The Effect of Calcium-Naloxone Treatment on Blood Calcium, β-Endorphin, and Acetylcholine in Milk Fever

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ABSTRACT

Milk fever is a postpartum syndrome of cows characterized by acute hypocalcemia, which reduces the release of acetylcholine (ACH), inducing flaccid paralysis and recumbency. Our aim was to evaluate the effect of calcium (Ca2+) combined with naloxone (Nx, an opioid antagonist; Ca2+-Nx) on plasma concentrations of ACH, β-endorphin (βE), and Ca2+ just before treatment (T0) and at 15, 30, and 90 min after treatment (T15, T30, and T90, respectively). Thirty cows were divided into 3 groups of 10 cows each. In group A1, cows affected by milk fever were treated (i.v.) with a combination of 0.2 mL/kg of body weight (BW) of Ca2+ borogluconate (20%) and 0.01 mg/kg of BW of Nx hydrochloride dihydrate. In group A2, cows affected by milk fever were treated (i.v.) with 2 mL/kg of BW of Ca2+ borogluconate (20%). In group C, healthy cows were treated (i.v.) with a combination of 0.2 mL/kg of BW of Ca2+ borogluconate (20%) and 0.01 mg/kg of BW of Nx hydrochloride dihydrate. Cows underwent treatments within 24 h of calving. Blood samples were collected at T0 and at T15, T30, and T90 for quantitative determination of ACH, βE, and Ca2+. The cows in groups A1 and A2 recovered within a mean of 20 ± 10 min, although 4 cows in group A2 underwent a relapse. Blood Ca2+ concentrations in group C increased slightly at T30 and at T90 (T30: 8.8 ± 0.6 mg/dL; T90: 8.7 ± 0.6 mg/dL) after treatment, whereas the response in groups affected by milk fever was similar, even though Ca2+ concentrations showed a sharp increase (A1: 8.9 ± 0.8 mg/dL; A2: 6.0 ± 0.7 mg/dL), particularly at T15 in group A1. Concentrations of βE showed a similar pattern in groups A1 and C, with an increase at T15 (A1: 8.2 ± 1.0 ng/mL; C: 2.7 ± 0.4 ng/mL) and a subsequent decrease until T90 (A1: 1.4 ± 0.3 ng/mL; C: 1.4 ± 0.4 ng/mL), whereas BE remained constant throughout in group A2. Concentrations of ACH in group A1 decreased significantly between T0 and T15, T30, and T90 (T0: 7.2 ± 1.1 nmol/L; T15: 4.2 ± 1.2 nmol/L; T30: 2.9 ± 0.8 nmol/L; T90: 3.1 ± 0.3 nmol/L), whereas in group A2, it did not change. In group C, concentrations of ACH decreased at T15 and increased again at T30 (T15: 1.1 ± 0.3 nmol/L; T30: 3.2 ± 0.7 nmol/L). Our results suggest that administration of Ca2+-Nx, which restored the physiological Ca2+ concentrations, might have an effect on nicotinic receptors by restoring the normal neuromuscular transmission at the motor endplate.

Key words: milk fever, acetylcholine, β-endorphin, calcium-naloxone

INTRODUCTION

Milk fever is one of the most common metabolic disorders in dairy cattle, with an incidence of 5 to 10% per lactation. In milk fever, the homeostatic mechanisms fail to maintain normal plasma calcium (Ca2+) concentrations (9 to 10 mg/dL; Goff et al., 1995). Plasma Ca2+ concentrations are controlled by the coordinated actions of calcitropic hormones, such as the parathyroid hormone and 1,25-dihydroxyvitamin D3, the concentration of which increases in response to hypocalcemia and acts to augment the pool of plasma Ca2+ (Horst and Reinhardt, 1983). Milk fever is associated with the sudden onset of lactation and usually occurs within 72 h of calving (Sorensen et al., 2002).

At parturition, cows have an increased Ca2+ requirement, and if they are unable to respond quickly to this demand, hypocalcemia develops. In most of the mammalian species, this condition is associated with a progressive increase in endogenous opioid peptides (EOP), which occurs during parturition (Petraglia et al., 1985; Sciorsci et al., 2001). These EOP, particularly β-endorphins (BE), activate their specific receptors, giving rise to many cellular responses, including blockade of the voltage-gated Ca2+ channels and opening of the K+ channels, which act synergistically, thereby inhibiting the release of acetylcholine (ACH) from the presynaptic membrane (Kim et al., 2005). This inhibition impairs normal neuromuscular transmission and muscle contraction (Sciorsci et al., 2000; Minoia and Sciorsci, 2001; Mayerhofer and Fritz, 2002).
Based on this knowledge, Sciorsci et al. (2001) demonstrated that administration of Ca\(^{2+}\) and naloxone (Nx), an opioid antagonist, together (Ca\(^{2+}\)-Nx) resulted in a faster and better recovery from milk fever than that achievable from administration of either Nx or Ca\(^{2+}\) alone. Thus, our aim was to evaluate the effects of Ca\(^{2+}\)-Nx on plasma concentrations of ACH, \(\beta\)E, and Ca\(^{2+}\).

**MATERIALS AND METHODS**

**Experimental Animals**

The study was performed between June 2005 and April 2006 on 30 Friesian cows (5 to 8 yr old). All the cows delivered their calves no more than 24 h before the treatments, with normal delivery and without placental retention. The animals had a mean BW of 600 kg (range 560 to 650 kg) and were maintained on farms in the south of Italy (Bari, Apulia). The animals were between the third and the fifth lactation, with average milk production ranging from 8,300 to 8,500 kg per lactation. Cows were restrained in tie stalls and fed hay, concentrate, and minerals, with access to water ad libitum. All cows underwent a clinical examination resulting in the diagnosis of milk fever (n = 20) within 24 h of calving. The diagnosis was performed by reference to the anamnesis, the evaluation of clinical symptoms (anorexia, recumbency, absence of rumination, tachycardia, tachypnea, and muscular spasms followed by flaccid paralysis) and plasma Ca\(^{2+}\) concentrations <6 mg/dL (Lindsay and Pethick, 1983). The cows were divided into 3 groups: groups A1 and A2, made up of 10 cows each that were affected by milk fever; and group C, made up of 10 healthy cows. In the first week after parturition, all the cows underwent a daily general health examination to evaluate their clinical status. All the procedures were carried out in accordance with the Italian Legislation on animal care (DL 116/92).

**Treatment and Blood Collection**

Groups A1 and C received the same treatment, consisting of a single i.v. infusion of 0.2 mL/kg of BW of Ca\(^{2+}\) borogluconate (20%; Fatro, Ozzano Emilia, Italy) and 0.01 mg/kg of BW of Nx hydrochloride dihydrate (Sigma, Milano, Italy) administered soon after the clinical examination. Group A2 was treated with a single i.v. administration of 2 mL/kg of BW of Ca\(^{2+}\) borogluconate (20%; Fatro; Sciorsci et al., 2001).

Blood samples were collected before (T0) and at 15 (T15), 30 (T30), and 90 (T90) min after treatments for the analysis of ACH, \(\beta\)E, and Ca\(^{2+}\) concentrations. All the blood samples were collected by jugular venipuncture with refrigerated Vacutainer tubes and were maintained at 4°C until taken to the laboratory within 2 h.

**ACH Determination**

The blood samples for ACH determination were collected in Vacutainer tubes containing lithium-heparin, with the addition of 2.5 mmol of physostigmine (Sigma). They were centrifuged at 1,620 \(\times\) g for 10 min at 4°C. The plasma was stored at −20°C in Eppendorf tubes (Eppendorf, Milan, Italy) with the addition of 0.25 mmol of physostigmine, until analysis. The analysis was performed by using HPLC-electrospray ionization mass spectrometry, with an 8 mpos dead-end path column (Superchrom, Milan, Italy). The HPLC method was a modified isocratic reversed-phase ion-pairing procedure. The mobile phase was prepared by adding 1 mL of heptafuorobutyric acid to 980 mL of water, followed by 20 mL of methanol. Luna C18 HPLC columns were used (3-μm particles, 2.0 × 150 mm; Phenomenex, Torrence, CA). The column was eluted isocratically at a flow rate of 0.3 mL/min and was maintained at 60°C. The mass spectrometer was operated either in the selected ion-monitoring mode or in the selected reaction-monitoring mode. The selected reaction-monitoring experiments monitored a collision-induced dissociation transition for each of the compounds. The transition producing the most abundant ion fragments was selected for the analysis (146+/87+ for ACH). Detection was performed by using a Thermo mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with the manufacturer’s heated capillary atmospheric pressure ionization interface operating in the electrospray ionization mode. The method detected ACH and its primary degradation product, choline, at the 10-fmol level, with an analysis time of less than 6 min. The intraassay CV was <2%.

**\(\beta\)E Determination**

The blood samples for \(\beta\)E determination were collected by using Vacutainer tubes containing EDTA and aprotinin (500 kIU; Sigma). The samples were centrifuged at 1,620 \(\times\) g for 10 min at 4°C and plasma was stored at −20°C in Eppendorf tubes containing 50 kIU of aprotinin until analysis. \(\beta\)-Endorphin determination was performed by using the Basic Robot Immunoassay Operator 4.54 (Radim, Pomezia, Italy), which relied on an immunoenzymatic method, and a Peninsula kit (Peninsula Laboratories Inc., San Carlos, CA) specific for bovine species (sensitivity 0.03 to 0.06 ng/mL). The intraassay CV was <5%.
Ca²⁺ Determination

Blood samples for Ca²⁺ determination were collected in serum Vacutainer tubes. The samples were centrifuged at 1,620 × g for 10 min at 4°C. The sera were stored at −20°C in Eppendorf tubes until analysis. Calcium determination was by a colorimetric method with a Seac kit (Radim; sensitivity 0.6 mg/dL). The intraassay CV was <0.81%.

Statistical Analysis

All values are expressed as mean ± standard deviation. The 1-sample Kolmogorov-Smirnov test was used to assess the normal (Gaussian) distribution of data. The statistical analysis was then carried out following a nonparametric approach with a 2-way Friedman nonparametric test. The Kruskal-Wallis test was applied to investigate the differences among the 3 groups. The Bonferroni correction was used to account for multiple comparisons. The statistical significance level was set at <5%. The statistical tests were performed by SPSS and Matlab software (Mathworks, Torino, Italy).

RESULTS

Cows in groups A1 and A2 recovered from milk fever within 20 min after treatment (able to stand, restoration of rumination, and displaying a normal heart rate and normal respiration), although 4 cows in group A2 subsequently relapsed. Figures 1, 2, and 3 show the concentrations and the profiles of ACH, βE, and Ca²⁺ concentrations in the 3 groups.

Serum Ca²⁺ in groups A1 and C increased from T0 to T15 (P < 0.01). It remained constant in A1 (Figure 1), whereas it increased at T90 in C cows. In group A2, Ca²⁺ concentration increased from T0 to T30 (P < 0.01), remaining constant from T30 to T90. At T0, mean Ca²⁺ concentrations were different between groups A1 and C and groups A2 and C (P < 0.01). At all other times, Ca²⁺ concentrations in group A2 were lower than in groups A1 and C (P < 0.01).

In groups A1 and C, βE concentrations found at T15 were greater than those found at T0, T30, and T90 (P < 0.01). No significant differences were observed in group A2 among the different times. Group A1 had greater βE concentrations than group C at T0, greater βE concentrations than groups A2 and C at T15, greater βE concentration than group C at T30, and lower βE concentration than group A2 at T90, peaking at T15 (Figure 2).

Acetylcholine concentration in group A1 was higher at T0 compared with T15, T30, and T90 (P < 0.01; Figure 3). In group A2, ACH decreased from T0 to T30. The concentration of ACH in group C at T15 was less than the concentrations found at T0, T30, and T90 (P < 0.01). The comparisons among the 3 groups clearly showed that at T0, ACH concentrations detected in the groups affected by milk fever were greater than that found in the healthy group. At T15, differences were noted between groups A1 and A2 and groups A1 and C.
At T30 and T90, ACH concentration in group A2 was greater than the ACH concentrations in groups A1 and C.

**DISCUSSION**

The combined Ca\(^{2+}\)-Nx treatment did not induce side effects in the animals treated. This study demonstrated the effect of this new therapeutic approach on the restoration of blood concentrations of ACH, βE, and Ca\(^{2+}\) and on the rapid clinical recovery of cows affected by milk fever.

Concentrations of Ca\(^{2+}\) in group A1 reached the same values as obtained in the healthy group, whereas in group A2, Ca\(^{2+}\) concentrations were lower than those found in the other groups. This demonstrated that administration of Ca\(^{2+}\)-Nx may be a more valuable therapeutic approach than Ca\(^{2+}\) alone for recovery from milk fever. The observed increase of Ca\(^{2+}\) in A1 may be due to the effect of the administration of exogenous Ca\(^{2+}\) and displacement of βE from receptors induced by naloxone, with a subsequent restoration of blood Ca\(^{2+}\).

Moreover, naloxone alone increased blood Ca\(^{2+}\) levels (Frago et al., 2007). Lower serum Ca\(^{2+}\) concentrations were found in group A2 than in group A1, notwithstanding a higher infusion rate of Ca\(^{2+}\). This finding can be explained in different ways. Our hypothesis is that EOP, found in greater concentrations in cows affected by milk fever, inhibit the release of the antidiuretic hormone (Grossman et al., 1980), thus impairing renal absorption of Ca\(^{2+}\) and allowing for a greater urine production and Ca\(^{2+}\) elimination.

Furthermore, EOP block Ca\(^{2+}\) channels, impairing its utilization by the cells (Sciorsci et al., 2001). Consequently, more Ca\(^{2+}\) is excreted than is retained by the cells. Moreover, administration of high doses of Ca\(^{2+}\) further contributes to increased urine production (Sansoè and Wong, 2007), and thus renal excretion of Ca\(^{2+}\). Finally, a high infusion rate of Ca\(^{2+}\) within minutes widely inhibits parathyroid hormone secretion, lowering blood Ca\(^{2+}\) concentrations (Fukagawa and Kurokawa, 2002).

Concentrations of βE were greater in groups A1 and A2 than in group C at T0, thus confirming the link between βE and milk fever suggested by Sciorsci et al. (2001). In group A1, the observed changes reflect a therapeutic effect of naloxone (half-life 15 to 20 min; Panerai, 1998), which antagonizes opioids on their membrane receptors. As a consequence, an increase in blood concentrations of βE occurred 15 min after the administration of Ca\(^{2+}\)-Nx, whereas the subsequent decrease was probably due to degradation exerted by the plasma proteases or to the fading of the pharmacological effects of naloxone, or both. These changes did not occur in group C because βE concentrations in the healthy group were significantly lower at T0 than βE concentrations in the affected groups. Thus, the increase was not as dramatic as the one observed in group A1 at T15.

In group A2, treatment with Ca\(^{2+}\) alone did not induce any change in concentrations of βE, thus suggesting βE were not displaced from their receptors. This condition may have been responsible for the relapses that occurred in this group, thus confirming the role of the EOP in the onset of milk fever.

Acetylcholine concentration at T0 was greater in cows affected by milk fever than in healthy cows, suggesting an association among greater concentrations of circulating ACH, low plasma Ca\(^{2+}\), and milk fever. Concentration of ACH in group A1 decreased from T0 to T15 through T90, reaching the values found in group C at T30, whereas it decreased from T0 to T30 in group A2. This difference highlights the effect of naloxone, which may enhance postsynaptic Ca\(^{2+}\) turnover, inducing the expression of nicotinic receptors (McManaman et al., 1981; Smilowitz et al., 1981). With the greater concentrations of ACH detected at T0 in the groups affected by milk fever, we hypothesize that ACH could be released from the presynaptic vesicles in the neuromuscular plaque but that postsynaptic receptor responsiveness may be inadequate.

Administration of Ca\(^{2+}\)-Nx, which restores the physiologic Ca\(^{2+}\) concentrations in cows with milk fever,
might have had an effect on nicotinic receptors, thus decreasing blood concentrations of ACH and restoring normal neuromuscular transmission, which was clinically manifested by the end of recumbency.

**CONCLUSIONS**

The results obtained suggest that in milk fever, the release of ACH may occur, even if it cannot adequately bind to its specific postsynaptic receptors. This confirms the efficacy of the therapeutic protocol consisting of the combination of Ca$^{2+}$ and Nx. Our study provides the basis for further research to better define the exact role of βE and ACH in the pathogenesis of milk fever.

**ACKNOWLEDGMENTS**

We dedicate this work to our late lamented, respected senior professor Paolo Minoia. We thank Lara Castellana for statistical support.

**REFERENCES**


