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Effects of a supplemental calcareous marine algae bolus on blood calcium concentration in dairy heifers

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ABSTRACT. Oral calcium (Ca) supplementation is common in dairy farms. The source and physical form of Ca greatly influence gastrointestinal absorption and consequently blood Ca responses. The aim of this study was to compare the effects of calcareous marine algae (CMA), calcium carbonate (CC) and calcium propionate (CP) on blood total Ca (tCa) and biologically active ionized form (Ca^{2+}) concentrations in dairy heifers. Holstein Friesian heifers (n = 6), 14.8 ± 0.75-month-old, were used in a 3 × 3 repeated Latin square design of treatments. For each treatment, a daily oral bolus was administered for 7 days by balling gun, and serial blood samples were obtained on days 1 and 7. Serum tCa at day 1 was higher for CMA (2.41 mmol/l) in comparison to CC (2.38 mmol/l) and tended to be higher in comparison to CP (2.39 mmol/l). In addition, the area under the curve in the first 60 min (AUC_{ab0-60}) was increased by CMA as compared to both CP and CC, without any difference between the other two sources. Oral Ca bolus supplementation did not improve plasma Ca^{2+} concentration. Effects on mean blood tCa concentration and AUC_{ab0-60} highlight the potential use of CMA as an oral Ca supplementation to promote a faster peak of blood tCa concentration than CC and CP.

Introduction

Hypocalcemia is a metabolic disorder in which homeostatic mechanisms fail to maintain normal blood calcium (Ca) concentrations at the onset of lactation. As a consequence, around 50% of fresh cows experience some degree of subclinical hypocalcemia, while the incidence of clinical symptoms is found in 5–7% of fresh cows (Reinhardt et al., 2011). Prophylactic oral Ca supplementation immediately after calving is a common preventive strategy in both Europe and the United States to maintain normal blood Ca concentrations at the onset of lactation (USDA, 2014; Venjakob et al., 2017). Several studies have highlighted its notable efficacy in preventing hypocalcemia with a significant potential economic return (McArt and Oetzel, 2015; Tadesse and Belete, 2015; Fikadu et al., 2016). In the last years, researchers have increasingly shown interest in new Ca sources that have a higher bioavailability (Walk et al., 2012). *Lithothamnium calcareum* is a calcified red alga of the Corallinacea family, whose main feature is the formation of Ca precipitate in its cell walls, and Ca is the main mineral present in products derived from *Lithothamnium calcareum*. Calcareous marine algae (CMA) are already used in bovine nutrition as a dietary buffer (Cruywagen et al., 2015) and have been reported to

lead to a higher milk protein yield (Wu et al., 2015). However, very little is known about the effects of CMA on blood Ca concentration in cattle. So, the aim of this study was to evaluate the calcium kinetics and metabolism in heifers, comparing the effects of CMA, calcium carbonate (CC) and calcium propionate (CP) on blood total Ca (tCa) and biologically-active ionized form (Ca^{2+}) concentrations, and to study the potential use of CMA, instead of CC and CP, in the diet to prevent the incidence of hypocalcemia.

Material and methods

Ethical statement

All procedures were approved by the Italian Ministry of Health (protocol no 936/2016-PR).

Animals, housing and feeding management

In April 2017, in total six, non-pregnant Holstein Friesian heifers from a single farm in Lodi, Italy were enrolled in the study. Heifers were weighed, transported (15 km) to the zootechnical research centre of the University of Milan and housed in an indoor 20-place experimental tie-stall barn with a controlled temperature of 15 °C. The stall had rubber-crumb mattress surfaces bedded with straw. At the beginning of the study, the heifers were 14.8 ± 0.75-month-old and weighed 535 ± 42 kg. Heifers were acclimated for 7 days prior to the beginning of the study and fed a fixed amount of the following diet for the whole trial period: 9 kg/head/day of ryegrass hay plus 5 kg/head/day of the following complimentary feedstuff, without calcium and phosphorous supplementation: cracked maize 57.42%, wheat bran 18.70%, beet pulp 10%, 48% crude protein soybean meal 10%, sugar cane molasses 2.50%, sodium bicarbonate 0.75%, sodium chloride 0.40%, magnesium oxide 0.08%, trace minerals and vitamin premix 0.15% (2.25 Mcal metabolizable energy (ME)/kg dry matter (DM); 133.1 g crude protein/kg DM; 455.2 g neutral detergent fibre (NDF)/kg DM; 198.3 g starch/kg DM; 333.0 g non-fibrous carbohydrates/kg DM; 30.4 g crude fats/kg DM; 3.3 g Ca/kg DM; 4.5 g phosphorous/kg DM; 1.9 g magnesium/kg DM) formulated to meet National Research Council requirements (NRC, 2001) for replacement heifers.

Experimental design

A 3 × 3 repeated Latin square design was performed, as summarized in Table 1. Each experimental period lasted 14 days and consist of 7 days of adaptation/washout and 7 days of treatment administration, data collection and measurements. Treatments were administered based on their inherent quantity of Ca related to 50 g of CMA obtained from *Lithothamnium calcareum* (15 g of elemental Ca, 30% Ca) (Acid Buf, Celtic Sea Minerals, Cork, Ireland). The Ca content (%) of the CMA was verified before its use by atomic absorption spectroscopy (Eurofins Food Testing Ireland Ltd. – Glanmire Industrial Estate-Glanmire Co., Cork, Ireland). After the start of the trial, the content of Ca in CMA product was verified every two weeks by atomic absorption spectroscopy, underlining strong stability of the product with low variability (average Ca % in the trial period: 29.98 ± 0.38) (Eurofins Food Testing Ireland Ltd. – Glanmire Industrial Estate-Glanmire Co., Cork, Ireland). Consequently, to maintain the same Ca levels, a total of 68 g of CP (22.06% of Ca) and 40 g of CC (37.5% of Ca) were used. The treatment powder was weighed and wrapped using a filter paper disk to create a homemade bolus, which was the same size as a prophylactic magnet for traumatic reticulitis. The boluses were administered for 7 days using a standard balling gun.

Table 1. Experimental design

Heifers	Experimental period[1] 1	
	days 1–7	Adaptation period
	days 8–14	Treatment[2] period 1:
A and B		CC
C and D		CP
E and F		CMA
	Experimental period 2	
	days 15–21	Washout period
	days 22–28	Treatment period 2:
A and B		CP
C and D		CMA
E and F		CC
	Experimental period 3	
	days 29–35	Washout period
	days 36–42	Treatment period 3:
A and B		CMA
C and D		CC
E and F		CP

[1] each experimental period is composed of 14 days: 7 days of adaptation/washout and 7 days of measurement period; [2] treatment: CMA – daily dose of an oral calcareous marine algae bolus providing 50 g of powder for 7 days, CC – daily dose of an oral calcium carbonate bolus providing 40 g of powder for 7 days, CP – daily dose of an oral calcium propionate bolus providing 68 g of powder for 7 days

Sampling and chemical analysis

Approximately 48 h prior to starting the treatments, a 14G × 150 mm polypropylene long-term catheter (Certofix Mono S415, BBraun Italia,

Milan, Italy) was inserted into the left jugular vein of each heifer. A 30-cm, multiple sample Luer-lock extension tube (Extension Set, CareFusion Italia, Milan, Italy) was connected to the catheter, and both devices were sutured to the skin with a USP 2 polyamide suture. Catheter patency was maintained using a 5-ml heparinized saline flush solution (one IU of sodium heparin/ml). Heifers were monitored by a daily clinical examination. On days 1 and 7, blood samples for the baseline determination of tCa and Ca^{2+} concentrations were collected at 08:50 (Tbasal), 10 min before bolus administration. One blood sample was then collected every 15 min for the first 90 min after bolus administration (T15min–T90min), after 120 min (T120min) and then every 60 min for the next 12 h (T3h–T12h) and finally 24 h after bolus administration. Briefly, for each blood sample, 10 ml of blood was collected in a disposable syringe and then discarded to prevent dilution of the saline solution within the blood sample collection device. After this procedure, 9 ml blood samples were collected in vacuum tubes without additives for the determination of the tCa serum concentration. Samples were allowed to clot and then centrifuged at 20 °C for 10 min at 900 *g*. Serum was transferred into an Eppendorf tube and stored at −80 °C until analysis. Blood samples were anaerobically collected, for plasma Ca^{2+} analysis, from the blood collection device in a disposable heparinized 2.5-ml syringe. After each blood sample, the catheter was flushed with 10 ml of saline solution. Ionized Ca concentrations were immediately determined using a blood pH gas-analyser (AVL Opti CCA, Idexx Italia, Milan, Italy). Total Ca concentrations were analysed by an automatic spectrophotometer using ortho-cresolphthalein complexone according to the manufacturer's instructions (BT3500 VET, Biotecnica Instrument, Rome, Italy).

Statistical analysis

Statistical analysis was performed for two separate moments of each experimental period of the study, on the first day (day 1) and at the end (day 7) of the treatment administration phase. Data distribution was evaluated using the Shapiro-Wilk test through a PROC UNIVARIATE (SAS ver. 9.4, SAS Institute Inc., Cary, NC, USA) and non-normally distributed data ($W < 0.98$) were log-transformed and back-transformed for presentation. The area under the curve (AUC) above the baseline of tCa and Ca^{2+} in the first 60 min after administration ($AUC_{ab0\text{-}60}$) was calculated following the trapezoidal rule:

$$AUC_{ab0\text{-}60} = 0.5 \times \Sigma(T_{i+1} - T_i) \times (C_{i+1} - C_i),$$

where: T_i – time at each single blood sampling and C_i – tCa or Ca^{2+} concentration at each time.

Residual estimated maximum likelihood was performed with a Proc Mixed (SAS ver. 9.4, SAS Institute Inc., Cary, NC, USA) using a mixed model for repeated measures that accounted for the fixed effects of treatment (CC, CP or CMA), sampling time (from 1 to 19), their interaction and treatment period (1, 2 or 3), and the random effects of the animal (heifer A, B, C, D, E or F) within the treatment period. The Tukey's post hoc test was applied to differentiate multiple comparisons.

Results and discussion

No signs of disease or distress were observed during the study. Overall blood tCa at day 1 was affected by Ca source ($P = 0.002$), while only a trend for time and Ca source interaction was evident ($P = 0.09$). Average overall serum tCa on day 1 was higher ($P = 0.001$) for CMA (2.41 mmol/l) compared to CC (2.38 mmol/l) and tended to be higher ($P = 0.07$) compared to CP (2.39 mmol/l), while no differences were observed between CC and CP ($P = 0.38$). The Ca source effect was also evident at day 7 ($P < 0.001$), where CMA showed a higher average tCa ($P = 0.03$) compared to CC (2.39 vs 2.37 mmol/l), without any difference between CMA and CP or CP and CC (Figure 1). On day 1, a lower magnitude of Ca source effect was evident for Ca^{2+} ($P = 0.10$), with a higher overall average of Ca^{2+} concentration for CMA (1.25 mmol/l) compared to CC (1.24 mmol/l) ($P = 0.08$) (Figure 2). No difference was recorded between CMA and CP ($P = 0.59$). No Ca source effect was evident at day 7 for serum Ca^{2+}. All the results, both for tCa ($P < 0.0001$ and $P = 0.0004$ at day 1 and 7, respectively) and Ca^{2+} ($P < 0.0001$ and $P < 0.0001$ at day 1 and 7, respectively), at day 1 and 7 were influenced by the time. Moreover, the difference in serum tCa and Ca^{2+} in the same time points was statistically evaluated, with a Tukey's post hoc adjustment. No significant differences were found, comparing serum tCa and Ca^{2+} at the same time points, indicating a correct correlation between those two parameters. The area under the curve in the first 60 min after administration ($AUC_{ab0\text{-}60}$) for tCa at day 1 was affected by treatment ($P = 0.02$) with CMA showing an increased $AUC_{ab0\text{-}60}$ compared with both CP ($P = 0.02$) and CC ($P = 0.04$), without any differences between the other two sources. A similar trend was evident at day 7 ($P = 0.05$), with CMA showing a greater $AUC_{ab0\text{-}60}$ for tCa compared to CP ($P = 0.06$) and

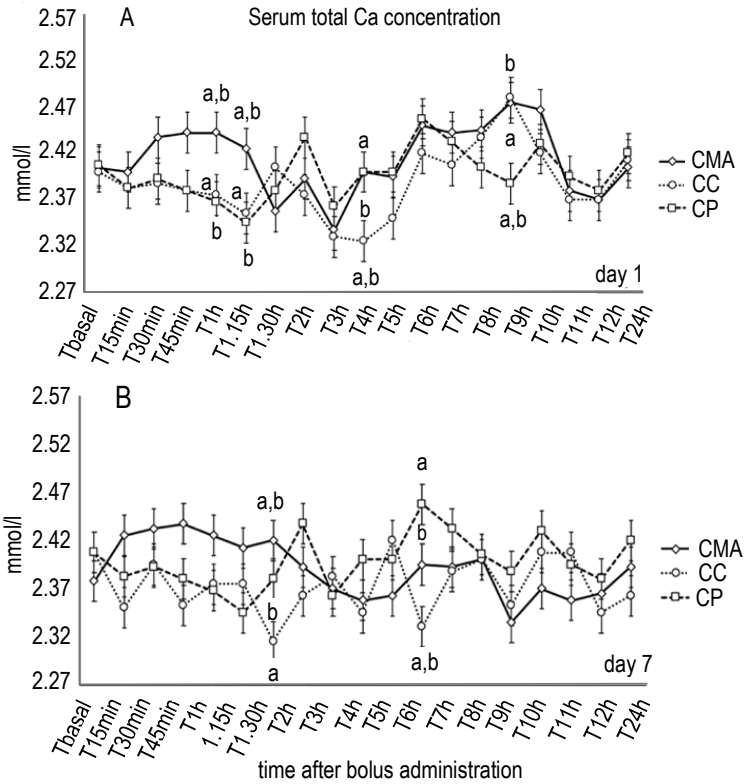

Figure 1. Mean serum total Ca concentration (mmol/l) in six dairy heifers at day 1 (A) and day 7 (B) after bolus administration

[a,b] – the same time points with different superscripts are significantly different between groups at $P < 0.05$. The effect of time was present at $P < 0.0001$ at day 1 and $P = 0.0004$ at day 7. CMA – daily dose of an oral calcareous marine algae bolus providing 50 g of powder for 7 days, CC – daily dose of an oral calcium carbonate bolus providing 40 g of powder for 7 days, CP – daily dose of an oral calcium propionate bolus providing 68 g of powder for 7 days.

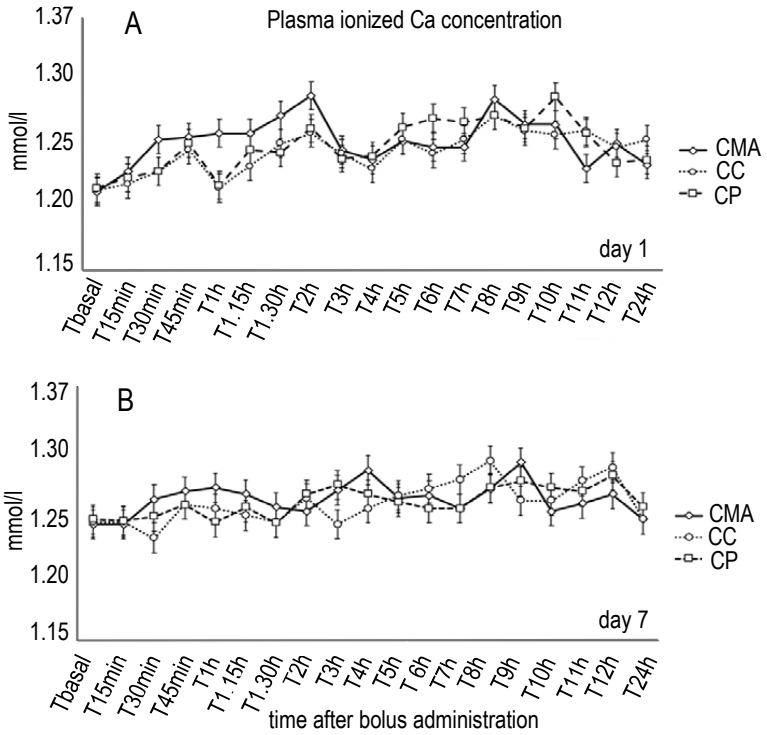

Figure 2. Mean plasma ionized Ca concentration (mmol/l) in six dairy heifers at day 1 (A) and day 7 (B) after bolus administration

The effect of time was present at $P < 0.0001$ at day 1 and $P < 0.0001$ at day 7. The overall effect of time was present at $P < 0.0001$. CMA – daily dose of an oral calcareous marine algae bolus providing 50 g of powder for 7 days, CC – daily dose of an oral calcium carbonate bolus providing 40 g of powder for 7 days, CP – daily dose of an oral calcium propionate bolus providing 68 g of powder for 7 days.

CC (P = 0.09). Due to the smaller magnitude of variation, no differences were evident for $AUC_{ab0\text{-}60}$ of Ca^{2+} concentrations both at days 1 and 7.

The oral Ca bolus increased the serum tCa concentration after treatment. Calcareous marine algae demonstrated a greater overall average for tCa at both days 1 and 7 compared with CC. In addition, at day 1 CMA was higher in AUC above the baseline in the first h after bolus administration. As expected, the serum tCa concentration reached a higher peak immediately after treatment in CMA fed heifers than CP and CC. Previous studies have demonstrated that the CMA is highly soluble at rumen pH (Cruywagen et al., 2015) and when compared to other Ca sources (Walk et al., 2012). This condition ensures a greater Ca ion concentration in gastrointestinal fluids, which may facilitate Ca flow into the blood by the passive paracellular routes (Goff, 2014). Oral Ca treatment, in fact, increases blood Ca concentration through passive transport which is directly related to the solubilities of the Ca sources used (Goff and Horst, 1993). Passive diffusion across the rumen-gastro-intestinal epithelia could explain the fact that the highly available Ca from CMA reached a significantly larger $AUC_{ab0\text{-}60}$ than CP and CC and an average higher blood tCa than CC. These results reflect Goff and Horst (1993), who found that CC needs a very low pH to dissolve, and therefore is unable to provide readily absorbable Ca ions. The pattern of bioavailability of tCa differed between times in the three treatments, with CMA showing a higher bioavailability, testified by the higher values of tCa, in the first T1.30h. This difference, however, vanishes from the second hour after administration. Indeed, from T2h to T24h, the tCa values practically overlap (Figure 1). This could be explained by the passage of calcium ions through either the rumen or intestinal wall. In fact, the initial passage of CMA is through the rumen wall, due to the solubility of CMA at rumen pH. The later flow into the blood is speculated to be through the intestinal wall, after the solubilisation of all test preparations in the abomasum. The flow of calcium ions through the rumen wall has been demonstrated by Schröder et al. (1997) in small ruminants. Another possible explanation is that supplemental dietary Ca has only marginal effects on blood Ca concentration in non-lactating cows because the demand for Ca is relatively low compared with the lactation period. Miltenburg et al. (2016) showed that in dairy cows treated with subcutaneous doses of Ca, cows with lower pre-treatment blood Ca concentration showed a more pronounced increase in blood Ca concentration than normocalcemic cows. During the non-lactating period, the blood mineral status is more easily maintained, while the urinary excretion rate seems more predictive of the absorption rate of supplemental dietary mineral sources (Leno et al., 2017). No differences were detected in blood Ca^{2+} concentrations in the current study. These results agree with those obtained by Oetzel and Miller (2012) who found no significant effect of oral Ca bolus supplementation on Ca^{2+} levels in early lactation dairy cows. In contrast, Sampson et al. (2009) reported short-term effects of Ca boluses on Ca^{2+} plasma concentration at calving and 12 h after calving in 10 dairy cows. Previous studies have suggested that the blood Ca concentration is finely regulated (Martín-Tereso and Martens, 2014). Relatively small alterations in organic fluid Ca^{2+} concentrations represent a life-threatening condition (Case et al., 2007). For this reason, the Ca^{2+} homeostatic system is an ancestral system in eukaryotic cells and maintains adequate Ca^{2+} concentrations also in extreme physical challenges (Case et al., 2007). This condition could explain the fact that no differences were observed in Ca^{2+} plasma concentrations after bolus administration because the animals used in our study were limited to healthy normocalcemic heifers which did not experience Ca redirection from the blood to the mammary gland during the experiment.

Conclusions

The study highlighted the ability of *Lithothamnium calcareum* to support the mean blood Ca concentration and ensured a faster peak of blood total Ca concentration compared to calcium carbonate (CC) and calcium propionate (CP). Our findings suggest that calcareous marine algae (CMA) obtained from *Lithothamnium calcareum* may be used as a prophylactic oral Ca supplementation. Future field trials should assess the effects of macrominerals contained in CMA in the prevention of subclinical hypocalcemia during the early-lactation period with a focus on the effects on health outcomes, and productive and reproductive performance.

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Conflict of interest

The authors declare that there is no conflict of interest.

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