Changes in plasma pH and blood and urinary macromineral concentrations in experimentally induced hypocalcemic cows with Na_2EDTA

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ABSTRACT. This study was performed to confirm the alterations of blood and urine parameters in artificially induced hypocalcemic cows. For a 2×2 cross-over design, four non-pregnant, non-lactating Holstein Friesian cows (623 ± 63 kg) were utilized. Cows in the treatment and control group were infused with ethylenediaminetetraacetic acid (Na_2EDTA) solution and normal saline through an intravenous catheter for 3 hr, respectively. Laboratory analyses included complete blood cell count, plasma chemistry, blood gas analysis and urine chemistry. During the hypocalcemic period, abnormal signs were not observed clinically, hematologically nor biochemically either in groups. But, plasma calcium and magnesium concentrations continued to decrease throughout Na_2EDTA infusion, and significant group differences ($P<0.05$ or $P<0.001$) were detected until 5 hr after the initiation of infusion. Urinary excretions of these minerals were significantly reduced compared to the control group by 6 hr (Ca, $P<0.05$; Mg, $P<0.001$). Moreover, there is a significant group difference in the change in plasma pH at 1 hr after Na_2EDTA infusion ($P<0.05$) and maintained a decreased level until 6 hr. Consequently, the blood pH was diminished simultaneously with hypocalcemia and hypomagnesemia induction in cows infused with Na_2EDTA. This phenomenon may be one of the mechanisms to recover normocalcemia including maximizing the effect of parathyroid hormone, however, further studies are needed to elucidate the mechanism to alter the blood pH in hypocalcemia.

KEY WORDS: blood analysis, blood pH, cattle, hypocalcemia, mineral

As in humans and other animals, the blood levels of macrominerals, such as calcium (Ca), phosphorus, and magnesium (Mg) are regulated relevantly in cows, and have an important role in regulating the body status and are maintained in consistent [1, 7, 11, 16, 18, 19, 23]. Abrupt changes in the mineral levels in the blood can affect the physiological condition, and if the balance is disrupted, serious diseases can result [21]. When the mineral balance in the body, especially the Ca balance, is disrupted around calving in dairy cows, it can lead to energy metabolic disorders, which can lead to suppressed immune function and increased peripartal diseases incidence such as mastitis or uterine infections [2, 7, 17].

Various factors are involved in the maintenance of homeostasis of minerals in the blood, especially hormones and overall body conditions regulate macromineral levels through several processes [1, 7, 11, 19, 23]. The parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 and calcitonin are known as endocrinal factors that regulate blood Ca levels [7, 11]. In particular, PTH contributes to an increase in blood Ca levels by suppressing the excretion of Ca through the kidneys when Ca levels are low [7, 12]. At the same time, PTH increases Mg levels in blood, lowers inorganic phosphate (iP) levels, and affects the activation of vitamin D_3 [7, 11]. This activated form of vitamin D_3 regulates the blood concentrations of Ca, Mg, and iP to increase [16]. In cows initiating lactation, the blood mineral levels change more rapidly than in other animals and other periods of life, and the adjustability to these changes affect productivity [1].

On the other hand, the pH is an important factor to function the body system normally and regulated tightly to sustain the

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physiological processes [4]. Thus, in activating a mechanism to return the body to normal in an emergency, the pH will provide a biochemically optimal environment. It was already assumed that body pH would also change as the Ca concentration in the blood decreased [7, 8]. In the recovery of hypocalcemia, it is known that the uptake of Ca increases when the pH of the blood is changed to the mild acidic status most suitable for the activity of PTH [7, 8]. However, to date, there are no reports investigating the change in the blood pH in cows with hypocalcemia induced artificially.

Therefore, the purpose of this study was to investigate the changes in parameters of complete blood cell count (CBC), blood biochemistry, and blood gas and including blood pH as well as urinary mineral excretion analysis in hypocalcemic cows infused with disodium ethylenediaminetetraacetic acid (Na_2EDTA) and to determine the relationship between blood Ca level and other altered parameters.

Four non-pregnant, non-lactating Holstein-Friesian cows were used in this experiment. The mean parity was 2.5 ± 0.6 and the body weight was 623 ± 63 kg. The cows were reared under the same conditions. Cows were fed the same amounts of hay and concentrated feed twice a day and allowed free access to water. All experimental protocols were validated by the Institutional Animal Care Use Committee of Seoul National University (SNU-181105-3).

The experiment was performed using a 2×2 cross-over design. One day before each administration, intravenous catheters (EQUIVET Hiflow long-term IV catheter 14 G × 5.25; KRUUSE, Langeskov, Denmark) were indwelled and fixed by suture in both jugular veins for infusion and blood sampling, respectively. The infusion for induction of hypocalcemia was performed after the cows were tied to the frame, and sampling was performed by restrainment to cow stanchion. Na_2EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 540 ml of 0.9% saline. The solution was pH 5.5 and sterilized before infusion. The dosage of Na_2EDTA was calculated as 40 mg/hr/kg [20], because the blood volume varies according to body weight and an average concentration of solution was about 13%. Two cows were infused with 540 ml of Na_2EDTA solution through intravenous catheter for 3 hr (treatment, TRE), and intravenous infusion of 540 ml of 0.9% saline (pH 5.5) was conducted in the other two cows (control, CON). Setting three days of washing period after the last sampling, the same administration protocol was repeated with changing the group.

Blood and urine samples were collected at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16, 20, 24, 48, 72 and 96 hr, and 0, 1, 3, 6, 12, 24, 48, 72 and 96 hr after the initiation of administration, respectively. The samples for CBC were collected in EDTA tubes (K_2 EDTA tube; BD Vacutainer, Franklin Lakes, NJ, USA), and heparin tubes (Lithium Heparin tube; BD Vacutainer) were used for the samples for plasma chemistry. Plasma was separated by centrifuging the heparinized blood samples at 2,000 × g for 15 min and stored at −70°C. The urine sample was collected by inducing urination by rubbing the lower part of the vulva or inserting the urinary catheter. Urine was dispensed into a plastic tube (Falcon® 15 ml PP tube; Falcon, Tewksbury, MA, USA), and supernatant was separated after centrifugation at 2,000 × g for 15 min and stored at −70°C. For blood gas analyses, the plasma samples of 0, 1, 3, 6, 12, 24, 48, 72 and 96 hr were used. Samples stored at −70°C were thawed to room temperature and centrifuged again before analyses to use supernatant.

Laboratory analyses included CBC (Hemavet® 950; Erba® Diagnostics, Miami, FL, USA), plasma and urinary chemistry (BS-400; Mindray, Shenzhen, China), and blood gas analysis (i-STAT; Abott, North Chicago, IL, USA). Immediately after blood sampling, CBC was performed using EDTA blood. In plasma chemistry, total protein (TP), albumin (Alb), total cholesterol (T-chol), glucose (Glu), creatinine (Crea), Ca, iP, and Mg, were included, and blood gas analysis was performed on pH. Although arterial blood is utilized in blood gas analysis, we used venous blood due to difficulty in repeated approach to an artery and the correlation with arterial pH [13]. Also, blood gas analysis was performed with the thawed plasma instead of whole blood due to the availability of analyzing equipment. Through our preliminary experiment before this study, plasma pH was confirmed to be approximately 0.2 higher than whole blood. But considering the differences in each individual, the results of pH were converted to delta pH (ΔpH, $\Delta pH = pH_{t=x\ hr} - pH_{t=0\ hr}$) for determining the pH change. In urinary chemistry, all results of minerals were converted to ratio with creatinine (Ca/creatinine, U-Ca; iP/creatinine, U-P; Mg/creatinine, U-Mg) to evaluate the excretion of minerals through the urine [15, 22].

Numerical data were expressed as mean ± standard deviation, and analyzed using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). One-way repeated measures analysis of variance followed by Holm-Sidak multiple comparison method was applied to determine the significance of posttreatment values in comparison with the pretreatment value (0 hr). Student's t-test was performed to compare the difference between the groups. The significance was set at $P<0.05$ for all analyses.

During the induction period in both groups, there were no significant events. In particular, clinical signs due to hypocalcemia like sternal or lateral recumbency were not observed in the TRE group. Also, in plasma chemical parameters including TP, Alb, T-chol, Glu and Crea, the normal state was confirmed by stable levels of parameters (data not shown). However, significant differences in Ca ($P<0.001$), iP ($P=0.009$) and Mg ($P<0.001$) within group were observed in TRE (Fig. 1). Among, plasma Ca and Mg concentrations continued to decrease throughout the Na_2EDTA infusion and were significantly lower until 5 hr than those of CON ($P<0.05$ or $P<0.001$). The lowest plasma iP concentration was identified at 3 hr like other minerals, but no significant groups difference was observed ($P=0.098$).

In urinary analyses (Fig. 2), levels of U-Ca and U-Mg in TRE decreased after infusion and remained at low levels until 6 hr after infusion (U-Ca, $P=0.004$; U-Mg, $P<0.001$). Significant group differences were also observed until 6 hr in U-Ca ($P<0.05$) and U-Mg ($P<0.001$). In comparison, the change in U-P was shown similar to other minerals but insignificant.

In the result of blood gas analysis using plasma, the alteration in Δ pH showed a significant difference in TRE ($P<0.001$, Fig. 3). The Δ pH in TRE at 1 hr was significantly lower than CON and remained low until 6 hr although not statistically significant.

The first objective of this study was to confirm the relationship among the Ca concentration and blood pH and other parameters
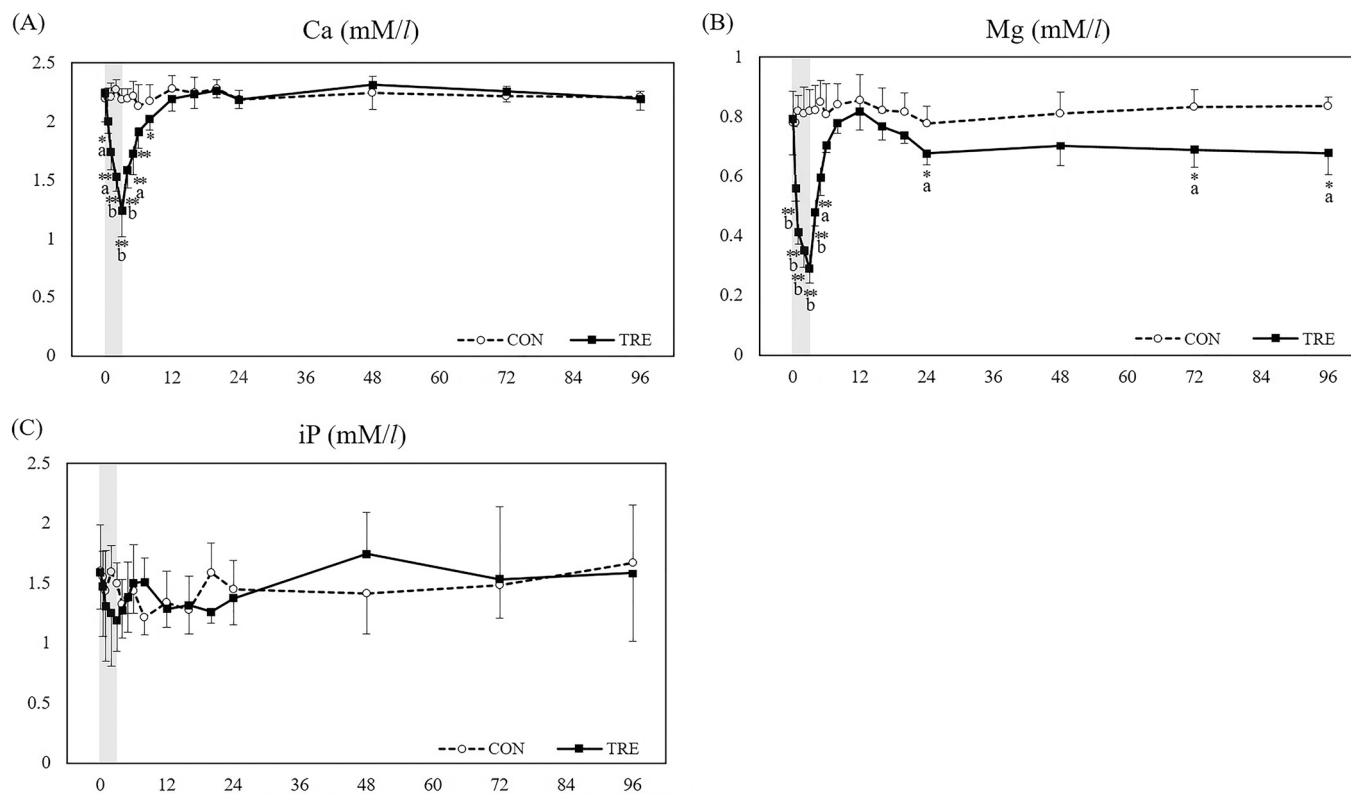
Fig. 1. Changes in plasma mineral concentrations; A, calcium (Ca); B, magnesium (Mg); C, inorganic phosphate (iP). Gray zone means Na_2EDTA infusion time. The values represent the means ± SD. Significant difference with the value at 0 hr is denoted as * ($P<0.05$) and ** ($P<0.001$). Significant difference with the control (CON) at the same time-point is denoted as a ($P<0.05$) and b ($P<0.001$).
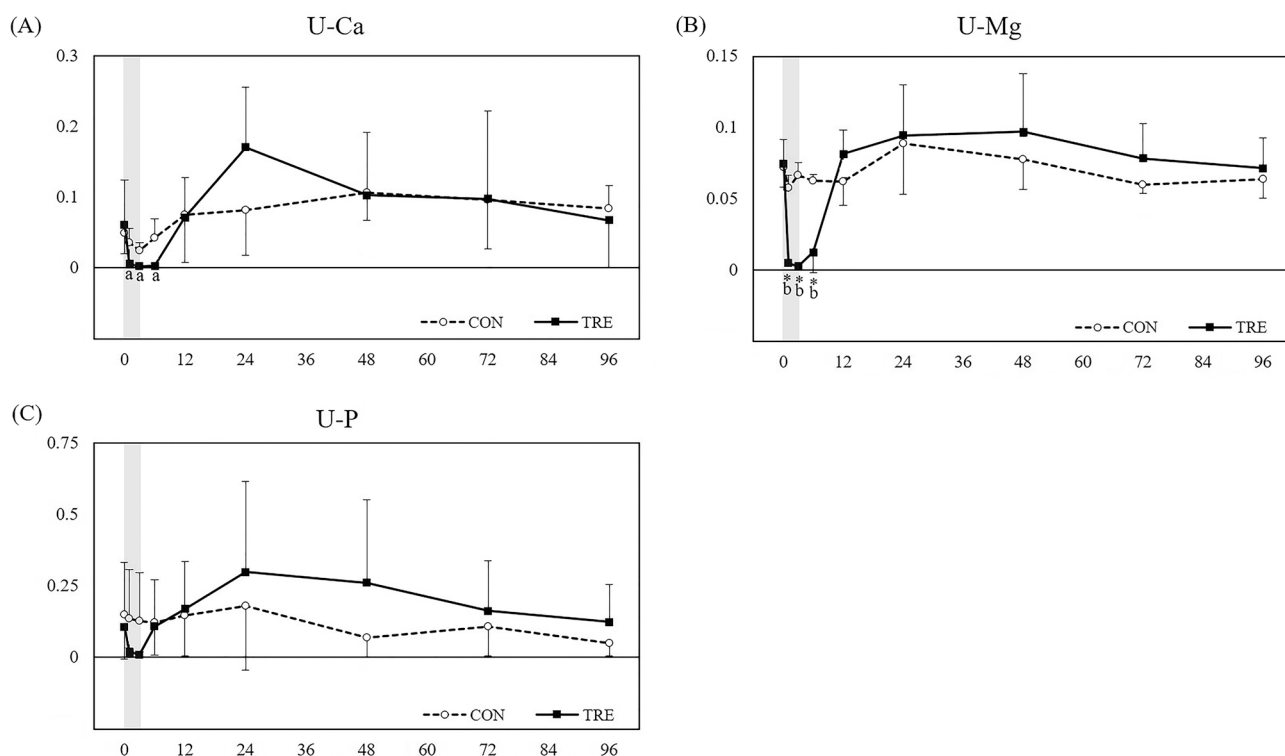

Fig. 2. The amounts of urinary excretion of minerals through the ratio of minerals and creatinine; A, total calcium: creatinine (U-Ca); B, magnesium: creatinine (U-Mg); C, inorganic phosphate: creatinine (U-P). The values represent the means ± SD. Significant difference with the value at 0 hr is denoted as * ($P<0.05$). Significant difference with the control (CON) at the same time-point is denoted as a ($P<0.05$) and b ($P<0.001$).
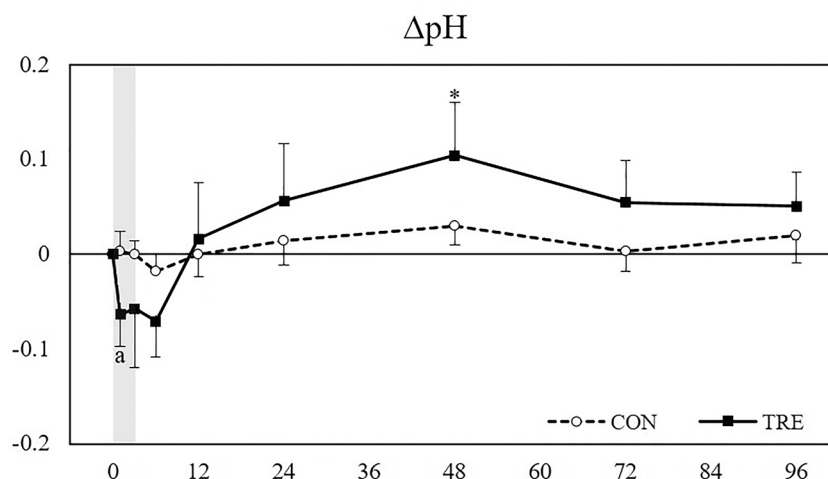
Fig. 3. Effects of induced hypocalcemia on the change in plasma pH (Δ pH). The values represent the means ± SD. Significant difference with the value at 0 hr is denoted as * ($P<0.05$). Significant difference with the control (CON) at the same time-point is denoted as a ($P<0.05$).

in dairy cows with induced hypocalcemia. In this study, Na$_2$EDTA infusion for 3 hr induced hypocalcemia for 6 hr, at that time, the TRE group showed negative Δ pH due to a decrease in pH. Here, if negative Δ pH was caused by the infusion of acidic solution, Δ pH should have been the lowest at 3 hr like plasma Ca concentration. However, Δ pH was significantly decreased at 1 hr and maintained the level until 6 hr. Thus, it seems that negative Δ pH was not induced due to infusing acidic solution but due to some mechanisms to lower pH against hypocalcemia. On the other hand, the Δ pH of TRE increased significantly at 48 hr compared to 0 hr ($P=0.05$). This seems to be caused by the small number of experimental animals. The Δ pH of 3 cows was less than 0.03, but 0.175 in one cow.

The mechanisms for regulating blood pH are known as the respiratory system and buffer system [3, 24]. The main mechanism to lower the pH in the body is to suppress the respiration for increasing CO$_2$ concentration in the blood. Here, blood gas analysis can be used to determine whether the pH of the blood is lowered through the respiratory mechanism by measuring pCO$_2$. However, in this experiment, since the sample was frozen and thawed, it was difficult to confirm the pCO$_2$ in fresh whole blood. Therefore, the mechanism of lowering pH could not be confirmed. Consequently, further study is needed to confirm the mechanism of lowering pH in the blood.

When blood Ca is lowered, PTH raises Ca levels by increasing Ca mobilization and reabsorption in bones and kidneys, respectively [7, 11]. In this study, when hypocalcemia occurred, the urinary excretion of calcium was simultaneously lowered, and it was estimated that the renal reabsorption of Ca was increased in hypocalcemic status by PTH.

The most significant result of this experiment is the decrease in plasma pH (negative Δ pH) in a hypocalcemia-induced group, which may be one of the processes of maintaining homeostasis of blood Ca concentration. In the induced hypocalcemia group, the plasma pH was drastically lowered with the decrease of the concentration of blood Ca. When the blood Ca level decreases, PTH is released earlier than vitamin D$_3$ or other factors [6, 7]. The secretion of PTH and its binding to receptors are activated in lower blood pH than normal [6, 7]. According to previous studies, when blood pH is approximately 7.35, PTH actively binds to the receptor and acts to target organs [6, 7]. In addition, when the pH of blood becomes alkaline, the structure of PTH receptor is denatured, so that the binding of PTH is abnormal, and thus it is difficult to stimulate Ca uptake. Accordingly, in this experiment, the plasma pH of TRE might decrease simultaneously with induction of hypocalcemia to maximize the function of PTH.

In previous studies investigating mineral metabolism, administration of Na$_2$EDTA was mainly used to induce the hypocalcemic status in cattle [5, 14]. Several studies that induced hypocalcemia by EDTA infusion have identified changes in Ca absorption in the intestine, resorption of Ca from bone and immune suppression [8, 14, 20]. In addition, the change in blood Ca concentration and other changes such as a liver enzyme, hormones and electrolytes were confirmed [5, 14, 17]. On the other hand, in some previous studies [5, 14, 20], normal saline and CaEDTA solution were considered as solutions to be infused to the control group, and we used normal saline for administration to CON. Because the purpose of this study was to identify changes in blood parameters, especially pH, in hypocalcemia and normal status, normal saline was used to minimize other physiological changes.

Another purpose of the study was to identify the changes in the concentrations of macrominerals released into the urine in induced hypocalcemic status. In the status of low concentrations of minerals in the blood, the macromineral/creatinine ratio in urine is lowered until these levels rise to normal levels. These results confirmed that reducing the excretion of minerals in urine is also the compensatory process to raise blood mineral concentrations to normal levels. However, unlike U-Ca and U-Mg, the level of U-P did not show a significant group difference between TRE and CON. This is because, unlike other macrominerals, iP is largely secreted through saliva or feces, and can be recycled [1]. Also, the factors regulating iP in blood and its mechanisms are not yet clear. Research on fibroblast growth factor 23 (FGF23), which is known as one of the regulators of iP, has been actively conducted

in experimental animals [9, 10]. However, it is difficult to discuss the function of FGF23 in cattle as it has not been identified as a useful method for measuring FGF23 in cattle. According to the studies in other species, FGF23 may have shown positive regulation with the decrease of iP in this study [9, 10], but it is estimated that FGF23 may be less potent than the function of PTH, and other factors may be involved in the regulation of iP level, so there may be no significant difference in U-P.

In this study, the pH of blood in hypocalcemia-induced cows was lowered immediately after the initiation of Na_2EDTA infusion and maintained at a low level until Ca levels in blood were recovered to the state before induction. This decrease in blood pH suggests that there is a physiological response to promote the secretion of PTH and to improve reaction among mechanisms for maintaining Ca homeostasis. However, further studies are needed to determine which reaction changes the blood pH spontaneously.

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