Biomarkers for the Activation of Calcium Metabolism in Dairy Cows: Elevation of Tartrate-Resistant Acid Phosphatase Activity by Lowering Dietary Cation-Anion Difference is Associated with the Prevention of 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 prepregnant period is safe and effective for preventing milk fever in multiparous cows. In the present study, several biomarkers, which might show activation of Ca metabolism, were analyzed using stored samples in the previous study to investigate the mechanism of the preventive effect on milk fever by lowering DCAD. Changes in bone-specific alkaline phosphatase activity, osteocalcin and insulin-like growth factor I concentrations in serum were almost the same among the three groups of multiparous cows with or without the oral administration of anion salts, while the levels of these serum biomarkers in the group of primiparous cows (heifer group) were much higher compared with those in the three multiparous groups throughout the experimental period. Urinary deoxyxypyrinidolone excretion was not a useful biomarker for dairy cows because it hardly changed during the peripartum period in all groups. However, serum tartrate-resistant acid phosphatase (TRAP) activity, which is known as a biomarker of osteoclast activity, was well associated with the administration of anion salts lowering DCAD because among the three multiparous groups, only the group of multiparous cows fed the anion salts (anion group) showed an increased level, which rose to the level in the heifer group, and was markedly higher than those in the other control groups of multiparous cows. The increased activity of serum TRAP in the anion group 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 was the best biomarker to monitor the activation of Ca metabolism in dairy cows fed anion salts.

KEY WORDS: dairy cow, dietary anion-cation difference, heifer, milk fever, tartrate-resistant acid phosphatase.

Milk fever or parturient hypocalcemia is a metabolic disorder that generally affects dairy cows around parturition [6, 17, 27]. This condition directly decreases the productivity of dairy cows or indirectly by increasing the incidence of many other diseases associated with practical sites such as dairy farms. This disease is directly caused by increased demand placed on the plasma Ca pool due to the sudden onset of lactation, but it has not been demonstrated why Ca is not smoothly mobilized to the plasma pool at parturition in cows affected with milk fever and where the most important Ca pool for quickly supplying Ca to the plasma pool. 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, 13, 14, 22]. Manipulation of dietary cation-anion difference (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 [11, 12, 33]. However, this strategy has some risks that too low DCAD induces severe metabolic acidosis resulting in a decline in dry matter intake and productivity [5, 16, 17].

In our previous study [18], 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 milk fever 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 probably due to increased responsiveness to Ca requirement. The mechanism of increased responsiveness to Ca was not demonstrated, but at least it was unrelated to the excretion of parathyroid hormone and 1,25-dihydroxyvitamin D. In addition, primiparous cows, which did not develop milk fever, had a high potential to respond to sudden Ca demand at parturition, and interestingly, their peripartum Ca metabolism was in some respects similar to that in multiparous cows fed anion salts. However, it could not
be determined from where Ca was smoothly mobilized to the plasma pool at parturition in primiparous cows and multiparous cows fed anion salts.

There are many biomarkers that show activation of Ca metabolism and these have been utilized mainly as bone metabolic indices in humans and animals [7, 8, 16, 21]. These biomarkers may include bone-specific alkaline phosphatase (BALP), osteocalcin, insulin-like growth factor-I (IGF-I) and tartrate-resistant acid phosphatase (TRAP) in serum, and also deoxypyridinoline excretion to urine. In the present study, we focused on these biomarkers to investigate the mechanism of the preventive effect on milk fever by lowering DCAD. For this purpose, these biomarkers were analyzed using stored samples from multiparous cows fed anion salts in our previous study [18], and compared with those in multiparous and primiparous cows not fed anion salts.

MATERIALS AND METHODS

Experimental design: In the present study, serum and urine that had been collected in our previous study [18], were used for analysis of several biomarkers that may indicate activation of Ca metabolism. Briefly, the experimental design of our previous study is described as below. 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 CaCO₃, 42 g of CaHPO₄, 65 g of MgSO₄·7H₂O and 80 g of CaCl₂·2H₂O 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 stomach catheter as in the anion group. The cows in the control and heifer group were not fed any supplemental salts. The prepartum DCAD calculated from the dietary and supplementary salts was +1.2, +14.6, +15.3 and +15.3 mEq/100 g of dietary dry matter in the anion, non-anion, control and heifer groups, respectively, and the postpartum DCAD was +17.6 mEq/100 g of dietary dry matter in all groups. 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. The cows were diagnosed as having severe hypocalcemia when their serum Ca concentration was less than 5 mg/dl, and moderate hypocalcemia when the concentration ranged from 5 to 7 mg/dl.

Analysis of biomarkers: Serum BALP activity was measured by enzyme immunoassay using Osteolinks BAP Kit (Quidel Corporation, San Diego, CA, U.S.A.). Serum osteocalcin concentration was measured by immunoradiometric assay (IRMA) using BGP IRMA Kit (Mitsubishi Kagaku Iatron, Tokyo, Japan). Serum IGF-I concentration was measured by IRMA using IGF-I IRMA Daiich Kit (Daiich Radioisotope Laboratories, Ltd., Tokyo, Japan). Serum TRAP activity was measured according to the method reported by Lau et al. [20]. Deoxypyridinoline concentration in urine was measured by enzyme immunoassay using Osteolinks DPD Kit (Quidel Corporation). Creatinine concentration in urine was measured using an automated biochemical analyzer (COBAS MIRA plus, Hoffmann-La Roche, Basel, Switzerland). Urinary deoxypyridinoline excretion was expressed as nmol of deoxypyridinoline per nmol of creatinine.

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, U.S.A.). Values of P<0.05 were considered significant. Marks indicating significance were used as followed: *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs heifer group. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs control group. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs non-anion group.

RESULTS

The change in serum BALP activity was almost the same in the anion, non-anion and control groups of multiparous cows (Fig. 1). The activity in the multiparous groups increased slightly on day 0, then decreased and returned to the prepartum level on day +7. The change in serum BALP activity in the heifer group was basically similar to that in the three multiparous groups with an increase only on day 0. However, the activity was significantly higher in the heifer group than in all other groups of multiparous cows through-
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Fig. 2. Change in serum osteocalcin 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.

Fig. 3. Change in serum insulin-like growth factor-I 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.

Fig. 4. Change in serum tartrate-resistant acid phosphatase activity 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 osteocalcin concentration was also almost the same among the three multiparous groups (Fig. 2). The concentration decreased slightly from day -40 to the last two weeks before parturition and decreased further on days 0 and +3, but started to increase on day +7. The change in serum osteocalcin concentration in the heifer group was basically similar to that in the three multiparous groups. However, the concentration was significantly higher in the heifer group than in all other groups of multiparous cows throughout the experimental period.

The serum IGF-I concentration in all groups gradually decreased during the prepurpartum period, then sloped sharply on day 0 and continued to decrease until day +7 (Fig. 3). The concentration was significantly higher in the heifer group than in all other groups of multiparous cows almost during the experimental period, but there was no significant difference (P=0.050) only between the heifer and non-anion groups on day +7.

The change in serum TRAP activity differed from those in other biomarkers and the difference was involved in the administration of anion salts (Fig. 4). The activity in the non-anion and control groups hardly changed and remained relatively low throughout the experimental period. The activity in the heifer group hardly changed either, but remained at a significantly higher level than that in the non-anion and control groups. However, the change in serum TRAP activity in the anion group was unique. The activity in the anion group increased slightly throughout the experimental period and gradually came up to the level in the heifer group. As a result, there were no significant differences between the anion and heifer groups on days -14 to +7, although the activity was significantly lower in the anion group than in the heifer group on day -40. The activity was significantly higher in the anion group than in the control or non-anion group on days -7 to +7.

The urinary deoxypyridinoline excretion tended to increase slightly on days 0 and +7 in all groups, but there were no significant differences among all groups (Fig. 5).

DISCUSSION

In the present study, BALP, osteocalcin, IGF-I and TRAP in serum, and deoxypyridinoline in urine were analyzed as...
candidate biomarkers that might show the activation of Ca metabolism in dairy cows in order to investigate the mechanism of the preventive effect on milk fever by lowering DCAD. In serum biomarkers, changes in BALP activity, osteocalcin and IGF-I concentrations were almost the same among the three multiparous groups, that is, the anion, non-anion and control groups, while the levels of these serum biomarkers in the heifer group, which did not develop milk fever [18], were much higher compared to those in the three multiparous groups throughout the experimental period (Figs. 1–3). These results showed that BALP, osteocalcin and IGF-I are involved in the activation of Ca metabolism in dairy cows in the peripartum period, but not the administration of anion salts lowering DCAD. In addition, urinary deoxypyridinoline excretion was not a useful biomarker for dairy cows because it hardly changed in the peripartum period in all groups (Fig. 5). However, only serum TRAP activity was well associated with the administration of anion salts because the level in the anion group alone increased among the three multiparous groups and came up to the level in the heifer group, which was much higher than those in the non-anion and control groups (Fig. 4).

TRAP has been recognized as a specific cytochemical marker in osteoclasts because TRAP is highly expressed in bone-resorbing osteoclasts and activated macrophages [15, 19]. An increase in TRAP activity seems to show activation of mature osteoclasts that can be induced by acidosis, but not the increased number of osteoclasts. Actually, Meghji et al. [23] demonstrated that net Ca release into the culture medium from mouse calvarial bones stimulated by HCO₃⁻ acidosis is almost entirely the result of osteoclast activity and also that HCO₃⁻ acidosis stimulates resorption by activating mature osteoclasts already present in calvarial bones, rather than by inducing the formation of new osteoclasts. Arnett and Dempster [1] reported that the numbers of osteoclasts and mononuclear cells were not altered by reduction of medium pH in an in vitro experiment. In another report, it was observed that the number of osteoclasts per microscopic field was smaller in dairy cows fed anion salts than in cows fed cation salts, even though there was an increase in eroded surface of trabecular bone in cows fed anion salts [25]. In the present study, an increase in TRAP activity in the anion group (Fig. 4) may be due to activation of mature TRAP-positive osteoclasts in bone, resulting from mild metabolic acidosis induced by the administration of anion salts. This may be why cows fed anion salts could respond highly to the Ca requirement at parturition in our previous study [18]. In primiparous cows in the heifer group, osteoclasts in bone seem to be previously activated because TRAP activity was much higher than that in multiparous cows not fed anion salts during the peripartum period.

Arnett and Spowage [2] investigated the effect of small changes in extracellular pH on the resorptive activity of rat osteoclasts in vitro. In this experiment, the relatively great change occurred by a pH difference of as little as 0.1 units near the physiological range. Over this narrow range, the average number of resorption pits formed on each bone wafer increased several-fold. Therefore, in the present study, even mild acidosis induced by the administration of anion salts lowering slightly DCAD could stimulate sufficient activation of mature osteoclasts in bone in the anion group, resulting in sensitivity to sudden Ca requirement at parturition. The increased activity of TRAP in the anion group may imply that osteoclasts in bone are ready to mobilize bone-bound Ca into the plasma pool.

BALP, which is only produced in bone and present in serum, has been considered to be a good biomarker showing bone formation and/or turnover and osteoblast activity [31]. Rodin et al. [29] reported that serum BALP was significantly higher in the last trimester of pregnancy than in early pregnancy in 40 healthy women and this elevation was still apparent at the end of the puerperium. This suggests that bone turnover is most activated at the end of puerperium in humans. In the present study, BALP was highest at parturition in all groups (Fig. 1), suggesting that bone turnover and osteoblast activity are accelerated markedly at parturition in dairy cows as in humans.

Osteocalcin has also been considered to be a specific and sensitive biomarker of bone formation or turnover, related by osteoblast function [6, 9, 10]. It was reported that there is a significant positive correlation between serum osteocalcin concentration and BALP activity in women [30]. This seems to be reasonable because they are similar biomarkers showing the status of osteoblast activity. However, in the present study, the change in osteocalcin concentration was definitely different from that in BALP activity (Figs. 1 and 2). The osteocalcin concentration in all groups tended to decrease in the peripartum period in the opposite direction from the BALP activity, and initially started to increase a week after parturition. Naito et al. [26] also reported a similar change in osteocalcin in dairy cows. In this experiment, serum osteocalcin concentration gradually decreased from 3
days before calving, then reached the bottom 1 day after calving and gradually recovered. The decreased concentration of osteocalcin in dairy cows observed in the present and other studies might be due to a clearance of osteocalcin in the placenta. Rodin et al. [29] suggested that the disappearance of osteocalcin during pregnancy could be due to degradation by a placental enzyme that catalyses an osteocalcin peptide. The recovery of osteocalcin concentration after parturition is likely due to the expulsion of placenta.

In the present study, BALP activity and osteocalcin concentration in serum were significantly higher in the heifer group than in the three multiparous groups throughout the experimental period (Figs. 1 and 2). There are animal and human studies indicating that these biomarkers are significantly higher in younger than in older individuals [24, 31]. These data show that bone formation and osteoblast activity in younger animals are accelerated compared with those in older ones. However, the common site of action following administration of anion salts may be osteoclast alone because this treatment affected a biomarker (TRAP) related to osteoclast activity but not biomarkers (BALP and osteocalcin) related to osteoblast activity.

In addition, the present study showed that IGF-I concentration was also much higher in the heifer group than in the three multiparous groups (Fig. 3). Therefore, IGF-I might be a useful biomarker showing certain functions like bone formation and osteoblast activity, although the role of this biomarker is not yet understood in relation to estimating the status of Ca or bone metabolism. However, the change in IGF-I concentration basically differed from changes in BALP activity and osteocalcin concentration, suggesting that IGF-I concentration is controlled by a different mechanism. Urinary deoxypyridinoline along with the related molecule, pyridinoline, has been used as a biomarker showing bone resorption [32], but there were no significant differences in level or change among groups, suggesting that it is not a useful biomarker in dairy cows.

In conclusion, it was demonstrated that TRAP is the best biomarker to monitor the activation of Ca metabolism in dairy cows fed anion salts. The increased activity of serum TRAP in cows fed anion salts suggested that Ca in the plasma pool might be mobilized smoothly from bone-bound Ca via mature osteoclasts at parturition, which might be activated beforehand under mild acidosis induced by slightly lowering DCAD.

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