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FULL PAPER

Clinico-pathological findings in peripartum dairy cows fed anion salts lowering the dietary cation-anion difference: Involvement of serum inorganic phosphorus, chloride and plasma estrogen concentrations in milk fever

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Abstract

In our previous study, it was demonstrated that the administration of anion salts, which slightly lower the dietary cation-anion difference (DCAD), in the prepartum period is safe and effective for preventing milk fever in multiparous cows. In the present study, several clinico-pathological constituents in serum and urine, which might be related to milk fever, were analyzed using stored samples from the previous study to identify clinico-pathological parameters for easily evaluating the efficacy of lowering DCAD and to further investigate the mechanism by which lowering DCAD prevents milk fever. Among the parameters analyzed in the present study, inorganic phosphorus (iP) was involved in milk fever because the serum concentration and urinary excretion of iP were significantly higher in the group of primiparous cows (heifer group), which did not develop hypocalcemia, than those in other groups of multiparous cows. Serum chloride concentrations in the heifer group and the group of multiparous cows fed anion salts (anion group) tended to remain higher than those in other control groups of multiparous cows suggesting that serum chloride concentration may be utilized for evaluating the status of metabolic acidosis and the efficacy of lowerng DCAD in dairy cows fed anion salts. In addition, plasma estradiol-17β concentration in the heifer group tended to be lower at parturition compared with that in other multipa-

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rous groups suggesting that estrogen known as a potent inhibitor of bone resorption may be involved in developing milk fever.

Key Words : chloride, cow, dietary anion-cation difference, phosphorus, milk fever.

Introduction

Milk fever or parturient hypocalcemia is a metabolic disorder that generally affects dairy cows around parturition, and decreases the productivity of dairy cows on dairy farms.[20,31,33] Because of the complex causes of milk fever, ideal protocols for preventing this disease have not been established, although a number of strategies have been proposed.[3,4,16,17,29] The difference in mEq of dietary cations and anions (DCAD) is most often expressed as $[(Na^+ + K^+) - (Cl^- + S^{--})]$. This equation implies that a mEq of chloride and a mEq of sulfate are equipotent in their ability to alter acid-base balance of the cow. Manipulation of DCAD in prepartum diets has also been one of the strategies proposed, and has been successful in lowering blood pH and reducing the incidence of hypocalcemia.[12,15,19] However, this strategy has some risks that excessive lowering of DCAD induces severe metabolic acidosis resulting in a decline in dry matter intake and productivity.[5,20,22]

In our previous study,[24] we examined the effect of slightly lowering DCAD on milk fever using multiparous and primiparous cows in a commercial dairy farm in Hokkaido, Japan. As a result, it was demonstrated that the administration of anion salts slightly lowering DCAD in the prepartum period is safe and effective for preventing hypocalcemia in multiparous cows. The total incidence of hypocalcemia in cows fed anion salts decreased to approximately half of that in control cows not fed any supplemental salts. Safe and mild metabolic acidosis induced by anion salts could be evaluated by urinary pH (6.8-7.0) and might keep serum total and ionized Ca concentrations relatively high at parturition. This successful outcome may be due to increased responsiveness to Ca requirement at parturition in cows fed anion salts, but it was unrelated to the excretion of parathyroid hormone and 1,25-dihydroxyvitamin D. In addition, primiparous cows, which did not develop hypocalcemia, originally had a high potential to respond to the sudden Ca demand at parturition, and interestingly, their peripartum Ca metabolism was in some respects similar to that in multiparous cows fed anion salts. Therefore, the administration of anion salts to multiparous cows may approximate Ca metabolism in multiparous cows to that in primiparous cows.

In our next study,[25] we focused on several bone biomarkers that might show the activation of Ca metabolism in order to investigate the mechanism by which lowering DCAD prevents milk fever. For this purpose, the biomarkers including tartrate-resistant acid phosphatase (TRAP), bone-specific alkaline phosphatase (BALP) and osteocalcin, were analyzed. As a result, serum TRAP activity, which is one of biomarkers showing activation of mature osteoclast activity and bone resorption, was well associated with the administration of anion salts because among the three multiparous groups, only the anion group fed anion salts showed increased activity of TRAP, which rose to a markedly high level in primiparous cows. This finding suggested that Ca in the plasma pool was mobilized smoothly from bone-bound Ca via mature osteoclasts at

parturition, which might be due to prior activation under mild acidosis induced by slightly lowering DCAD. Therefore, TRAP is the optimal biomarker to monitor the activation of Ca metabolism in dairy cows fed anion salts. However, a special laboratory technique[27] and relatively high cost is necessary to measure TRAP activity.

In the present study, we focused on clinico-pathological constituents in serum and urine in turn, which are easily measured even at commercial sites at a relatively low cost, and also examined estrogen, a known inhibitor of Ca resorption in bone.[34] These constituents were analyzed using stored samples from multiparous cows fed anion salts in our previous study,[24] and compared with those in multiparous and primiparous cows not fed anion salts, in order to identify clinico-pathological parameters for easy evaluation of the efficacy of lowering DCAD and to further investigate the mechanism by which lowering DCAD prevents milk fever.

Materials and methods

Experimental design

In the present study, serum and urine that had been collected in our previous study[24] were used to analyze several constituents that may be related to milk fever. Briefly, the experimental design of our previous study is described as followed. Thirty pregnant multiparous Holstein cows were divided into three groups of ten animals each, i.e., anion, non-anion and control groups. Ten pregnant primiparous cows (heifer group) were also used. All cows were fed the same standard diet until calving. The cows in the anion group were given anion salts along with Ca via catheter every day from 21 days before the expected date of parturition until the actual date of parturition. The supplemental salts consisted of 115 g of $CaCO_3$, 42 g of $CaHPO_4$, 65 g of $MgSO_4 \cdot 7H_2O$ and 80 g of $CaCl_2 \cdot 2H_2O$ as a daily dose for each cow. The cows in the non-anion group were given only the high Ca supplement but without sulfate and chloride salts via catheter as in the anion group. The cows in the control and heifer group were not fed any supplemental salts. DCADs (mEq/100 g of dietary dry matter) are +1.2, +14.6, +15.3 and +15.3 in the anion, non-anion, control and heifer groups, respectively before parturition, and +17.6 in the all groups after parturition. Venous blood and urine were collected 40, 14, 7 and 3 days before the expected date of parturition (days −40, −14, −7 and −3), and on 3 and 7 days after parturition (days +3 and +7). The sample at parturition (day 0) was collected immediately after parturition.

Analysis of clinico-pathologic variables

The concentrations of magnesium (Mg), iP and creatinine in serum and/or urine were measured using an automated biochemical analyzer (COBAS MIRA plus, Hoffmann-La Roche, Basel, Switzerland). Urinary Mg and iP excretions were expressed as mg of Mg or iP per mg of creatinine. Serum Cl concentration was measured using an ion-selective electrode method. Plasma steroids were extracted using diethyl ether as described previously[21] with a slight modification. In brief, for the measurement of estradiol-17β, 4 ml of plasma was shaken with 12 ml of diethyl ether for 15 min. The mixture was frozen in acetone with beads of dry ice for 2-3 min. The ether phase was decanted into a 15 ml-glass tube, evaporated by a centrifugal evaporator and redissolved in 200 μl of 0.04 M phosphate-buffered saline (pH 7.2) supplemented with 0.1% bovine serum albumin (BSA-PBS). The glass tube was rinsed with 2 ml of diethyl ether and again evaporated. For defatting, the extracted sample was shaken with 0.5 ml

of acetonitrile and 1 ml of hexan for 3 min. The hexan phase was discarded, then 1 ml of hexan was added again and again discarded after shaking. This procedure was repeated three times. After removal of the hexane phase, the acetonitirile phase was evaporated and dissolved in 200 μl of BSA-PBS. This solution was used for assay of estradiol-17β. Estradiol-17β was determined using competitive double antibody enzyme immunoassay as described previously.[21] The primary antiserum against estradiol-17β was anti-estradiol-17β-6-CMO-BSA (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan). The secondary antiserum was goat anti-rabbit serum (Seikagaku Co., Tokyo, Japan). The assay sensitivity was 1.0 pg/well for estradiol-17β. The intra-and inter-assay coefficients of variations were 5.7% and 4.9% for estradiol-17β. Overall steroid recoveries from serum were 93.1 ± 8.6% for estradiol-17β.

Statistics

Statistical analysis was performed using 1-way factorial analysis of variance with post hoc tests (Tukey method). These analyses were carried out on a computer using a statistical software package (SYSTAT, Evanston, IL, USA). Values of $P<0.05$ were considered significant. The following marks were used to indicate significance: $^{\alpha}P<0.05$, $^{\beta}P<0.01$, $^{\gamma}P<0.005$, $^{\delta}P<0.001$ vs heifer group. $^{a}P<0.05$ vs control group. $^{A}P<0.05$ vs non-anion group.

Results

The change in serum Mg concentration was similar in all groups (Figure 1A). The concentration was increased only on day 0 and then rapidly decreased on day +3 or +7 to slightly less than the prepartum level. Urinary Mg excretion was also highly similar in all groups, and the level hardly changed throughout the experimental period although it tended to decrease on days −3 and 0 (Figure 1B).

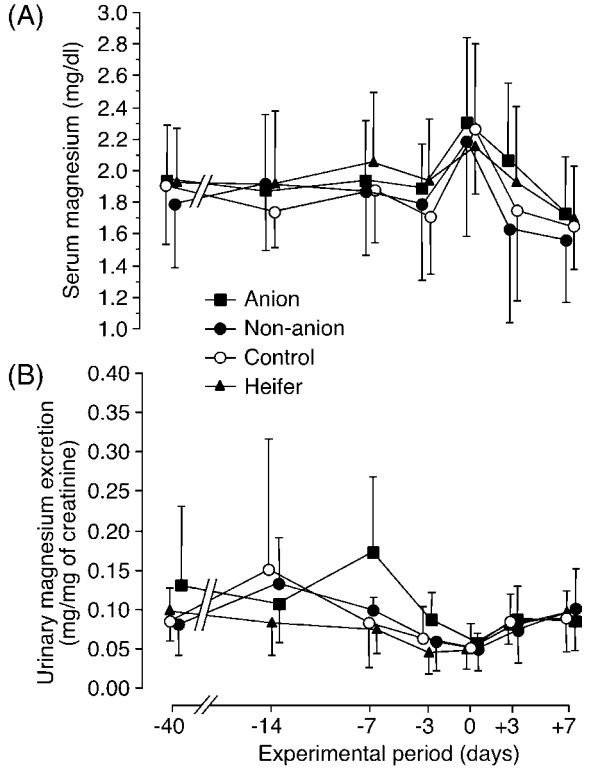

Figure 1. Changes in serum magnesium concentration (A) and urinary magnesium excretion (B) during the pre and postpartum period. Day 0 indicates the time immediately after parturition. Data represent the mean ± standard deviations. The details of each group and the marks used to indicate significance are explained in Materials and Methods.

The change in serum iP concentration was almost the same in the three multiparous groups, i.e., the anion, non-anion and control groups (Figure 2A). The serum iP concentration in these groups did not change in the prepartum period, then decreased rapidly on day 0 and returned to the prepartum level on day +3 or +7. However, the change in the heifer group differed from that in the multiparous groups. In the prepartum period, serum iP concentration in the heifer group remained markedly higher than that in all other groups of multiparous cows, and occasionally there

was a significant difference between the heifer and multiparous groups. Serum iP concentration in the heifer group also decreased on day 0, but the level was still significantly higher than that in the non-anion and control groups. After parturition, the concentration in the heifer group rapidly increased and peaked on day +3, which was similar to that in the multiparous groups in the prepartum period.

Urinary iP excretion in three multiparous groups remained very low especially in the prepartum period, but the level in the anion and control groups slightly increased after parturition (Figure 2B). However, urinary iP excretion in the heifer group remained markedly high throughout the experimental period, compared with that in all other groups of multiparous cows, but decreased temporally on days −3 and 0. There was a significant difference between the heifer and all multiparous groups from days −40 to 0.

Serum Cl concentration in all groups gradually increased throughout the prepartum period, then reached a peak on day 0, and returned to the initial level by day +7 (Figure 3). However, the change in the anion and heifer groups apparently differed from that in the non-anion and control groups. The concentrations in the anion and heifer groups tended to remain higher compared with those in the non-anion and control groups. This tendency was obvious especially from the prepartum period until parturition. On day −14, there was a significant difference between the anion and non-anion groups and between the heifer and non-anion groups, and the P-value was relatively low (P = 0.052) between the

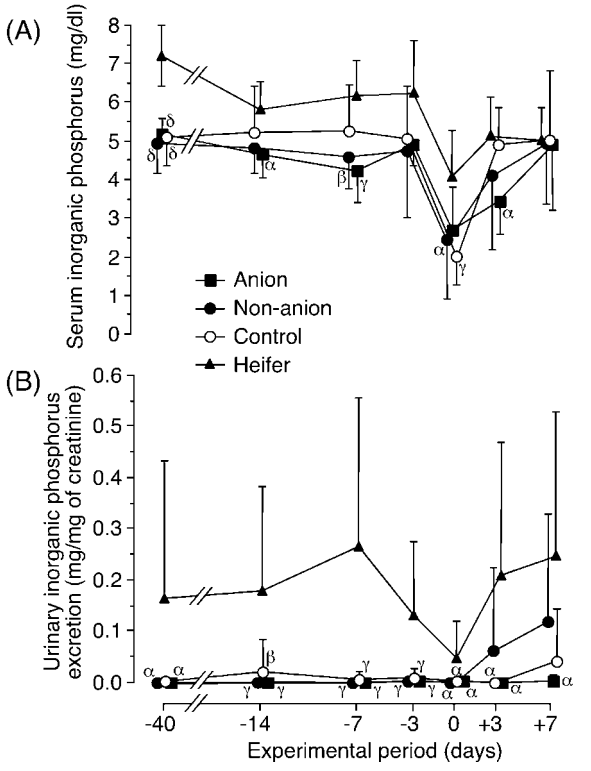

Figure 2. Changes in serum inorganic phosphorus concentration (A) and urinary inorganic phosphorus excretion (B) during the pre and postpartum period. Day 0 indicates the time immediately after parturition. Data represent the mean ± standard deviations. The details of each group and the marks used to indicate significance are explained in Materials and Methods.

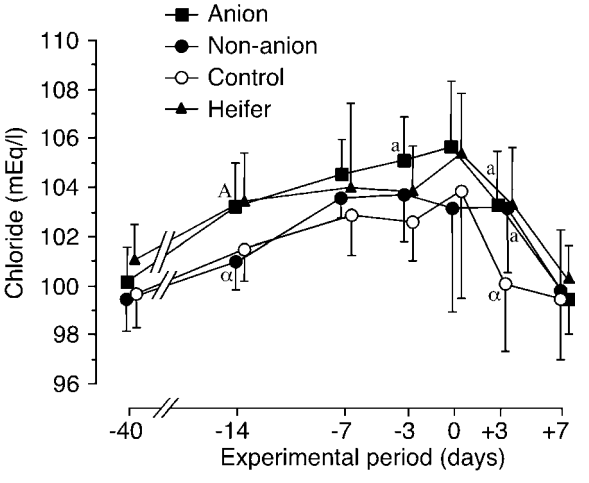

Figure 3. Change in serum chloride concentration during the pre and postpartum period. Day 0 indicates the time immediately after parturition. Data represent the mean ± standard deviations. The details of each group and the marks used to indicate significance are explained in Materials and Methods.

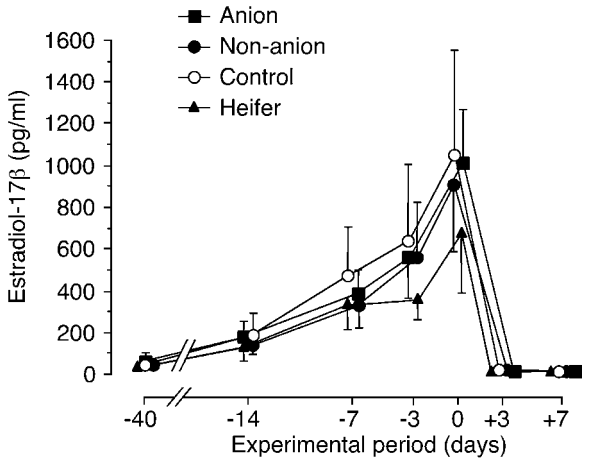

Figure 4. Change in plasma estradiol-17β concentration during the pre and postpartum period. Day 0 indicates the time immediately after parturition. Data represent the mean ± standard deviations. The details of each group and the marks used to indicate significance are explained in Materials and Methods.

heifer and control groups. In addition, a significant difference between the anion and control groups was observed on days −3 and +3.

Plasma estradiol-17β concentration in all groups started to increase on day −14, peaked on day 0, then rapidly decreased to a very low level on day +3 (Figure 4). However, plasma estradiol-17β concentration in the heifer group tended to be lower than those in the multiparous groups on days-3 and 0, although there were no significant differences between them. The P-value was relatively low (P=0.088) for the comparison on day-3 between the heifer and control groups.

Discussion

In the present study, serum Mg concentration was increased only on day 0 in all groups (Figure 1A). A change like this has been reported in other research using not only normal cows but also paretic cows.[2,14,31,37] At parturition, serum Mg may be mobilized from bone through stimulation by parathyroid hormone (PTH).[23,28,35] However, PTH concentration in the heifer group did not increase as it did in the three mutiparous groups,[24] suggesting that serum Mg at parturition may be mobilized from other sources. The transiently increased concentration of serum Mg may be due to absorption from the gastrointestinal tract, a shift from the intracellular compartment and/or reabsorption in the renal tubules. In the present study, urinary Mg excretion slightly decreased on days −3 and 0 (Figure 1B). This might result from reabsorption of Mg in the kidney and contribute to the increased concentration of serum Mg at parturition. However, changes in serum and urinary Mg were not involved in milk fever because they did not differ among the groups.

In the present study, there was no specific difference in serum iP concentration or urinary iP excretion among the three multiparous groups, i.e., the anion, non-anion and control groups, although a transiently decreased concentration of serum iP was observed at parturition in these groups (Figure 2). This finding agreed with that in the other research concerning DCAD[7,30] suggesting that metabolic acidosis induced by lowering DCAD does not affect the metabolism of iP in dairy cows. However, serum iP concentration in the heifer group was maintained at a higher level in the prepartum period and until 3 days after parturition, compared with that in all other groups of mutiparous cows. Also, urinary iP excretion in the heifer group remained higher throughout the experimental period compared with that in the multiparous groups. In our latest study,[25] serum BALP and osteocalcin, which are biomarkers related to bone formation or turnover and osteoblast activity,[6,9,10,38] were markedly higher in the heifer group than in the multiparous groups and were not affected by lowering DCAD. In this respect, iP is similar to BALP and osteocalcin. Therefore, iP may be a biomarker involved in

milk fever, but not a useful biomarker to monitor the efficacy of altering DCAD as an alternative to TRAP, which is a biomarker showing bone resorption and osteoclast activity.[18,26]

Cl concentration in plasma or serum is increased in accordance with dietary supplement of Cl in dairy cows.[1,8] In the present study, serum Cl concentration in the anion group was maintained at a higher level compared with that in the non-anion and control groups (Figure 3). This is probably the result that cows in the anion group were administered anion salts containing 80 g of $CaCl_2 \cdot 2H_2O$ once a day from days −21 to 0.[24]. Elevated serum Cl requires hydrogen ions to maintain electroneutrality and the increased hydrogen ions in serum reduced blood pH.[8] In addition, interestingly serum Cl concentration in the heifer group also tented to be as high as that in the anion group even though heifer cows were not fed anion salts. It suggested that heifer cows might have the potential to up-regulate serum Cl concentration without any supplemental dietary anion salts in order to down-regulate blood pH and increase the response to Ca demands in the peripartum period. Multiparous cows might also have a potential similar to that in heifer cows because serum Cl concentration in the non-anion and control groups increased gradually in the prepartum period. However, the potential of multiparous cows may be too weak to auto-regulate serum Cl concentration and blood pH without the administration of anion salts. In our previous study,[24] the safe and mild metabolic acidosis induced by anion salts can be evaluated practically by determining urinary pH in a range from 6.8 to 7.0. The serum Cl concentration may be utilized along with urinary pH for evaluating the status of metabolic acidosis in dairy cows fed anion salts.

Estrogens including estradiol-17β are potent inhibitors of bone resorption,[34] and thus estrogens are used for inhibiting bone resorption and improving calcium balance in human patients with postmenopausal osteoporosis.[13] In dairy cows, several studies suggested that the higher concentration of serum estrogen induces a greater risk of developing milk fever.[19,32] However, there were other studies that failed to demonstrate the relation between estrogens and milk fever.[11,36] Thus, the role of estrogen in the development of milk fever is unclear. In the present study, plasma estradiol-17β concentration rose markedly in the prepartum period and reached a peak at parturition in all groups (Figure 4). The estradiol-17β concentration in heifer cows that did not develop hypocalcemia,[24] tended to be lower on days −3 and 0 compared with that in other groups of multiparous cows. This might suggest that estrogen may be involved in developing milk fever not lowering DCAD.

In conclusion, from the data in the present study, it was suggested that serum iP, Cl and plasma estradiol-17β concentrations are involved in milk fever, and change in the serum Cl concentration is also associated with the preventive effect of anion salts on milk fever. The serum Cl concentration may be a good parameter for evaluating the efficacy of lowering DCAD in dairy cows.

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