor for the development of displaced abomasum (Massey *et al.,* 1993), decreases the chewing activity and the rumen and abomasum muscle contraction (Daniel, 1983; Hansen *et al.,* 2003) and reduces the rate of pregnancy (Martinez *et al.,* 2012). Moreover, subclinical cases are not detected, so it is much more important to invest in prevention instead of treatment.

Adjusting the DCAD can alter the blood acid-base balance, making it possible to improve the calcium homeostatic mechanisms. The induction of metabolic acidosis by adding anionic salts to the diet lowers the incidence of hypocalcemia in dairy cows (Block, 1984), but it has low palatability (Horst *et al.,* 1997) and they are not always successful (Romo *et al.,* 1991). Also, preparing an special ratio for prepartum cows difficult the management of the farm. On the other hand, reducing the calcium intake prior to calving stimulates the secretion of calcitropic hormones (Horst *et al.,* 2005; Goff, 2008), but it is difficult to implement and the formulation of ruminant

feed does not allow such low concentrations of calcium. Oral administration of high volumes of calcium salts to promote diffusion of calcium to blood during the puerperal period reduces hypocalcemia incidence (Oetzel, 1988), but causes mouth and gastrointestinal ulcers (Horst *et al.,* 2005) and can induce a metabolic acidosis so severe that results in loss of appetite (Goff and Horst, 1993).

Other preventive strategies take on the calcium homeostasis mechanisms. It is controlled by three main hormones: PTH, $1,25(OH)_2D_3$ and calcitonin. PTH is released when blood calcium is low (DeGaris and Lean, 2008) and acts on osteoblasts dissolving bone mineral (Block, 1994) and in kidney promoting renal absorption (Horst *et al.,* 1994). When PTH increases, $25(OH)_2D\text{-}1\alpha$ increases in kidney, converting more 25-hydroxycolecalciferol to its active form, $1,25(OH)_2D_3$ (Goff, 2008), enhancing calcium absorption from the intestines (Horst, 1986). Calcitonin exerts the contrary effect; it inhibits reabsorption from bone and increases urinary calcium excretion (Murray *et al.,* 2008) in order to diminish blood calcium concentrations in response to hypercalcemia (Austin and Heath, 1981; Findlay and Sexton, 2004) and to food intake (Roos *et al.,* 1980).

Administration of Vitamin D3 metabolites prevents puerperal paresis for 10-14 days and then the animals develop the illness again (Horst, 2003). Administration of PTH is very dangerous, because the effective dose is close to its toxic dose (Clarck *et al.,* 2001). None of these approaches provide a solid and easy strategy to apply solution to cow hypocalcemia. However calcitonin has not been studied so far as a target to modulate hypocalcemia. If this hormone is inhibited in rats, blood calcium levels increase (Kalu *et al.,* 1976; Roos *et al.,* 1980). Also, patients with postmenopausal osteoporosis have neutralizing antibodies against calcitonin causing hypercalcemia, and exogenous calcitonin has to be administered (Grauer *et al.,* 1995). Besides, the hormonal profiles of PTH and calcitonin show an inverse correlation between these hormones (Kovacs and Kronenberg, 1997; Shappell *et al.,* 1987). Therefore, the neutralization of calcitonin by passive immunization could be a valid strategy to prevent hypocalcemia in dairy cow. Antibody neutralization has been successfully used in animal science for the treatment and prevention of infectious diseases (Lopez *et al.,* 2007; Monger *et al.,* 2006). This strategy has also been used to

neutralize inhibin, increasing FSH secretion and stimulating follicular growth and ovulation in goats (Medan *et al.,* 2003). The objective of this study was to obtain neutralizing antibodies against calcitonin and to test its neutralization potential *in vitro* and *in vivo* in order to assess its preventive potential for cow hypocalcemia.

2. MATERIALS AND METHODS

2.1. Sample analysis and hormones

After recovering the serum from the blood samples, calcium was analyzed using a colorimetric method (SPINLAB 100) for the polyclonal assay and atomic absorption spectrophotometry for the B10 *in vivo* assay. Calcitonin in plasma was analyzed by a radioimmunoassay developed at the animal physiology department of the Universitat Autònoma of Barcelona (UAB, Barcelona, Spain) using the polyclonal serum. Bovine calcitonin was synthesized in the Proteomics Department of Universitat Pompeu Fabra (Barcelona, Spain).

2.2. Polyclonal antibodies

Rabbit immunization was performed at the antibody service (SCAC) of the Universitat Autònoma of Barcelona (UAB, Barcelona, Spain). Two rabbits were inoculated subcutaneously with bovine calcitonin conjugated to ovalbumin in complete Freund's adjuvant. At 3, 6 and 9 weeks after the first inoculation they received booster injection with the same preparation, except that incomplete Freund's adjuvant was used. Exsanguination was performed 2 weeks after the last booster injection and polyclonal serum was collected. Purification and concentration of the antibodies was performed using a SulfoLink Immobilization Kit for Peptides (Thermo Fisher Scientific) following manufacturer's instructions.

2.3. Indirect ELISAs

In a 96-well plate, 50µL of 10µg/mL antigen in PBS were plated and incubated at 4^{o}C o/n. After two washes with PBS 200µL of 3%BSA in PBS was applied per well as blocking buffer. The microtiter plate was incubated for 2h at RT and washed twice

with PBS. The polyclonal antibody (rabbit) or B10 ScFv was serially diluted and 100µL added in blocking buffer to the wells. Then a 2h incubation was performed at RT followed by 4 washes with PBS. For the rabbit polyclonal antibodies, 100µL of 1:50000 anti-rabbit-HRP (Sigma-Aldrich, St. Louis, MO) were added as a secondary antibody. For the B10 ScFv, 100µL of 1:5000 anti-FLAG-HRP (Sigma-Aldrich, St. Louis, MO) were added as a secondary antibody. A final incubation of 1h at RT and 4 washes with PBS were applied. Then, 100µL of TMB (Sigma-Aldrich, St. Louis, MO) were added in each well. After sufficient color development, 100µL of stop reagent for TMB substrate (450nM, SIGMA) was added to the wells and absorbance was read at 450nM.

2.4. Calcitonin activity in T47D cells

Calcitonin activity assays in-vitro were performed with T47D cells, expressing calcitonin receptor, through measuring receptor activation by cAMP (Lamp *et al.,* 1981). T47D breast cancer cells were purchased from the European Collection of Cell Cultures (Salisbury, UK) and cultured at a density of 10^5 cells/mL in 96 well plates in DMEM media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and dexamethasone 100nM (Sigma-Aldrich, St. Louis, MO). After an overnight incubation at 37°C (5% CO_2) culture media was aspirated and 100µL of the calcitonin sample, blank or standard were applied to each well and incubated for 90 min. Supernatant was aspirated and 200µL of 0.1M HCl were applied to each well in order to lysate the cells. After 15 minutes, cells were scraped with a pipette tip and recovered into a previously blocked eppendorf (3% BSA incubated overnight at 4°C). Cell extracts were frozen and further analyzed for cAMP (Lamp *et al.,* 1981) using a commercial ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA).

2.5. Polyclonal assay in rats

A preliminary assay to establish the functional concentration of bovine calcitonin (bCT) in rats was performed using 32 male Wistar rats (125-150g body weight to the reception) purchased from Charles River and housed in groups of 4 animals per cage. There were 4 treatments: saline, bCT 10, bCT 100 and bCT 1000. Saline treatment

was the control treatment and bCT treatments included 10, 100 or 1000 nanograms in 250µL of saline solution. Products were administered (saline and calcitonin) at t=0 by intravenous injection (coccygeal vein). Blood was collected at t=-20, 15, 60, 180, 360min and 22h through the saphenous vein. After the last sampling, rats were euthanized by exposure to CO_2 atmosphere. The polyclonal assay was conducted using 48 male Wistar rats (125-150g body weight to the reception), purchased from Charles River and housed in groups of 4 animals per cage. The different treatments were prepared as described in table 1 and solutions were incubated for 1h at 4°C and constant stirring prior to administration. Products were administered (saline, antibody, antibody + calcitonin and calcitonin) at t=0 by intravenous injection (coccygeal vein). To minimize variability due to circadian variations in the concentration of plasma calcium, administrations took place between 09:30 and 10:00. For 12d, each day 3-4 animals (1 of each experimental group) were processed, assigned on a rotating basis within each box. Blood was collected at t=-20, 15, 30, 60 and 240min through the saphenous vein. After the last sampling rats were euthanized by exposure to CO_2 atmosphere. In both assays the samples were collected in Microvette heparinized tubes (Sarstedt, CB 300) and then centrifuged at 2000g during 5min to obtain the serum.

Treatment	Product 1	Product 2	Volume
Control	--	--	250µL
1	--	Antibody (100µg)	250µL
2	--	Antibody (3µg)	250µL
3	Calcitonin (1000ng)	Antibody (100µg)	250µL
4	Calcitonin (1000ng)	Antibody (3µg)	250µL
5	Calcitonin (1000ng)	--	250µL

Table 1. Different treatments of the polyconal assay in rats with the amount of product 1 (calcitonin), product 2 (antibody) or both administered to Wistar rats.

2.6. Phage selection and ScFv screening

The phage selection was done with the John McCafferty library, kindly handed over by Dr. Brian Kay (University of Illinois at Chicago, Department of Biological Sciences, Chicago). First, 100µL of Dynabeads M-280 Streptavidin magnetic beads

(Invitrogen) were put in a 2mL tube. With a magnetic separator, storage buffer was withdrawn. Beads were washed 3 times with 500µL of PBS and blocked with 500µL 5% BSA in PBS rotating for 1 h. Beads were washed 3 times more with 500µL of PBS. A total of 18µg in 500µL of target peptide (Bovine calcitonin or Lactoferrin as positive control) was added to the magnetic beads and mixed in the rotator for 1 h. Beads were washed 3 times with 500µL of PBS. Then 500µL of PBS with 100µg/µL of streptavidin and $1x10^{12}$ phages from the McCafferty library were added and tumbled for 1h in the rotator. Beads were washed 3 times with PBS and finally resuspended in 100µL of 100µg/mL trypsin in PBS and incubated for 10 minutes with rotation to elute the phage. Trypsin was removed with the eluted phage and added to 750µL of *Escherichia coli* TG-1 cells grown to OD_{600}=0.6. Cells were then plated on 150x15 mm 2xYT + carbenicillin plates and incubated o/n at 30°C.

The plates with phages were scraped into 10mL of 2xYT media. After recovery, 50µL were added to 50mL of 2xYT + Cb and grown at 37°C until OD_{600}=0.4. After growth, 10mL were removed and $5x10^{10}$ KM13 phages were added and incubated at 37°C without shaking for 30 minutes. The tube was spun down for 10 minutes at 3000g, supernatant was discarded and cells were resuspended in 50mL of 2xYT with Cb 50µg/mL and Kan 50µg/mL and then incubated o/n at 25°C and 250 rpm shaking. The overnight culture was spun down at 6000g for 15 minutes. Supernatant was added to clean tubes and spun again 15 min at 6000g. A solution with 10% PEG8000 and 1.2M NaCl was filter sterilized and added to the supernatant in order to achieve a final concentration of 5% PEG8000 and 0.6M NaCl. Then, the supernatant was put on ice to precipitate the phages for 1 hour. The precipitated phages were spun down at 10000g for 20 min. The pellet was briefly rinsed with PBS. Pellets were resuspended in 1mL of sterile 1% casein in PBS and centifuged for 7 minutes at 6000g. The supernatant can be used in the next round of selection by adding it to the beads with the target protein. In order to analyze the binding affinity of the phages, a phage ELISA was performed as follows. After the third round of selection the phage-infected cells were diluted and plated in 2xYT-Cb in order to obtain isolated colonies. Each colony was picked, and put in a single well of a 96-deep well plate with 100µL of 2xYT and incubated at 37°C for 2.5 hours. Then, 100µL of 2xYT with

$2x10^{10}$ KM13 were added and incubated at 37°C without shaking. The 96-well plates were spun down at 3000g for 5 min, supernatant was removed, 400µL of 2xYT + Cb + Kan were added and incubated o/n at 30°C with 250 rpm shaking. Two 96-well plates were coated with 50µL of 10µg/mL of streptavidin or with PBS (background assessment) and incubated o/n at 4°C. Plates were then washed 3 times with PBS and blocked with 200µL of 1% casein in PBS and incubated with shaking for 1 h. Plates were washed 3 times with PBS-Tween 0.05% (PBST) and 50µL of 10µg/mL of the biotinilated target protein were added. Plates were then incubated with shaking for 30 min and washed 5 times with PBST. Deep well plates from the o/n expression were spun down at 3000g for 5 min. The phage in the supernatant was added by pipetting 50µL to the corresponding well in the target peptide and the background plate and incubated for 1h without shaking. Plates were washed 5 times with PBST and 50µL of 1:5000 anti-M13-HRP in PBS was added to all wells. Plates were then incubated for 1 hour with shaking and then washed 5 times with PBST. Fifty µL of ABTS substrate in 50 mM Sodium Citrate with 0.03% hydrogen peroxide was added and microtiter plates were then read at 405nM.

2.7. ScFv Production

The more specific ScFv plasmids from the phage library were digested using NcoI and NotI and ligated into pKp300DIII, a plasmid containing a PhoA promoter, a His-tag and a FLAG-tag. The ligated plasmid was then electroporated with 40µL of TG1 electrocompetent cells. In order to produce the selected ScFv, 200mL of low phosphate medium supplemented with carbenicillin (50µg/mL) was inoculated with 50µL of an overnight culture of transformed TG1 cells. Composition per liter of low phosphate media was ammonium sulfate (3.57 g); sodium citrate dihydrate (0.71 g); potassium chloride (1.07 g); yeast extract (5.36 g); and, Hy-Case SF casein acid hydrolysate (5.36 g) from bovine milk (Sigma–Aldrich). The solution's pH was adjusted to 7.3 with KOH. The media was autoclaved, cooled, and MgSO4 (1 M, 7mL) and glucose (1 M, 14mL) were added. After 18–20h of ScFv expression at 30°C and 250–280 rpm, the culture was centrifuged at 6000g for 10 min at 4 °C.

The cell pellet was lysed on ice with lysis reagent (3mL/50mL of original culture): Composition of lysis reagent was BugBuster 10x reagent (Novagen, Madison, WI) diluted to 1x final concentration using 1x Tris buffered saline, supplemented with Benzonase Nuclease (3µL/3mL; Novagen, Madison, WI) and complete, EDTA-free protease inhibitor cocktail (1x concentration, 1 tablet/10mL of lysis buffer; Roche Applied Science, Indianapolis, IN). The lysed cells were incubated at room temperature on a shaking platform at 40rpm for 20min, followed by centrifugation at 6000g for 10min, at 4 °C, to pellet the cell debris. The clarified cell lysate was used for protein purification. Agarose resin was prepared by washing 100ul of the Clontech His60 agarose bead slurry (60mg/ml binding capacity) twice with PBS. Cleared cell lysate was added to the resin and incubated at 4°C o/n while tumbling. Resin was then spun down for 2 minutes at 1000g, supernatant was removed and resuspended in 1mL of wash buffer containing 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4, 10mM Imidazole. This was spun down again at 1000g and the wash was repeated 3 times. Finally, antibodies were eluted in 250uL of filter sterilized Elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4, 300mM Imidazole) for about 10min. Elution was repeated with the same resin with an additional 250uL of elution buffer. Then, the 500µL of eluted antibody were dialyzed against PBS.

2.8. Calcitonin neutralization with B10 ScFv in calves

Forty-eight calves with an average BW of 87.56 ± 8.59 kg were distributed in three treatments with 16 animals each: saline solution as the control treatment (SAL), B10 ScFv in a molar relationship calcitonin:ScFv of 1:250 (B250) and 1:100 (B100). The calves were fed and had water *ad libitum* throughout the night and at 8am the feed was retired, blood was sampled and the treatment was subcutaneously injected on the back of the neck. Blood was sampled right before applying the treatment, and at 3, 4 and 8 hours post-injection. After the last sampling, feed was restored. Blood samples were centrifuged at 4000 g for 10 minutes and the serum was transferred to 1.5mL eppendorfs at -20 °C until calcium and calcitonin analysis were performed.

2.9. Calcitonin neutralization with B10 ScFv in cows

A previous study was performed in order to establish the incidence of subclinical hypocalcemia of the commercial farm and a 69% was determined as the percentatge of subclinical hypocalcemia at parturition in 347 cows. Moreover eleven cows were sampled at 0700h, 1400h and 2000h at calving in order to evaluate the fluctuations of blood calcium through the day. Blood samples were collected from the tail vein, centrifuged at 4000 g for 10 minutes, the serum was transferred to 1.5mL eppendorfs only to be frozen at -20 °C until calcium analysis.

A total of 132 healthy primiparous and multiparous Holstein cows were randomly distributed in a 2x2 factorial design according to subclinical hypocalcemia (SHC) at parturition and ScFv treatment. SHC was established at calcium concentrations <8mg/dL. Half of the animals with SHC at parturition were treated with saline solution (SAL) and the other half were administered the ScFv B10 in saline solution (B10). The treatments were injected intramuscularly on the posterior back after parturition in a final volume of 5mL. The dose of B10 was adjusted to the animal's blood amount calculated accordingly the BW to obtain a molar relationship ScFv : calcitonin of 250:1. The BW was calculated from the chest circumference of the cow, measured with a tape and transformed into BW (kg) through the HG method (Heinrichs *et al.,* 1992; 2007). Average calcitonin concentration in blood was assumed to be 54pg/mL from previous studies (Rodriguez *et al.* submitted). Blood samples were collected from the tail vein right after parturition before applying the treatment and 4 hours later. Samples were centrifuged at 4000 g for 10 minutes and the serum was transferred to 1.5mL eppendorfs. Tubes were frozen at -20 °C until calcium analysis.

2.10. Blood calcitonin measurements

Plasma calcitonin was measured by a double-antibody RIA using the rabbit polyclonal antibody. Synthetic calcitonin was iodinated with [^{125}I]Iodine (PerkinElmer) following the chloramine T method (Greenwood *et al.,* 1963) and used as the tracer. Plasma samples were mixed with 1.5 volumes of acetone (Sigma, Spain) and incubated on ice. After centrifugation, the liquid phase was dried up by a

nitrogen stream, and the wet material reconstituted with assay buffer (bovine albumin 0.25%; Triton X-100 0.1%; EDTA 25 mM; sodium phosphate 50 mM; pH 7.4). All samples that were compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 5.6%.

2.11. Statistical analysis

Data were analyzed in SAS using a mixed-effect model with repeated measures; the model included treatment, time and interaction as fixed effects and animal as random effect. Correlations between parameters were analyzed in JMP using bivariate fit lines between treatments. Values with $P<0.05$ were considered significant. Values with $P<0.10$ were considered as a tendency to be significant. In the experiment of calcitonin neutralization in cows data were analyzed with an analysis of variance considering ScFvB10 treatment and subclinical hypocalcaemia (SCH) and their 2-way interaction as fixed effects.

3. RESULTS

3.1. Antibody production, calcitonin-neutralizing ability *in vitro* and *in vivo*

The rabbit polyclonal antibodies were evaluated against synthetic calcitonin ELISA giving a titer ($1/IC_{50}$) between 13000 and 15000 (Figure 1a). The neutralizing potential of the polyclonal antibodies against calcitonin was assessed *in vitro* by experiments with T47D cells. The antibodies suppressed synthetic bovine calcitonin activity in T47D cells ($P<0.001$). It neutralized up to a 99.26% the calcitonin activity using a 1:3 serum dilution (Figure 1b) corresponding to a molar ration of 1:1 IgG: calcitonin. Even with a 1:96 dilution, the polyclonal antibodies neutralize 97% of the calcitonin activity. The control rabbit serum before calcitonin immunization, also purified through the SulfoLink column, did not affect calcitonin activity when evaluated with T47D cells.

Plasma of adult cows containing wild calcitonin also produced an increase in the cAMP production of T47D. The polyclonal antibody significantly reduced the wild

calcitonin activity as well as the synthetic bovine calcitonin (P<0.001) while the incubation with control rabbit serum do not produced any effect (Figure 1c).

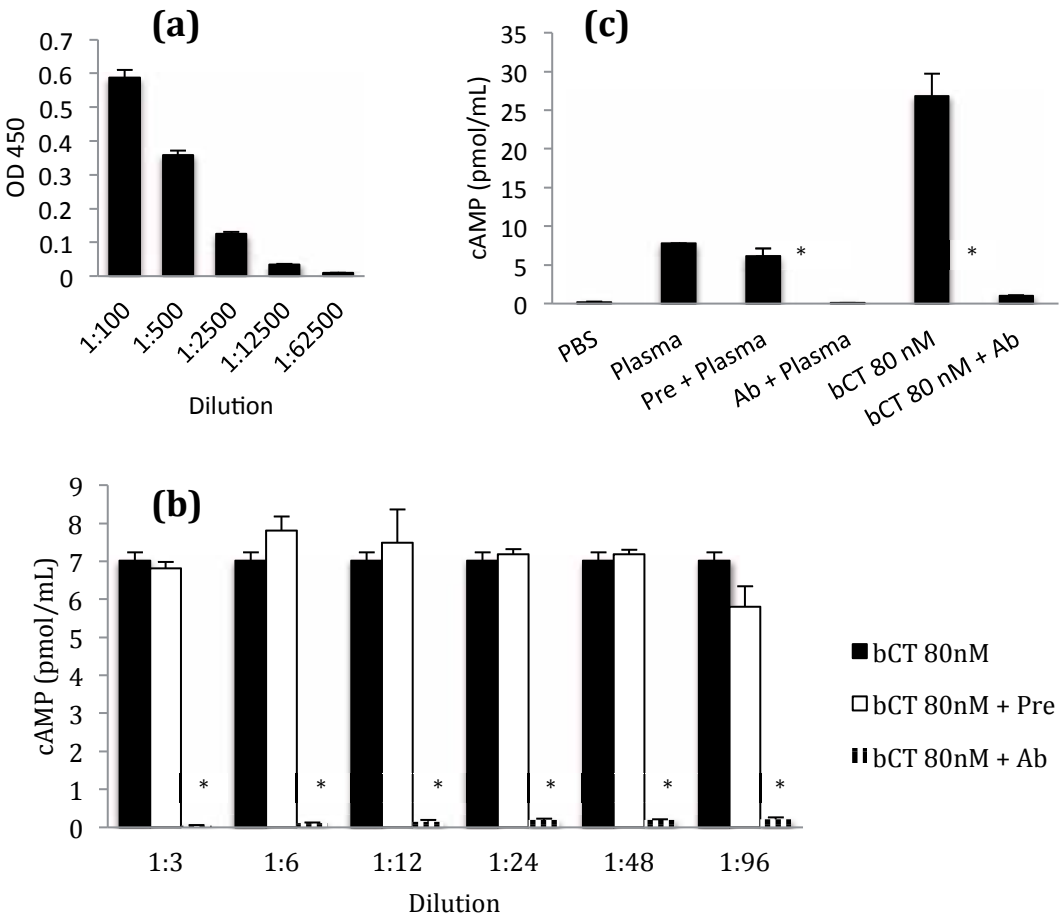


Figure 1. Performance of rabbit polyclonal sera against calcitonin. (a) Least squares means (±SE) of OD at 450nM obtained with different dilutions of polyclonal rabbit serum against calcitonin in an indirect ELISA. (b) Least squares means (±SE) of cAMP produced in T47D cells (pmol/mL) when synthetic bovine calcitonin was added alone (bCT 80nM), with pre-immunized serum (Pre) or with polyclonal neutralizing antibodies (Ab) at different dilutions. (c) Least squares means (±SE) of cAMP produced by T47D cells (pmol/mL) when wild bovine calcitonin in plasma (Plasma) was added alone, with pre-immunized serum (Pre) or with polyclonal neutralizing antibodies (Ab). A comparison with synthetic bovine calcitonin and its neutralization was also performed. PBS was the negative control. Data was compared between all treatments and samples treated with synthetic calcitonin alone. *P < 0.001.

The calcitonin neutralization was also evaluated in rats. A preliminary assay was performed to set up a concentration of synthetic bovine calcitonin that reduced blood calcium in rats (Figure 2a). The treatments with 10 and 100 g concentrations of bovine calcitonin did not produce any reduction in the blood calcium of the rats in

comparison to saline control. However a 1000ng dose reduced significantly the blood calcium at 15 minutes (P<0.05). In the calcitonin neutralization assay in rats (Figure 2b), the 1000ng of bovine calcitonin reduced the blood calcium concentration respect the rest of groups (P<0.05). The saline and antibody treatments without calcitonin did not show any differences in the calcium profile. In the animals treated with calcitonin plus polyclonal antibody diluted 1:96 (theoretical molar ratio of IgG:calcitonin corresponding 0.1:1) the calcitonin effect was abolished and there was not any differences regarding the saline negative control. Finally, in animals treated with calcitonin and polyclonal antibody less diluted (1/3, molar ratio of of IgG:calcitonin corresponding 3:1) presented calcium concentration greater than the control group (P<0.05).

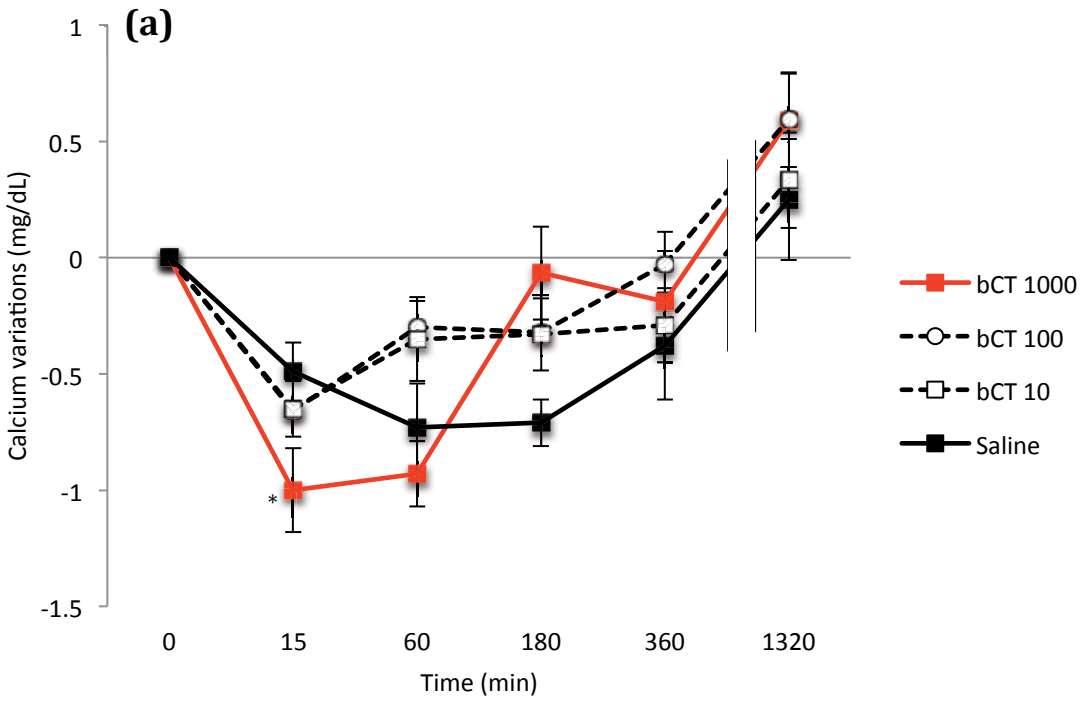
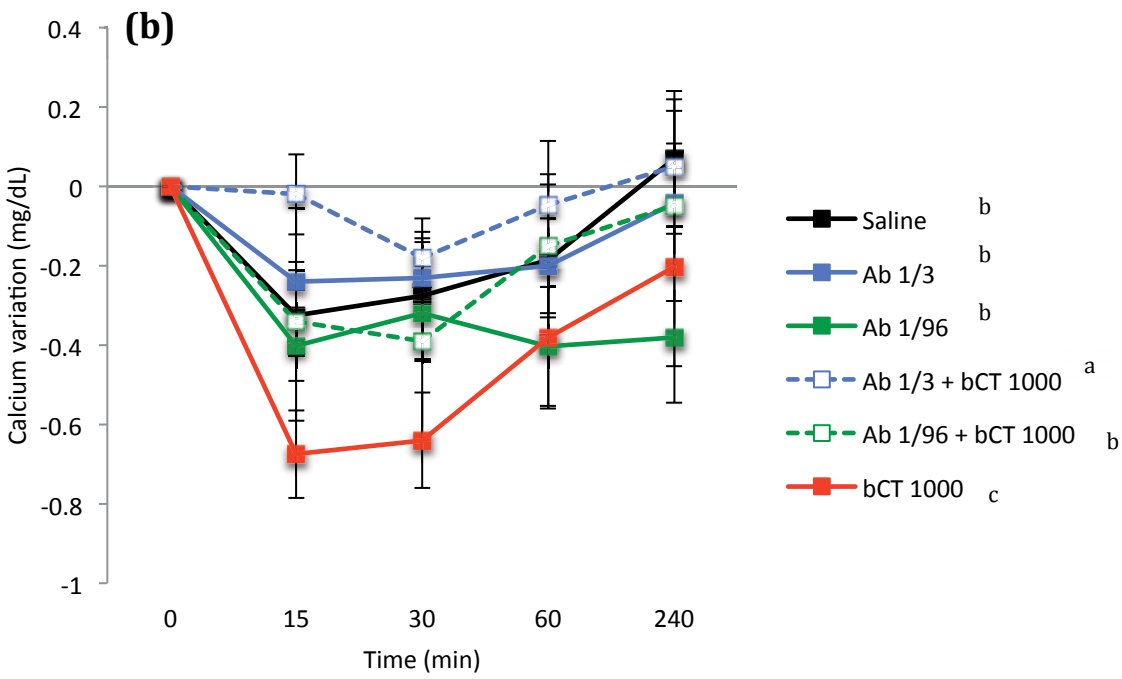
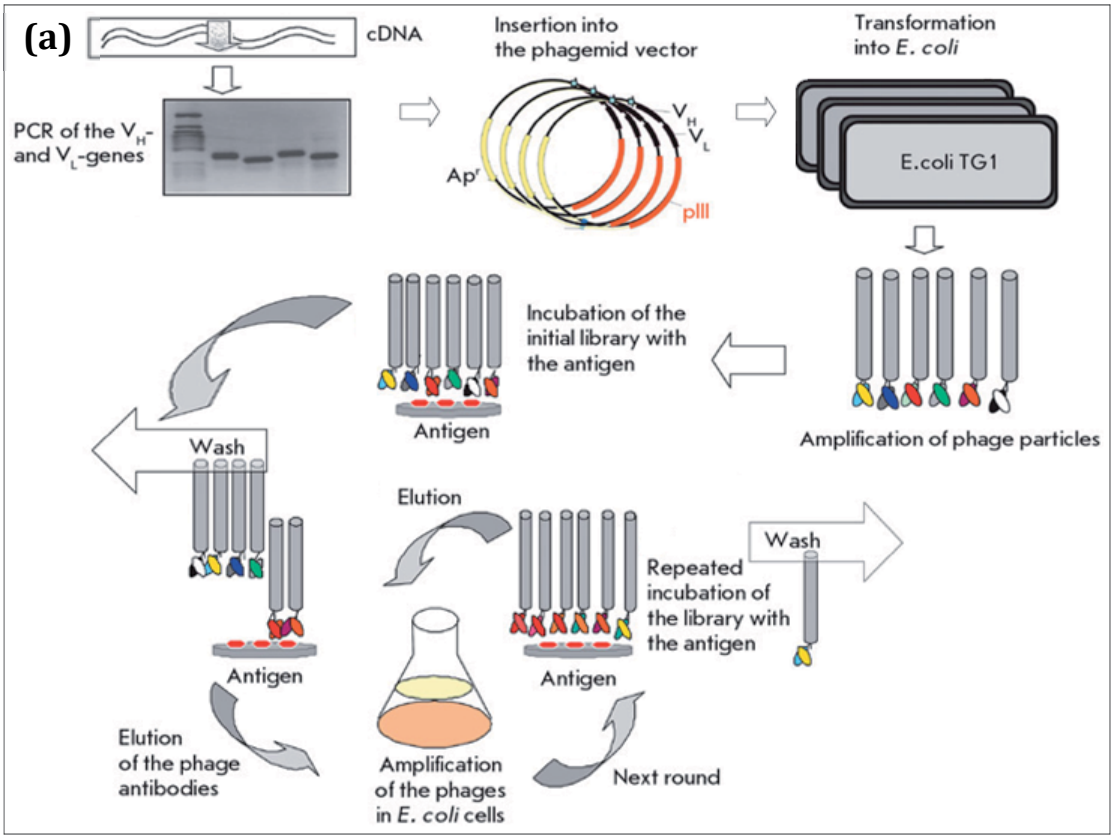


Figure 2. Bovine calcitonin neutralization in rats. (a) Least squares means (±SE) of rat blood calcium variations (mg/dL) with different amounts of synthetic bovine calcitonin (bCT). Saline solution was used as a negative control. Samples were collected from 0 minutes to 1320 minutes (22 hours). (b) Least squares means (±SE) of rat blood calcium variations (mg/dL) with 1000ng of synthetic bovine calcitonin and different dilutions of neutralizing polyclonal antibodies (1/3 Ab or 1/96 Ab). Saline solution was used as a negative control. Samples were collected from 0 minutes to 240 minutes. Different letters show a P < 0.05 between them.



3.2. Naïve library, monoclonal antibody screening and production

The massive production of antibodies was carried out to assess *in vivo* assays in cattle. As a first step, McCaferty human naïve ScFv phage library was screened in order to find positive control antibodies (Figure 3a). An ScFv against lactoferrin (LF) was used as the positive control. After 3 rounds of screening, two positive monoclonal ScFv antibodies against calcitonin were obtained: G5 and B10 (Figure 3b). The ScFv antibody B10 was chosen, as the specific signal was closer to the positive control. Through sequencing and western blot, the molecular weight of B10 was determined as 15 kDa (data not shown). The DNA encoding the B10 ScFv was successfully cloned into a producing system based on TG1 *E.coli* and expressed in low phosphate media, under the control of the PhoA promoter, an endogenous *E.coli* promoter induced when phosphate is depleted, with a His-tag and a FLAG-tag. Production of the ScFv antibody in *E.coli* with the posterior purification and dialysis in PBS yielded 5.35mg for each L of original culture. The purified recombinant antibodies in the indirect ELISA against calcitonin showed recognition of the monoclonal ScFv antibody against bovine calcitonin (Figure 4a).



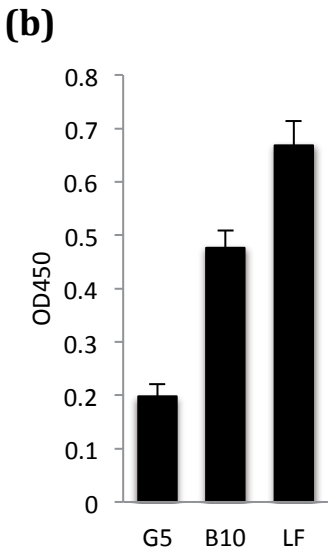


Figure 3. ScFv isolation and characterization. (a) Schematic representation of the construction of an antibody fragment phage library and the consequent biopanning procedure needed in order to obtain the target antibody. Adapted from Tikunova and Morozova (2009). (b) Least squares means (±SE) of OD at 450nM obtained in indirect ELEISA with ScFv isolated against calcitonin (G5 and B10) and Lactoferrin (LF) through biopanning of the phage library.

3.3. B10 neutralization assay *in vitro* and *in vivo*

The calcitonin neutralization potential of B10 ScFv was tested in T47D cells and approximately 0.5µg of ScFv/well with a molar proportion ScFv:calcitonin of 63:1 reduced up to 85% the calcitonin activity (Figure 4b).

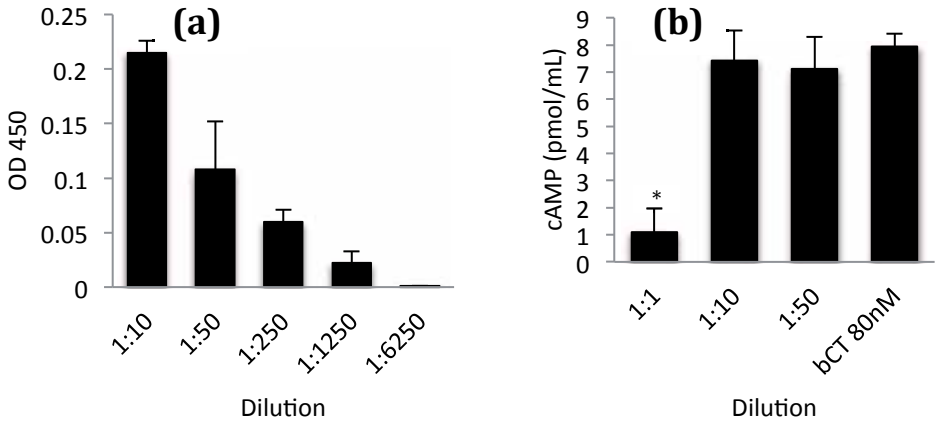
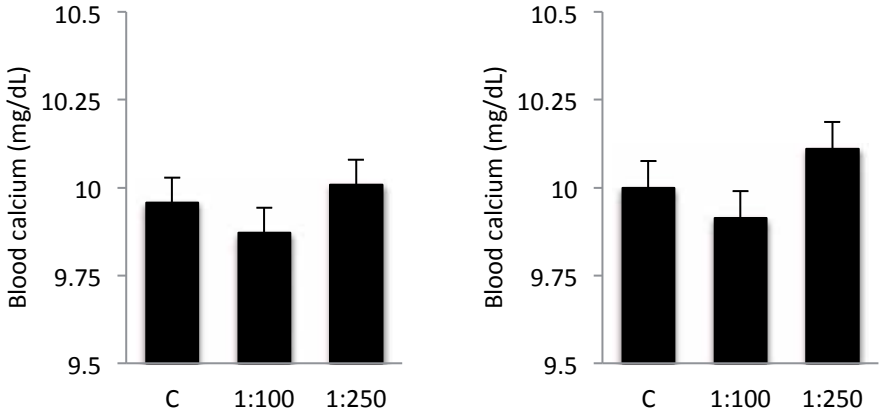


Figure 4. (a) Least squares means (±SE) of OD at 450nM obtained in ELISA against calcitonin with different dilutions of B10. (b) Least squares means (±SE) of cAMP produced in T47D cells (pmol/mL) when synthetic bovine calcitonin was added alone or with B10 in PBS at different dilutions. *P ≤ 0.01.

The treatment of cattle with B10 did not affect the levels of blood calcium of the animals after 4 (P=0.15) or 8h (P=0.32) after the injection of the antibody (Figure 5a, 5b). We also evaluated the serum calcitonin at 0h and 3h in the RIA assay to evaluate if a decrease in the RIA efficiency could be indication of the ScFv binding to calcitonin or to any type of neutralization. However the variability was too high and there were no statistically significant differences (Figure 5c).

Before trying the calcitonin neutralization in cows, the calcium fluctuations along the day and the incidence of hypocalcemia was assessed in the farm of interest (Murucuc, Spain). There were no significant differences between times 0700, 1400 and 2000 related to calcium levels (*P*=0.84) with mean values ± SEM of 7.200 ± 0.36, 7.015 ± 0.36 and 7.306 ± 0.36mg/dL of blood calcium respectively. Subclinical hypocalcemic values were found in 69% of the samples (data not shown).

(a) **(b)**



(c)

	Treatments[1]								
	SAL		B100		B250		P-value[2]		
	0h	3h	0h	3h	0h	3h	ScFv	T	ScFv x T
Serum calcitonin, pg/mL	63 ± 21.6	78 ± 22.9	97 ± 20.5	89 ± 21.6	143 ± 24.5	93 ± 24.5	0.173	0.419	0.342

[1] SAL=calves treated with saline solution; B100=calves treated with B10 ScFv at ratios 100:1 ScFv:calcitonin; B250=calves treated with B10 ScFv at ratios 250:1 ScFv:calcitonin;
[2] ScFv= effect of treating cows with B10 ScFv at parturition; T= effect of time on calcitonin; ScFv x T= interaction between treating cows with ScFv B10 and time.

Figure 5. Neutralization of calcitonin in calves. Least squares means (±SE) of cattle's blood calcium (mg/dL) after administration of ScFv B10 in a relationship calcitonin:antibody of 1:100 or 1:250 after 4 hours (a) or 8 hours (b) post-injection. Saline solution was administered as negative control. (c) Evaluation of serum calcitonin concentration at 0h and 3h post-injection through RIA quantification (least square means ± SEM).

Afterwards, a total of 66 cows with SCH and 66 normocalcemic cows were treated with saline solution (66) or the ScFv B10 in saline solution (66). The concentrations of calcium in serum did not differ between SAL and B10 cows at t=4h post-treatment neither in SCH nor normocalcemic animals (Table 2).

Table 2. Blood calcium values of the cows treated with ScFv anti-calcitonin B10 or with saline solution (SAL) at calving.

	Treatments[1]							
	SAL		B10			P-value[3]		
	NH	SHC	NH	SHC	SEM[2]	ScFv	H	ABxH
Serum Ca conc., mg/dL	8.58	7.36	8.60	7.76	0.190	0.266	<0.001	0.330

[1] SAL=cows treated with saline solution at parturition; B10=cows treated with anti-calcitonin antibodies at parturition; NH=cows with calcium levels greater than 8mg/dL at parturition; SHC=cows with calcium levels lower than 8mg/dL at parturition

[2] standard error of the mean

[3] ScFv= effect of treating cows with anti-calcitonin ScFv at parturition; H= effect of calcium levels at parturition; ScFv x H= interaction between treating cows with anti-calcitonin ScFv and calcium levels at parturition.

4. DISCUSSION

The aim of the present study was to evaluate the potential of antibodies against calcitonin in order to study the calcitonin neutralization as a strategy to increase blood calcium and prevent cow hypocalcemia.

To perform the proof of concept of the study, a polyclonal rabbit antibody rinsed against synthetic bovine calcitonin was used. The antibody not only recognized the synthetic peptide in an indirect ELISA but also completely neutralized the activity of synthetic calcitonin and wild type calcitonin present in cow plasma when tested in T47D cells. The T47D assay was developed by Lamp *et al.* (1981) and based on the detection of cAMP, a secondary messenger whose intracellular concentration increases when calcitonin binds to its receptor.

Calcitonin has a conserved structure across species but the receptor of calcitonin is even more conserved structurally. Consequently, calcitonin can bind to calcitonin receptors of different species and still exert its effect (Pham *et al.,* 2005). Therefore, rat or human receptors can recognize bovine calcitonin. The potential to modify the blood calcium concentration by polyclonal antibodies was tested in rats. The first preliminary study concluded that 1000ng bovine calcitonin was able to decrease the

blood calcium in rats. Interestingly, there was a significant increase of the calcium concentration 180min after the injection with all three doses. A plausible explanation is that the rat is receiving exogenous calcitonin lowering its blood calcium, and the rat homeostatic mechanisms respond to that calcitonin overdose producing PTH and, soon after, $1,25(OH)_2D_3$. The calcium concentration was stabilized at t=360min.

The neutralization of calcitonin with different dilutions of the polyclonal antibodies not only neutralized the action of calcitonin; when the dilution of the antibodies was 1/3 (molar relationship of 3:1 IgG:calcitonin) it increased the blood calcium concentration. Bovine calcitonin decreased the rats' blood calcium, thus activating its homeostatic mechanisms to increase the levels of calcium. The antibodies, in its most concentrated dose, neutralized the action of exogenous calcitonin while the homeostatic mechanisms were still boosting the release of calcium and it provoked an overall increase in the levels of blood calcium of the animal.

The next step was to mass-produce a neutralizing antibody to test the potential of calcitonin neutralization of cows. Using recombinant antibodies allows us to obtain large amounts of antibody at a low cost. Recombinant antibodies retaining the specific properties of the parent antibody (Ning *et al.,* 2003; Corisdeo and Wang, 2004, Kim *et al.,* 2001), are easier and faster to produce (Holt *et al.,* 2003) and its stability can be improved by mutagenesis (Pansri *et al.,* 2009). We selected the antibody from the Mccaferty naïve library, obtaining a monoclonal ScFv produced by *E.coli* named B10. This monoclonal recombinant antibody reduced the action of the bovine calcitonin *in vitro* in an 85%. However the efficiency of ScFv B10 *in vitro* was lower than polyclonal antibody because the dose (moles) of B10 had to be 63 times higher than polyclonal antibodies to achieve similar efficiencies of neutralization, probably affected by different half-life and neutralization potential. In rats, the polyclonal sera gave the best results when used at molar ratios 3:1 of IgG: calcitonin. So, if we would assume that neutralization and homeostasis of bovine calcitonin in rats and cattle is similar we should maintain a ratio of at least 189 ScFv: calcitonin in calves or cow. This prompted us to use a dose of 250 ScFv: 1 calcitonin in cattle. In a preliminary study, calves were used as a model to study the ScFv effects. However

no effects were observed and since cows are affected by hypocalcemia at the beginning of the lactation we reasoned that the neutralization of calcitonin would be more relevant in animals with hypocalcemia than in normocalcemic animals. Subclinical hypocalcemia incidence in the farm Murucuc was really high (69%) so we could include in the experiment 66 SHC and 66normocalcemic animals treated with saline or ScFv anti-calcitonin. The analyses of blood calcium along the day showed no significant fluctuations indicating that it would no be importantly affected by the time of the treatment, which would be subjected to the calving. No effects on blood calcium concentrations after 4 hours of treatment were observed in comparison with saline animals although animals were hypocalcemic and the results confirmed the findings of calves experiment. As the calcitonin RIA assay is based on the recognition of the calcitonin polyclonal antibody, the linking of serum calcitonin to an ScFv *in vivo* could be translated into a reduction in the final quantification of serum calcitonin in the RIA. However, no differences of calcitonin quantification between 0 and 3h were found in calves' sera, confirming that probably the dose used was not enough to block the calcitonin hormone. It is important to stress that the lifetime of a whole antibody is generally greater than that of an ScFv, which can be removed rapidly from blood circulation by renal mechanisms (Tikunova and Morozova, 2009). Hence, in this context, further experiments to study the lifetime of B10 and its biodistribution along with the effect of polymers, liposomes or mutagenesis increasing its half life and the effective dose, could be relevant to deeply characterized the real insights of the proposed strategy.

5. CONCLUSIONS

In conclusion, this study demonstrate that using polyclonal antibodies or ScFv against bovine calcitonin it is possible to neutralize its activity *in vitro* and abolish its hipocalcemic effects in rats with a polyclonal sera. However no effects have been observed so far with the recombinant ScFv B10 *in vivo* when injected at equivalent ratios in cattle or in cows under hypocalcemic or normocalcemic enviroment. Further experiments focused on the modulation of recombinant antibody half-life

and ScFv biodistribution will be of relevant importance to evaluate the real potential of calcitonin neutralization by recombinant antibodies as a preventive strategy to prevent subclinical cow hypocalcemia.

6. ACKNOWLEDGEMENTS

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Chapter VI – Study 4

New flow-through analytical system based on ISFETs with optimized Ca selective photocurable membrane for bovine serum analysis.

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1. INTRODUCTION

The history of the cow's milk fever study began in the twenties when analysis of ionic compounds of cow's serum sample confirmed the hypothesis that this disease provokes decrease of calcium concentration level for these animals (Godden *et al.,* 1935). Milk fever, which is a severe form of hypocalcemia, in dairy cows is an economically important disease and significantly increases a cow's susceptibility to mastitis, retained fetal membranes, displaced abomasum, dystocia and ketosis, which can reduce a cow's productive life (Reindhart *et al.,* 2011). Currently, producers can readily identify animals with milk fever through the observation of overt clinical signs, including dull appearance, lethargy, cold ears, or a down cow (Jawor *et al.,* 2012). In this connection, the development of portable, easy to used, semiautomatic measuring system for serum calcium concentration might be very beneficent for small and middle farms, because 47% of all cows in their second lactation or greater had varying degrees of subclinical hypocalcemia that in some cases is severe enough to alter physiological and immune functions (Kimura *et al.,* 2006).

Traditionally, assessment of an animal's calcium status is based on evaluation of the total calcium concentration (tCa). The tCa concentration is assumed to be directly proportional to ionized calcium (iCa), which is the biologically active form of calcium and is regarded as the gold standard for determination of calcium status (Sharp *et al.,* 2009). Normal serum calcium concentrations in healthy midlactation cows range from 2.1 to 2.8 mmol/L and from 1.6 to 2.6 mmol/L during the week after calving among cows with no subsequent clinical disease (Quiroz-Rocha *et al.,* 2009). Subclinical hypocalcemia can be more difficult to diagnose, because it is characterised by low serum calcium concentration in the absence of clinical milk fever symptoms. Values of ≤1.8 mmol/L for serum calcium in the first week after calving have been proposed as a suitable threshold for the diagnosis of subclinical hypocalcemia (Goff and Horst, 1997(.

Earlier we have reported about application of an analytical system based on optimized flow-through cell and ion selective field effect transistors (ISFETs) with

membranes sensitive to sodium, potassium and chloride ions for blood serum ion analysis (Abramova *et al.,* 2009). Application of photocurable polymers and photolithography technique for ISFET encapsulation, as well as for ion-sensitive membrane formation (Ipatov *et al.,* 2008) showed the feasibility of this approach that helps to make the process of ISFET fabrication more technologically favourable. To enhance the performance and the lifetime of cation sensitive polymer membranes a copolymerisable plasticizer di-(n-hexyl)-itaconate have been used. The developed system permits to determine ion concentration of sodium, potassium, chloride ions and pH in blood serum samples with a standard deviation of 3-5% (Abramova *et al.,* 2009).

The novelty of the present work lies in optimisation of the composition of previously proposed (Bratov *et al.,* 2000) calcium ion selective photocurable polyurethane membranes in terms of their stability and selectivity and application of the optimised Ca-ISFETs within a semiautomatic flow-through measuring system for analysis of serum calcium concentration of animals in cattle-ranches.

2. MATERIALS AND METHODS

2.1. Reagents and solutions

Commercial calcium Ionophore II, plasticizer bis(2-ethylhexyl)sebacate (DOS), lipophilic additives potassium tetrakis(p-chlorophenyl)borate (K-TpClPB) and ETH 500, as well as hexafluorobutyl acrylate HFBuA were purchased from Fluka. Copolymerisable plasticizer di-(n-hexyl)-itaconate (DHI) was synthesised in the laboratory (Abramova *et al.,* 2009). Aliphatic urethane diacrylate (oligomer Ebecryl 270) and cross-linker, hexanediol diacrylate (HDDA), were from UCB Chemicals. Photoinitiators 2,2'-dymetoxyphenylacetophenone (IRG 651) and 2-hydroxy-2-methyl-1-phenyl-propane-1-one (DAR 1173) were from Ciba-Geigy. All other chemicals used were analytical-reagent grade. Standard solutions were prepared with deionised water.

2.2. Preparation of ion-selective membranes

Photocurable membrane composition was prepared as presented elsewhere (Bratov *et al.,* 2000). The main polymer composition was prepared by mixing together the aliphatic urethane diacrylate oligomer Ebecryl 270 reactive diluent HDDA and photoinitiator Irgacure 651 in an 81:17:2 w/w ratio. In the case of membrane composition MCa1 (see Table 1) the main polymer composition was as reported earlier (Bratov *et al.,* 2000) and consisted of Ebecryl 270, DHI and photoinitiator DAR 1173 in a 59:36:5 w/w ratio. Then, 0.3 g of the main polymer composition were dissolved in 0.2mL of tetrahydrofuran and to this solution plasticizer if need (35-40% w/w), ionophore and lipophilic salts were added. The mixture was thoroughly stirred in an ultrasonic bath until homogeneous and then left for several hours to evaporate the solvent. Compositions of studied Ca selective membranes are given in Table 1.

N	Ionophore ETH 129, wt%	Lipohilic additieves, mol%	Plasticizer, wt%	Exposition time, sec	$-\log K_{Ca/Na}$
MCa0	1.0	K-TpClPB, 56	DOS, 40	15	3.5
MCa1	1.8	K-TpClPB, 56 ETH 500, 21	DHI, 36	200	Anionic influence
MCa2	1.7	K-TpClPB, 58	DHI, 36	60	1.3
MCa3	1.0	K-TpClPB 58	DHI, 41	200	2.1
MCa4	1.8	K-TpClPB, 71	DHI, 37	200	1.7
MCa5	1.1	K-TpClPB, 53	DOS, 20 DHI, 20	90	1st day-3.4 15 - 2.4 29 - 1.9 43 - 1.6 164- 0.8
MCa6	1.0	K-TpClPB, 57	DOS, 10 DHI, 30	60	1st day-3.4 15 - 2.5 29 - 2.3 111 - 1.1 164- 1.0
MCa7	1.0	K-TpClPB, 53	DOI, 39	60	0.6
MCa8	1.0	K-TpClPB, 53	DMHI, 40	75	0.4
MCa9	0.9	K-TpClPB, 56	HFBuA, 36	60	Hi resistance
MCa10	1.0	K-TpClPB, 54	HFBuA, 38	20	0
MCa11	1.0	K-TpClPB, 57 ETH 500, 20	DOS, 20 HFBuA, 20	20	1st day-3.6 8 - 3.6 27 - 3.6 86 - 3.3

Table 1. Different membrane composition for Ca^{2+}-selective ISFETs

2.3. Sensor fabrication

Sensors were made using n-channel ISFETs with SiO_2 gate insulator. After scribing and wire bonding ISFETs were encapsulated with photocurable polymer composition, as presented elsewhere (Muñoz *et al.,* 1996). To enhance the adhesion of the acrylated urethane polymer ISFET devices were preliminarily silylated by exposure to a 10% (v/v) (methacryloxy)propyltrimethoxysilane solution in methanol with a subsequent heat treatment during 1 hour under 80^{o}C in an oven. The membrane composition was then delivered by a microsyringe into the well formed by encapsulated layer over the gate region of an ISFET and was exposed to UV using UV Curing Light Lamp System PC-5000 (Dymax) with irradiance of 62 mW/cm^2 at the wavelength of 365nM. Exact values of exposure time are presented in Table 1. This resulted in a polymer membrane formation with the thickness of 150-200 μm.

2.4. Evaluation of chemical response

Initially, for optimisation of membrane compositions individual ISFET sensors were used. At least 4 devices were studied to determine the properties of photocurable membranes. These experiments were performed in batch under stationary conditions by addition of prepared stock solutions of $CaCl_2$ in the concentration range from 10^{-7} to 10^{-2} M. Selectivity coefficients for sodium, as the most interfering ion in serum analysis, were determined by the mixed solution method on the background of 0.1M concentration of interfering ion.

ISFET devices have been measured in a conventional manner in a constant drain current mode (I_D=100μA, V_D=0.5V) using a computer controlled experimental set-up. A double-junction Ag/AgCl reference electrode (Orion 90-02) was used as an external electrode with a 0.1M solution of lithium acetate as a salt bridge.

2.5. Flow-through system

The designed automatic system is shown schematically in Figure 1. To pump solutions and deliver them to the flow cells (4) a syringe pump (1) is used, while a multiposition valve (3) permits to select among various solutions (calibration solutions or samples) and to collect them in the holding coil (2) before injecting into

the flow cells. The high precision syringe pump used operates in the flow rate range of 1-500 µl/sec and with a sample volume of 1-1000 µl with a ± 0.1 µl precision.

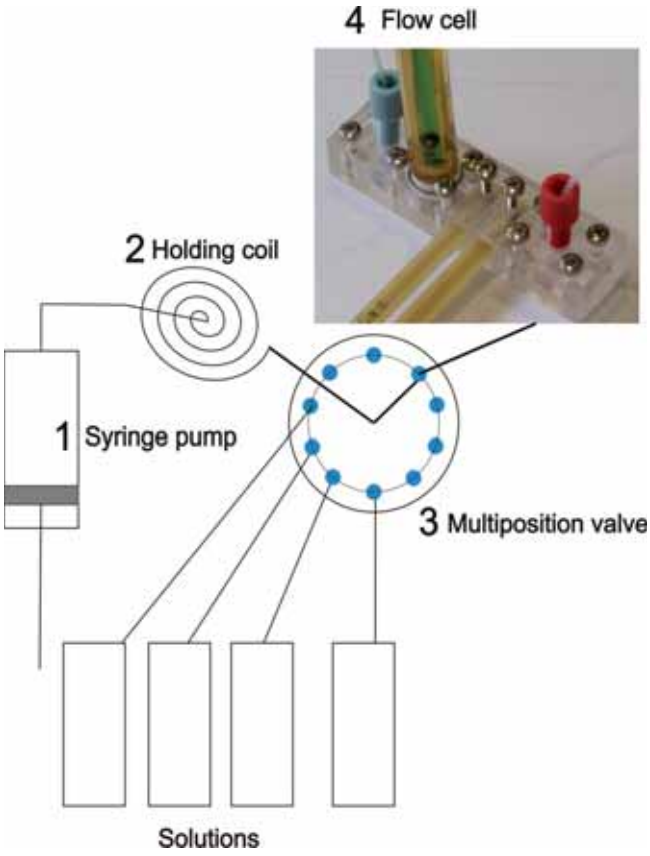

Figure1. The designed automatic flow-through system

A flow cell that incorporates two sensors and the reference electrode was developed and fabricated from a polymethylmethacrylate (PMMA) plate by micromilling (Stepfour GMbH, Salzburg, Austria). Different milled parts of the PMMA were glued together with methacrylic acid. The inner fluidic channels were height – 0.5 mm high and 0.8 mm wide with the total length of 50 mm. The external dimensions of the cell were 12 x 81 x 21 mm^3. Flow cell contains two separate cameras for each sensor (3 mm in diameter and 0.5 mm high). Standard 0.8 mm inner diameter tubing for flow injection analysis were connected to the inlet and outlet by ¼''-28 UNF connectors. For tight contact between the flow cell and sensors polydimethylsiloxane gasket was used and the cell was fixed by screws.

Calibration of the system under the flow rate of 20 µl/sec and solutions volume of 1000 µl lasts about 5-7 min and automatic analysis for each unknown sample takes about 2 min.

2.6. Recollection of serum samples

Thirteen healthy Holstein lactating cows from IRTA experimental farm (Monells, Spain) were used. Blood samples of the tail vein from each cow were collected in Vacutainer tubes (ref. BD367896) without additives or in tubes containing thrombin (ref. BDV368923, Beckton Dickinson, Rutherford, NJ, USA). From thrombin tubes serum was directly recovered after coagulation. Serum from blood samples without additives was recovered after centrifugation at 2000 x g for 15 minutes. Serum samples were stored at -20ºC until further analysis.

2.7. Blood serum measurements in a flow-through system

For the determination of total calcium in blood serum the procedure published by P. Anker *et al.* (1981) was applied. Calibration solutions contained 1, 2 and 9 mM Ca^{2+} in the presence of 140 mM Na^{+}, 4.5 mM K^{+} and 0.8 mM Mg^{2+}. The serum samples as well as calibration solutions were diluted with a 20-fold volume of acetate buffer with pH 3.5. The buffer was prepared by mixing 0.382M of acetic acid, 0.02M of NaOH and deionised water in a 1000mL flask. The slope of the sensors response was determined by measuring in 1 and 9 mM Ca^{2+} diluted solutions before and after serum sample measurements. The 2 mM Ca^{2+} diluted solution was measured in alternation with a blood serum sample also diluted with a buffer. The total calcium concentration was determined from the potential difference between the 2mM Ca^{2+} calibration solution and serum sample taking into account the actual slope of the ISFET sensor.

2.8. Inductively coupled plasma optical emission spectrometry (ICP-OES)

As an independent method of total calcium determination inductively coupled plasma optical emission spectrometry (ICP-OES) was used. 100 µl of serum was diluted with 2mL of solution containing 0.05% EDTA in 0.5% NH_4OH. The measurement were carried out on ICP-OES spectrometer (Perkin-Elmer, model Optima 4300DV) in the department of Chemical Analysis of Autonomous University of Barcelona (UAB).

3. RESULTS AND DISCUSSION

3.1. Optimisation of Ca-selective photocurable membrane.

Initial experiments performed under flow conditions showed that ion selective membranes prepared as reported earlier (Bratov *et al.,* 2000) (MCa0, Table 1) have limited lifetime and within a week lost their mechanical solidity due to more rapid, compared to stationary conditions, leaching of the plasticizer. Attempts to exclude the plasticizer from the membrane composition and variation of the polymer/cross-linker (aliphatic urethane diacrylate/hexanediol diacrylate) ratio were not successful due to high electrical resistance of the resulting membranes.

Taking into consideration that earlier studied K^+ and Na^+ selective membranes with copolymerisable plasticizer di-(n-hexyl)-itaconate (Ipatov *et al.,* 2008) showed promising results, the first membrane composition with this plasticizer (MCa1, Table 1) was prepared using the same main polymer composition (Ebecryl 270, DHI and photoinitiator DAR 1173 in 59:36:5 w/w ratio) together with lipophilic additives and the ionophore. Unfortunately, obtained calibration curve was nonlinear and starting from $5 \cdot 10^{-4}$M of $CaCl_2$ strong anionic influence was observed. Also for this composition (MCa1) the required polymer curing time was longer (200 sec) than in the case of normal plasticizer like DOS (see Table 1). This prolonged UV exposure may result in photobleaching and decomposition of K-TpClPB (Abramova and Bratov, 2009), which may affect the selectivity of membranes, as the selectivity of potentiometric sensors for double charge ions highly depends on a molar relation between the ionophore and lipophilic additives. To reduce the curing time 9% of HDDA (w/w) was added to the membrane composition (MCa2) and although this permits to decrease the curing time from 200 seconds to 60 and obtain linear calibration curve in a 10^{-5} to 10^{-1}M concentration interval, the selectivity of this membrane was insufficient (Table 1).

Assuming that the loss of selectivity may be related with another type of photoinitiator (DAR 1173) used and/or with the products of photochemical reactions, we went back to initial composition MCa0 using copolymerisable plasticizer DHI instead of DOS (composition MCa3). This substitution provokes increase in

membrane curing time till 200 seconds. The selectivity was better than in the case of MCa2 composition, but not sufficient for serum analysis. Increase in the ionophore/lipophilic additive molar ratio up to 71 mol% of K-TpClPB (MCa4) did not improve the selectivity.

It must be noted that the absence of free plasticizer within the membrane matrix may cause two problems. Firstly, the solubility of an ionophore and lipophilic additives in the membrane mixture will be decreased, which may seriously affect the sensor parameters like sensitivity, selectivity and the limit of detection. Secondly, this will affect the mobility of charged species within the membrane phase, which will result in a very high membrane impedance (50–200 MΩ) (Abramova and Bratov, 2009). As we reported earlier, copolymerisable plasticizer DHI may successfully used for sodium, potassium and pH ion-selective membranes (Abramova and Bratov, 2010), however in the case of Ca^{2+} ion selective membranes the results were not favourable. It is possible to enhance the selectivity in front of sodium ions by using the mixture of two plasticizer DOS and DHI in 1:1 and 2:1 w/w ratio (membrane compositions MCa5 and MCa6, respectively), but starting from the second week of the constant contact with water solutions selectivity coefficient deteriorate (see Table 1).

An improvement of the selectivity coefficient may be achieved by increasing the length of the alkyl chains of plasticizer (Eugster *et al.,* 1994). For this di-(n-octyl)-itaconate and di-(methylhepyl)-itaconate were synthesised and introduced in membrane compositions MCa7 and MCa8, respectively, but unfortunately the desired effect was not achieved and selectivity of sensors with these membranes was very low.

Another way to alter the selectivity of polymer ion-selective membranes is by changing the nature of polar moieties in the polymer structure. For example, by introduction of cyano, nitroether, amide, keto or alkylester groups in polydimethylsiloxane membranes sensors with good response to nitrate ions were obtained (Antonisse *et al.,* 1996). However, the synthesis of these siloxane monomers and polymers seems to require too much effort. The sensors selective to

K^+ and Ca^{2+} with good analytical performance were obtained by UV copolymerisation of commercially available oligodimethylsiloxane methacrylate with comonomers carrying polar moieties (Edelmann *et al.,* 2005). So, on the next step of our investigations we introduced one of comonomers studied in this work, hexafluorobutyl acrylate (HFBuA). This introduction of 36% (w/w) of HFBuA as a copolymerisable plasticizer (MCa9) resulted in membrane with very high resistance (more than 10 MΩ) that provoked the oscillation of ISFET-meter circuitry (Abramova and Bratov, 2009). To reduce membrane resistance we eliminated the crosslinker HDDA from the main polymer composition (MCa10), which allowed us to obtain the calibration curve of the sensor but the selectivity of these membranes were very low. Taking into account the behaviour of membranes MCa5 and MCa6, the mixture (1:1) of HFBuA and DOS was used for membrane composition MCa11. In this case sensors with Nernstian sensitivity of 28.3 ± 0.3 mV/pCa, linear response in the $0.1x10^{-5}$M concentration range and the detection limit of $3{\cdot}10^{-6}$M were obtained. Sensors showed very good selectivity and stable parameters of calibration curves that permitted us to apply them for the determination of total calcium in bovine blood serum.

Starting from the paper of Fogt *et al.* published in 1985, it is universally (Pranitis *et al.,* 1992) assumed that polymer membrane ISFETs with a membrane deposited directly over a silicon oxide or silicon nitride gate inevitably suffer from the interference caused by penetration of the carbon dioxide and organic acids from a water solution to the membrane organic phase. In the case of photocurable membranes as we have shown (Abramova *et al.,* 2010) the presence of CO_2 and acetate ions in test solutions has no effect on ISFET sensors response. This means that no water layer is formed at the membrane/dielectric interface where acidification, affecting an ISFET signal, may occur due to penetration of CO_2 or acetic acid. This may be attributed to technological peculiarities of the sensor design (Muñoz *et al.,* 1996), when the membrane composition is deposited into a well formed by a polymer encapsulating layer which prevents possible lateral attack of water at the membrane/insulator interface. It also should be mentioned that

adhesion of photocured polyurethane membranes to the silylated surface of silicon dioxide is very good.

The influence of the solution pH changes on calcium selective ISFETs response was tested in a 5×10^{-2}M TRIS buffer containing 5×10^{-3}M $CaCl_2$. The solution pH was changed by adding drops of a solution containing 1M HCl and 5×10^{-3}M $CaCl_2$. Results show that studied polyurethane membrane ISFETs do not have any pH response in the 2.0-5.5 pH range and small pH sensitivity of 2-3 mV/pH between 5.5 and 9.3 values. These results coincided with previous data obtained for calcium selective membrane with DOS as a plasticizer (Bratov *et al.,* 2000). The absence of pH influence in the 2.0-5.5 pH range permits calibration of the sensors and sample measurements in the presence of acetate buffer (see Experimental Section) with good precision. The sensors response slope obtained with calibrations solutions of 1, 2 and 9 mM Ca^{2+} in the presence of 140 mM Na^{+}, 4.5 mM K^{+} and 0.8 mM Mg^{2+} was 28.3 ± 0.3 mV/pCa . The same solutions diluted with 20-fold volume of acetate buffer yield a response with the slope of 28.8 ± 0.4 mV/pCa.

3.2. Analysis of serum samples

Typical sequences of calibration solutions and serum samples used in the total calcium measurement cycle in a flow through cell with two Ca^{2+}- ISFET sensors are shown in Figure 2. Obtained results along with total calcium concentration determined by optical emission spectrometry (ICP-OES) are given in Table 2.

Each serum sample listed in Table 2 was measured by potentiometric method three times and, as can be seen from the presented results, the precision of total calcium determination is very high. Typical accuracy of a potentiometric method for double charge ions concentration measurement according to the Nernst equation is 8-12 % and depends on the stability of a sensor potential and junction potential of the reference electrode (Kost and Hague, 1996). Comparing results obtained by potentiometric method and ICP-OES we may conclude that the accuracy of calcium measurements with ISFET sensors is within 3 - 7 %, which shows really high selectivity and stability of the developed ion selective membranes and the flow-through system as a whole.

Sample	ISFETs	ICP-OES*
10	2.35±0.16	2.25
18	2.23±0.09	2.07
26	2.14±0.08	2.15
41	2.21±0.12	2.13
89	2.14±0.09	2.25
96	2.51±0.14	2.40
100	2.19±0.12	2.30
136	2.37±0.14	2.25
560	2.24±0.11	2.28
561	2.14±0.07	2.10

*Precision of the OES method is 0.8-1.4%

Table 2. Total calcium concentration in bovine blood sera as obtained by two different methods (in mmol/L).

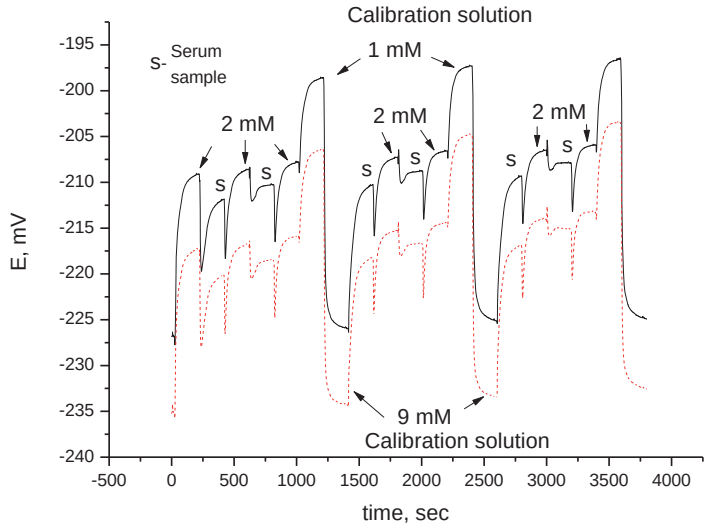

Figure 2. Typical sequences of calibration solutions and serum samples.

Table 3 presents results of the total calcium concentration determined in the flow-through system in serum samples obtained by centrifugation and by coagulation in thrombin test tubes. As follows from the presented results there are no differences between calcium total concentrations in samples with added coagulant and without it. This means that in the case of potential application of the developed measuring system in cattle farms it will be possible to avoid the process of centrifugation of blood samples and to obtain serum samples by coagulation, which is more economic and easier method.

Serum sample	Centrifugation	Thrombin
134	2.02 ± 0.09	1.86 ± 0.11
138	1.78 ± 0.12	1.82 ± 0.11
142	1.92 ± 0.10	1.94 ± 0.11

Table 3. Total calcium concentration determined in serum samples obtained by centrifugation and by coagulation in thrombin test tubes.

4. CONCLUSIONS

In this work application of the ISFET sensors with membrane sensitive to calcium ions for analysis of bovine serum is reported. To enhance the performance and the lifetime of calcium ion sensitive polymer membranes different type of copolymerisable plasticizers were synthesised and investigated. Optimised membrane composition contains a mixture of free (dioctyl sebacate, DOS) and copolymerisable (hexafluorobutyl acrylate, HFBuA) plasticizers, shows Nernstian response and sufficient selectivity in front of sodium, potassium and magnesium ions present in their physiological concentrations. Analytical system based on sequential injection analysis was developed and optimized in terms of the flow conditions, measurement sequence, and calibration solutions composition. The developed system permits to determine ion concentration of total calcium in blood serum samples with standard deviation of 3-7% and may be easily adapted as a handheld instrument for field measurements directly at a farm.

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Chapter VII – General discussion

The goal of this thesis was to study the importance and the real implications of cow subclinical hypocalcemia (SCHC) along with the exploration of new approaches to prevent or mitigate the disease. The consequences of hypocalcemia and, especially subclinical hypocalcemia, are not well elucidated and the strategies to prevent it do not accomplish the needs of the sector. Finding the physiological mechanisms underlying the onset of hypocalcemia, monitoring the incidence of this metabolic disease and setting up new possible strategies to prevent cow hypocalcemia are of great economical importance in the dairy industry.

In the **chapter III** of this thesis, SCHC is defined as a blood calcium (Ca) drop below 8.5mg/dL in cow at the beginning of the lactation. We have demonstrated that it is an illness of great importance for dairy industry since it affects more than 75% of the cows, in contrast with the 50% of dairy cows found by Goff *et al.,* 2014. If the cows have 2 or more parturitions, the risk of suffering from this illness is greatly increased.

We have also described that milk production of SCHC cows is higher than normocalcemic cows (Table 1, chapter III). When analyzed by quartile distribution, the differences affect cows with Ca concentrations lower than 7.399mg/dL (Table 3, chapter III). The higher milk production implies higher Ca requirements and thus it is reasonable that is associated to a greater risk to suffer hypocalcemia. A greater milk production is correlated by the amount of days dry, which is also higher in SCHC cows. A longer dry period would increase the production of the cows (Pezeshki *et al.,* 2010).

When the cow becomes affected by clinical hypocalcemia, it is more susceptible to metabolic and infectious diseases (Goff, 2008; Murray *et al.,* 2008). So far there is not an accurate study of the relationship between subclinical hypocalcemia and other periparturient diseases in dairy cow. In **chapter III** we found a correlation between subclinical hypocalcemia and several periparturient afflictions. To begin with, a high SCC from the first measure is correlated with SCHC and, at the same time, with the incidence of mastitis. Cows with SCHC tend to be 1.5 and 2.56 times more likely to suffer intramammary infections and metritis respectively (Table 2, chapter III). As previous studies suggested (Goff and Horst, 1997; Chamberlin *et al.,*

2013), mastitis can be caused by low Ca concentrations in blood, because they affect contraction of smooth and skeletal muscles, thus affecting the teat sphincter and myometrium. The same affectation in the uterine myometrium at post-partum could also be responsible for the metritis and retained placenta incidence. With retained placenta, the difference becomes clear between healthy cows and cows incurring in severe SCHC (blood Ca < 7.399mg/dL) with thrice the incidence (Table 3, chapter III). It has been described that these two last complications negatively affect fertility (Maizon *et al.,* 2004) and metabolic disorders delay the first detected oestrus and reduce submission rate to AI (Roche, 2006). The fertility of SCHC cows was assessed too and although no differences were found in the AI needed to get the cow pregnant, the heat was definitely delayed. A 61.7% of normocalcemic cows had its first zeal within the two first months after parturition while only 29.4% of SCHC cows did (Table 1, chapter III).

Along with reduced general muscle activation, it has been demonstrated that induced SCHC decreases abomasum muscle contraction (Daniel, 1983; Hansen *et al.,* 2003). Agreeing with these results, in **chapter III** cows with SCHC were 2.71 times more likely to suffer from LDA than normocalcemic cows (Table 2). We also found that ketosis is more likely to happen in SCHC cows, the same as in clinical hypocalcemic cows (Curtis *et al.,* 1983).

From our recent work it is clear that hypocalcemia, clinical or subclinical, is an important illness affecting the welfare and production of dairy cows. In order to understand the exact mechanisms orchestrating subclinical hypocalcemia and to find new targets to settle a new treatment or a preventive strategy, it is very important to study and compare the homeostasis of Ca in subclinical, clinical and normocalcemic animals. Under normal circumstances, there are 3 main hormones regulating blood Ca concentrations: calcitonin, PTH and $1,25(OH)_2D_3$. A decrease in blood Ca triggers the parathyroid gland to secrete PTH, which increases renal Ca reabsorption and induces the expression of 1-a-hydroxylase in the kidney, responsible for producing $1,25(OH)_2D_3$ (Wasserman and Fullmer, 1995). One of the most important effects of $1,25(OH)_2D_3$ is to stimulate Ca absorption through the active transport across intestinal epithelial cells (Perez *et al.,* 2008). In addition,

$1,25(OH)_2D_3$ stimulates, in conjunction with PTH, osteoclastic resorption activity of bone Ca (Horst *et al.,* 2003). By contrast, calcitonin inhibits the resorption of bone Ca and increases blood Ca losses through urinary excretion (Murray *et al.,* 2008). Blood calcitonin concentrations increase in response to hypercalcemia (Austin and Heath, 1981; Findlay and Sexton, 2004) and in response (or even in advance) to feed intake (Roos *et al.,* 1980).

Accordingly, it is known that PTH and $1,25(OH)_2D_3$ increase after a subclinical decrease of blood Ca around parturition (Wasserman and Fullmer, 1995). However the profile and importance of calcitonin during the onset of cow subclinical hypocalcemia is still unknown. In **chapter IV** we demonstrate that when subclinical hypocalcemia starts there is a clear and significant PTH release followed by a compensatory feedback of calcitonin. The calcitonin rise was not affected by the severity of subclinical hypocalcemia, but it was influenced by days from calving since there was a significant interaction with the time post-partum, showing greater calcitonin concentrations in severe subclinical hypocalcemic cows (HSH, Ca levels between 7.5-6.5mg/dL) than in cows with mild subclinical hypocalcemia (LSH, 8.5-7.6mg/dL) at calving (day 0) and at day 2 postpartum (2.25 and 1.89 folds more calcitonin, respectively). Calcitonin causes a decrease in blood Ca concentration, which would accentuate even more the Ca drop in HSH animals. Hence, the difficulties to recover from hypocalcemia are greater in HSH animals and moreover PTHR activation by PTH is not enough to activate $1,25(OH)_2D_3$ and reestablish blood Ca levels. This hypothesis was supported by the fact that PTH concentrations tended to be greater in HSH than in LSH cows while blood levels of $1,25(OH)_2D_3$ were lower ($P < 0.05$) in HSH than in LSH cows (Figure 1, chapter V). This findings support that the production of $1,25(OH)_2D_3$ is not only controlled by PTH and the consequent regulation of 1-a-hydroxylation of $1,25(OH)_2D_3$, but also by PTHR. Previous authors hypothesized an increased sensitivity of the tissues to PTH (Phiilippo et al 1994; Goff *et al.* 2014). Overall, it seems clear that in severe subclinical hypocalcemia, the deficiency of the homeostatic mechanisms relays on the inability of PTH to stimulate the enzyme 1-a-hydroxylation to produce $1,25(OH)_2D_3$, probably due to the amount

and/or sensitivity of the PTHR and to the higher secretion of calcitonin accentuating the Ca drop after parturition.

It has been demonstrated that most prepartum diets based on forages are high in cations and may induce metabolic alkalosis, promoting hypocalcemia (Goff *et al.,* 1998). Although this is not a feasible strategy, induction of metabolic acidosis through the manipulation of the acid-base status creates a negative metabolic balance of Ca and could prevent hypocalcemia at calving (Goff, 1998; Goff 2008). However, the exact physiological mechanisms involved in the prevention of hypocalcemia through metabolic acidosis are not well understood. A deep study of the physiological and molecular changes involved in metabolic acidosis could complement our recent findings and help us identify possible targets to modulate and prevent hypocalcemia using a strategy of easy applicability on the field.

This study (**chapter IV**) indicates that metabolic acidosis enhances the activity of PTH in cattle through an increase of the expression of its receptors PTHR in the glomerular cells of the kidney, potentially leading to an increased coupling of the hormone with its receptor and. In consequence, there would be an increased synthesis of $1,25(OH)_2D_3$, which would ultimately trigger an hypercalcemic response. These results are in agreement with an *in vitro* study performed with rat osteoblast-like cells, where the number of PTH receptors increased under metabolic acidosis (Disthabanchong *et al.,* 2002). Moreover, the results demonstrated that inducing metabolic acidosis impairs the activity of calcitonin *in vitro*, probably by affecting its stability or its binding to the receptor. Therefore, an impairment of calcitonin activity in combination with an increase in the number of PTHR could foster a hypercalcemic status under metabolic acidosis.

Calcitonin appears to be an active hormone involved in the onset and prevention of subclinical hypocalcemia (**chapter IV**). In consequence, in the next chapter of this thesis (**chapter V**), a strategy based on passive immunization to neutralize and control calcitonin activity after parturition was tested. A polyclonal rabbit antibody, obtained through immunization with calcitonin, completely neutralized the activity of synthetic calcitonin and wild type calcitonin *in vitro*. Since the calcitonin receptor

is well conserved across different species, bovine calcitonin can be tested in rats or in human cells conserving its effect (Pham *et al.,* 2005). Synthetic bovine calcitonin was tested in rats to obtain its effective dose. A total of 1µg of hormone decreased the blood Ca in rats in the first 15 minutes but surprisingly increased after 180 minutes. A plausible explanation can be that exogenous calcitonin is rapidly degraded, but still activates the rat hypercalcemic hormones increasing the total blood Ca. When the bovine calcitonin was administrated with polyclonal antibody at a molar ratio 1:3 respectively, the calcitonin activity was completely neutralized and induced an increase in blood Ca. As when using calcitonin alone, the same explanation can be applied here. Part of exogenous calcitonin activates the response of the homeostatis mechanisms to boost the release of Ca, but in this case it doesn't exert its hypocalcemic function because the antibodies neutralize most of the hormone.

To make this a viable strategy in cows, we needed a cheap way to mass-produce a neutralizing antibody. The solution was to produce recombinant antibodies. These antibodies are easier and faster to produce (Holt *et al.,* 2003) and its stability can be improved by mutagenesis (Pansri *et al.,* 2009). A single chain variable fragment (ScFv) was selected from the McCafferty naïve library and cloned into *E.coli*. Using this system, we could produce our anti-calcitonin monoclonal ScFv: B10. The same tests were tried with B10 as with the polyclonal antibodies. However the efficiency of ScFv B10 *in vitro* was lower than polyclonal antibody because the dose (in moles) of B10 had to be 63 times higher than polyclonal antibodies to achieve slightly lower efficiencies of *in vitro* neutralization (85% vs. 99%), probably affected by different half-life and neutralization potential.

Since the cow is a very large animal and its treatment requires a high production of ScFv, preliminary experiments were performed in calves using 2 dosis of ScFv B10. Taking into account the efficiency of polyclonal antibody in rats and the relationship between polyclonal and ScFv antibodies *in vitro*, we decided to maintain a ratio of at least 189:1 (ScFv:calcitonin) in cattle. This prompted us to use a dose of 250:1 ScFv:calcitonin and compare its efficiency with 100:1 ScFv:calcitonin in calves. However, no differences in Ca concentration were observed in calves in any of the

tested concentrations through 8h post-treatment. Although the negative results obtained in this *in vivo* experiment, we decided to go further and test ScFv at 250:1 in cows because we reasoned that the neutralization of calcitonin would be more relevant in the target animals, animals with hypocalcemia, than in normocalcemic animals. However, although animals were hypocalcemic, no effects on blood Ca concentrations of cows after 4 hours of treatment, were observed in comparison with the saline control. At this stage, further experiments to study the half-life of B10 and its biodistribution in cows, along with the effect of polymers, liposomes or mutagenesis to modulate the effective dose, could be relevant to deeply characterize the real insights of the proposed strategy.

While a preventive strategy for subclinical hypocalcemia is developed, the set up of a diagnostic system could be very useful to treat hypocalcemic cows and prevent health negative consequences. Usually, to assess an animal's Ca status, the total Ca concentration is measured. Total Ca is assumed to be directly proportional to ionized Ca, which is the biological active form of Ca. Hence, the development of a diagnostic tool would imply a portable and reliable Ca analysis. In **chapter VI** we optimized ISFETs with a Ca ion selective photocurable polyurethane membrane (Ca-ISFETs). Moreover, a semiautomatic flow-through measuring system was also designed to analyze several serum samples in a row. Measuring each sample by a potentiometric method three times, the precision of total Ca was very high. With a previous typical accuracy of 8-12% for double charge ions, our Ca-ISFET's accuracy was within 3-7%, showing a high selectivity and stability of the whole flow-through and measurement system. We also tried the evaluation of serum samples obtained by coagulation in thrombin tubes to skip the centrifugation step and make it more field-oriented. The same results were obtained with or without coagulant, indicating that the centrifugation step is avoidable and the ISFET-based diagnostic can be applied directly to unprocessed blood samples conferring a quick analysis with a quick and reliable response. With the Ca-ISFETs we can have a tight control in the farm and the hypocalcemic animals can be detected and treated.

In summary, this thesis helps to further understand hypocalcemia and, more specifically, SCHC. We also studied its consequences and possible treatments. SCHC

affects 75% of the dairy cows and has health consequences on the welfare and productivity of the animals. It could be divided into mild or severe SCHC, as the afflictions related to each degree of SCHC are significantly different. Calcitonin plays an important role in the homeostasis mechanism of hypocalcemia, especially at the beginning of lactation, the onset of the illness. Neutralizing calcitonin by passive immunization could be a valid strategy, but further experiments need to be done in order to assess its full potential in bovine. A portable and quick measuring system based on Ca-ISFET chips was developed to be able to tighten the control of the illness in the field.

Chapter VIII – Conclusions

1. Subclinical hypocalcemia is an important disease with an incidence of 75% when an upper calcium threshold of 8.5mg/dL is used

2. Subclinical hypocalcemia is associated with strong consequences in the health of the cows since there is an increased risk to suffer ketosis, LDA, mastitis, metritis and retained placenta. It also affects the fertility of the cows by delaying the zeal apparition.

3. Calcitonin has an active role in the onset of subclinical hypocalcemia, mainly in animals suffering severe subclinical hypocalcemia, because it is released as a compensatory feedback of PTH increase and difficulties even more the blood calcium recovery.

4. The hypercalcemic PTH effect mediated through the binding to PTHR is miss-controlled in cows with severe subclinical hypocalcemia.

5. The expression of PTHR is up-regulated in the kidney under metabolic acidosis increasing the effect of PTH and inducing increased $1,25(OH)_2D_3$ concentrations.

6. In an acidic environment such as metabolic acidosis, there is an impairment of calcitonin activity.

7. The ScFv B10 against bovine calcitonin has been successfully isolated from a naïve library and recombinantly produced in *Escherichia coli* at a yield of 5.35mg/L

8. Polyclonal rabbit antibodies against calcitonin or B10 ScFv neutralize bovine calcitonin activity *in vitro* up to rates of 99% and 85% respectively.

9. Polyclonal rabbit sera against calcitonin, neutralize the hypocalcemic effect of bovine calcitonin in rats and increase the total blood calcium levels, when pre-incubated and administrated at molar rates of 3:1 IgG:calcitonin.

10. No effects on blood calcium have been observed with B10 ScFv when injected at molar rates of 250:1 ScFv:calcitonin in calves or cows under a subclinical hypocalcemic or normocalcemic status.

11. An ISFET sensor with a membrane sensitive to calcium ions can be used to measure calcium in bovine serum and could be adapted for field measurements.

12. A semiautomatic analytical system based on sequential injection analysis was developed and optimized permitting a total calcium determination in blood serum samples with a standard deviation of 3-7%.

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