Improving the accuracy of estimating blood calcium concentration in Holstein cows using electrocardiographic variables

Megumi Itoh1)* Yasuhiro Nakajima2) Kouki Kuwano3) Daisuke Maeda2) Yoshie Sakurai3)
Yoshitaka Matsui4) Satoshi Kawamoto5)

1) Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan
2) Industrial Research Center, Hokkaido Research Organization, Kita 19, Nishi 11, Kita-ku, Sapporo, Hokkaido 060-0819, Japan
3) Animal Research Center, Hokkaido Research Organization, Sintoku, Hokkaido 081-0038, Japan
4) Dairy Research Center, Hokkaido Research Organization, Asahigaoka, Nakashibetsu, Hokkaido 086-1135, Japan
5) School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimati, Bunkyo-ku, Ebetsu, Hokkaido 069-8501, Japan

*Correspondence: Megumi Itoh
E-mail: itohmg@obihiro.ac.jp

RUNNING HEAD: BLOOD CALCIUM ESTIMATION USING ECG
ABSTRACT

We previously reported the possibility of using the electrocardiogram variable to estimate blood calcium (Ca) concentration in dairy cows based on the strong positive correlation between the blood Ca concentration and the inverse of the corrected ST peak interval (STc\(^{-1}\)).

To improve the accuracy of the estimation of blood Ca concentration, we investigated the relationship between blood Ca concentration and \(\text{STc}^{-1}\) for each postpartum day and available variables other than \(\text{STc}^{-1}\). We measured multiple variables (milk yield, calving number, age, body temperature, etc.), including serum total Ca concentration (tCa), blood ionized Ca concentration (iCa) and \(\text{STc}^{-1}\) in 462 Holstein cows on days 0, 1, 2, 3, 5, and 7 postpartum. A very high correlation was observed between iCa and tCa. The association between tCa and \(\text{STc}^{-1}\) for each postpartum day had a high coefficient of determination of 0.61–0.79 postpartum 0–2 days but decreased after the third day. In the investigation using the data from postpartum days 0–2, \(\text{STc}^{-1}\), heart rate interval, calving number, and age were highly correlated with tCa.

In addition, a multiple regression equation was obtained with tCa as the objective variable and \(\text{STc}^{-1}\) and calving number as explanatory variables. The estimation accuracy was improved as compared with the simple regression equation using only \(\text{STc}^{-1}\) as the explanatory variable. This multiple regression equation was used for 11 cows suspected of having hypocalcemia, and it was able to correctly detect cows requiring early treatment, except for one cow.

Keywords: calving number, cow, electrocardiogram, hypocalcemia, multiple regression equation
INTRODUCTION

Parturient hypocalcemia (milk fiver, parturient paresis) is a major peripartum disease in dairy cows. When cows begin lactating, large amounts of calcium (Ca) are transferred to milk. However, immediately after parturition, cows have insufficient reabsorption of Ca in the kidneys, absorption of Ca from the intestine, and resorption of Ca from bones [24]. As a result, the blood concentration of Ca decreases. Initial clinical signs are restlessness and anorexia, and in severe cases, cows can experience recumbency, lose consciousness, and may die [21]. Injection of Ca is the most common treatment, and properly treated cows have a good prognosis. Prolonged hypocalcemia resulting from delayed treatment leads to gastrointestinal hypomotility, sequelae, and decreased milk yield. In addition, long-term recumbency causes downer cow syndrome and increases the culling rate [10, 26]. Therefore, early detection and early treatment of this disease are important. The definitive diagnosis of hypocalcemia relies on measuring blood Ca concentration. However, collecting blood sample by a skilled person such as a veterinarian is necessary. Bringing blood samples to the examination center for measurement is a common practice; therefore, the results cannot be clarified in real time. Although there are portable Ca measuring devices, it is often challenging to measure it in the field because the cartridge used for measurement is expensive and measuring at low temperature in winter or high temperature in summer to maintain accuracy is not possible. Many dairy farmers and veterinarians have determined and treated hypocalcemia based on the clinical signs of cows.

We previously reported a strong positive correlation between STc⁻¹ (the inverse of ST peak intervals corrected by Bazett’s formula) and blood Ca concentration, and we found that hypocalcemia in dairy cows could be detected easily and noninvasively using an electrocardiogram (ECG) [14]. In this report, we created a formula to estimate blood Ca concentration using the data from recumbency cows within 24 hr postpartum and healthy cows.
within 1 week postpartum. The plot diagram used to create the estimation formula was widely dispersed in the high Ca region, and the estimated Ca value tended to be lower than the measured value in the high Ca region [14]. Because actual parturient hypocalcemia occurs within 72 hr postpartum [21] and there are factors other than blood Ca concentration that change the ECG [16, 20], we considered that factors other than blood Ca concentration affected the ECG with the passage of postpartum days, which contributes to the wide dispersion of plots in the high Ca region. In addition, because there are factors other than STc\(^{-1}\) that are related to the detection of hypocalcemia [7, 8, 23], it is highly possible that the blood Ca concentration can be estimated more accurately using multiple regression analysis than with simple regression analysis.

In this study, we clarified the relationship between blood Ca concentration and STc\(^{-1}\) for each postpartum day and added variables other than STc\(^{-1}\) that can be used estimate the blood concentration of Ca. We aimed to further improve the accuracy of the simple estimation of blood Ca concentration in dairy cows.

**MATERIALS AND METHODS**

A total of 462 Holstein cows (199, 100, 33, 47, 33, and 50 cows on days 0, 1, 2, 3, 5, and 7 postpartum, respectively) kept at 20 dairy farms in Hokkaido were used in this study. ECG was recorded for 30 sec by AB lead using an electrocardiograph (Harada Electronic industry Ltd., Sapporo, Japan), and STc (=ST peak interval/SS peak interval\(^{0.5}\)) (sec) was measured. Tail vein blood was collected into 5 ml tubes containing heparin lithium (Neotube, Nipro, Osaka, Japan) and into 10 ml plain tubes (Venoject II, Terumo, Tokyo, Japan). Blood ionized Ca concentration (iCa) (mmol/l) was measured in the heparinized samples by ion selective electrodes using a blood gas/electrolyte/hematocrit analyzer (Chiron 348, Siemens AG, Munich, Germany). All iCa values were adjusted to pH 7.4. Nonheparinized blood samples
were separated by centrifugation at 3,000 rpm for 15 min at 4°C, and serum total Ca concentration (tCa) (mg/dl) was measured by o-cresolphthalein-complexone method using an automated chemistry analyzer (AU480, Beckman Coulter, CA, USA). We also investigated the following other variables: ECG recording time of day (hr), time from milking to ECG record (hr), milk yield immediately before (kg), calving number (times), age (years), body temperature (°C), heart rate interval (sec; detected automatically by ECG), standing (yes: 1, no: 0), foraging or rumination (yes: 1, no: 0). Samplings were performed from 5:00 to 20:00.

First, a correlation analysis between iCa and tCa was performed. The normality of the data was confirmed by the Shapiro–Wilk test, and the correlation coefficient (r) and p-value were clarified by the Pearson’s correlation test. When a very high correlation (r > 0.9, p-value < 0.01) [3] was confirmed between iCa and tCa, studies 1–3 were conducted with tCa as the objective variable.

As study 1, linear regression analysis of Stc-1 and tCa was performed for each postpartum day, and the respective regression equations, coefficient of determination (R²), and p-value were calculated. The goodness of fit of linear regression was considered acceptable at R² > 0.5 and significant at p-value < 0.05, referring to the previous report [5, 22].

As study 2, we used the data on the days after parturition that confirmed the similar regression equation and the acceptable coefficient of determination in Study 1. Correlation analysis was performed using tCa, Stc-1 and other variables. The normality of the data was confirmed by the Shapiro–Wilk test, correlation coefficient (r) and p-value were clarified by for the normal distribution and Spearman’s rank correlation test for the others. The moderate or more correlation (r < −0.5 or r > 0.5) [3] and p-values of <0.05 were considered significantly correlated. Subsequently, a multiple regression model was created using the tCa as the objective variable and the others as the explanatory variables. The select of explanatory variable was performed by a forward–backward stepwise selection method based on AIC.
(Akaike’s Information Criterion) [1], and a multiple regression equation for estimating the tCa was calculated.

As study 3, we confirmed the validity of the multiple regression equation created in study 2. Eleven Holstein cows on postpartum 0–1 day that suspected hypocalcemia by veterinarians based on clinical findings such as ambulatory and skin temperature were used and compared the estimated value of tCa with the measured value of tCa.

All statistical analyses were performed using statistical software R (version 3.1.0). This study was approved by the ethics committee of the Hokkaido Research Organization Animal Research Center, Japan (No. 1312).

RESULTS
Mean and standard deviation values of milk yield immediately before, calving number, age, body temperature, and heart rate interval of the cows were 23.9 ± 9.9 kg, 3.3 ± 1.7 times, 4.6 ± 2.1 years, 38.9 ± 0.5°C, and 0.640 ± 0.192 sec, respectively.

A significant and very high correlation was confirmed between iCa and tCa (r = 0.91, p-value < 0.01). Therefore, estimation formula was created using the tCa as an objective variable.

Study 1
For the relationship between STc\(^{-1}\) and tCa for each postpartum day, the p-value was significantly associated on all days. The coefficient of determination was R\(^2\) > 0.5 on days 0–2 postpartum, but it was low at 0.35, 0.21, and 0.12 on days 3, 5, and 7 postpartum, respectively. On days 0, 1 and 2 postpartum, each simple linear regression equation had a regression coefficient (slope) of 4.42 ± 0.25, 5.31 ± 0.28, and 4.64 ± 0.60 and a constant (intercept) of −5.44 ± 0.72, −7.92 ± 0.80, and −5.88 ± 1.69, respectively. On the other hand, on postpartum day 3 or greater, it was far from this value (Fig. 1, Table 1). Therefore, in study 2, we decided...
to use data from 0 to 2 days after parturition.

**Study 2**

We performed a correlation analysis using 332 samples collected on postpartum days 0–2. tCa were significantly correlated with STc⁻¹, heart rate interval, calving number, and age, and correlation coefficients were \( r = 0.81, -0.52, -0.59, \) and \(-0.57\), respectively (Table 2). In the multiple regression analysis with tCa as an objective variable, the following multiple regression equation was obtained, with STc⁻¹ and calving number as explanatory variables.

\[
tCa \, [\text{mg/dl}] = a + b \times \text{STc}^{-1} \, [\text{sec}^{-1}] + c \times \text{calving number} \, [\text{times}]
\]

\[
a = -4.47 \pm 0.59, \quad b = 4.34 \pm 0.18, \quad c = -0.20 \pm 0.03
\]

where the coefficient of determination \( (R^2) = 0.79 \), \( p \)-value < 0.01, and standard error = 0.78.

On the other hand, when using only STc⁻¹ as the explanatory variable, \( R^2 \), \( p \)-value, and standard error were 0.74, <0.01, and 0.85, respectively.

**Study 3**

In the plot diagram of the estimated value of tCa with the measured value of tCa, the correlation coefficient was \( r = 0.90 \) and the standard error of the residuals was 0.96. When 6.5 mg/dl tCa, which indicated moderate or higher hypocalcemia [13], was used as the boundary, cows with hypocalcemia requiring early treatment could be detected, except for one sample near the border region (Fig. 2).

**DISCUSSION**

Our previously reported formula for estimating blood Ca concentration (iCa and tCa) using STc⁻¹ was able to avoid overlooking hypocalcemic cows. However, it was possible that cows without hypocalcemia would also be administered Ca. This is associated with a risk of cardiac arrest due to hypercalcemia [12] and should never be recommended. In dairy cows immediately after parturition, hypocalcemia might be a major factor that affects the STc⁻¹, but
other factors are expected to be involved over time. For example, postpartum fever, which
develops a few days after parturition, causes tachycardia and shortens the heartbeat interval [2].
Further administration of Ca to cows after milk fever treatment shortens QTc and causes
arrhythmia [18, 19]. Cows with downer cow syndrome have low blood K levels, and
hypokalemia prolongs the QT interval [4, 17]. The QTc and QT interval are synonymous with
the STc and ST interval, respectively [14]. There is an association between milk yield and
tachycardia [11]. In our previous report, we used data from cows within 1 week postpartum to
create the regression formula, and this might have caused the large error. Thus, examining data
from each postpartum day was considered effective.

tCa is generally used to clinically diagnose hypocalcemia in cows. On the other hand,
only iCa is physiologically active during skeletal or cardiac muscle contraction [9]. Therefore,
consistent with our previous report [14], we measured not only iCa but also tCa confirmed the
presence of a strong correlation between them and decided to create an estimation formula for
tCa. In this study, a statistically significant correlation was observed between the tCa and iCa.
In cows with hypoalbuminemia, the Ca ionization rate increases because of the reduction of
the protein-bound Ca proportion [25], thus the estimated Ca value may be higher than its
measured value. However, because the tCa obtained by this formula is an estimated value for
cows with normal albuminemia, in our opinion it does not present a risk for clinical
misdiagnosis.

In the regression equation created for each postpartum day, the regression coefficient
showed similar a value from 0 to 2 days but decreased sharply after 3 days. This was because
there were few cows with hypocalcemia, and some cows had normal blood Ca levels despite
prolonged STc. We suspected other factors affecting ECG, but no cows on 3–7 days
postpartum confirmed having tachycardia due to postpartum fever, arrhythmia due to further
administration of Ca and abnormal ECG due to hypokalemia. Since the goodness of fit
(coefficient of determination) drastically decreases when adding the data after the third day of parturition to the regression equation, we recommended estimating the blood Ca concentration using the ECG variables for cows on days 0–2 postpartum. Hypocalcemia in dairy cows mainly occurs from 1 day prepartum to 2 days postpartum, especially at 0 days [21]. We considered that hypocalcemia could be mostly detected by inspection in cows at 0–2 days postpartum.

In this study, in our determination of a new parameter to add to the regression formula, we examined the items that the dairy farmer himself could input and assumed that the dairy farmer would use in the field. Correlation analysis revealed a high correlation between tCa and STc\(^{-1}\), heart rate interval, calving number, and age. However, because we observed a high correlation between STc\(^{-1}\) and heart rate interval as well as between calving number and age, STc\(^{-1}\) and calving number were ultimately selected as explanatory variables. The calving number is a numerical value that can be grasped in advance and is an easy-to-use parameter. It is well known that cows with a high calving number tend to exhibit parturient hypocalcemia [6], and it was also applicable to estimate tCa. A prior study reported that blood Ca concentration decreases gastrointestinal motility [10] or milk yield [15], but it was difficult to use these as variables to estimate tCa. The multiple regression equation with STc\(^{-1}\) and calving number added to the explanatory variables increased the coefficient of determination by 5 points, decreased the standard error by 7 points, and improved the estimation accuracy of tCa as compared with the simple regression equation using only STc\(^{-1}\). Even in a verification experiment using cows suspected of having hypocalcemia, this multiple regression equation was sufficiently effective in estimating the tCa.

In the future, we would like to consider adding variables that require equipment and experience, such as rumen movement, body surface temperature, and pupillary reflex. If the farmers can utilize more variables under the guidance of veterinarians, we have confidence
that the accuracy of estimating blood Ca concentration will be improved further.

CONFLICTS OF INTEREST

The authors have no conflicts of interest directly relevant to the content of this article.

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Fig. 1. Relationship between STc$^{-1}$ and serum total Ca concentration for postpartum days 0, 1 and 2 (left side), and 3, 5 and 7 (right side).

STc$^{-1}$: The inverse of ST peak intervals corrected for heart rate by Bazett’s formula.
Fig. 2. Relationship between estimated value of serum total Ca concentration and measured value. The solid line shows the relationship of $y = x$. Values below the dotted line indicate serum total Ca $<6.5$ mg/dl.
Table 1. Regression coefficient (slope), constant (intercept), coefficient of determination, and $p$-value of the regression equation for each postpartum day.

| Postpartum day | Number of samples | Regression coefficient (slope) | Constant (intercept) | Coefficient of determination | $p$-value |
|----------------|-------------------|--------------------------------|----------------------|-------------------------------|-----------|
| 0              | 199               | 4.42 ± 0.25                   | -5.44 ± 0.72         | 0.61                          | < 0.01    |
| 1              | 0.2               | 5.31 ± 0.28                   | -7.92 ± 0.80         | 0.79                          | < 0.01    |
| 2              | 33                | 4.64 ± 0.60                   | -5.88 ± 1.69         | 0.71                          | < 0.01    |
| 3              | 47                | 2.85 ± 0.60                   | -0.02 ± 1.79         | 0.35                          | < 0.01    |
| 5              | 33                | 1.63 ± 0.58                   | 4.45 ± 1.73          | 0.21                          | < 0.01    |
| 7              | 50                | 1.82 ± 0.71                   | 3.96 ± 3.96          | 0.12                          | < 0.05    |

*: mean ± standard error
Table 2. Correlation matrix of all variables

|                  | tCa  | STc⁻¹ | Heart rate interval | ECG recording time of day | Times from milking to ECG | Milk yield | Calving number | Age | Body temperature | Standing | Foraging or rumination |
|------------------|------|-------|---------------------|---------------------------|---------------------------|------------|----------------|-----|------------------|----------|------------------------|
| tCa              | 1    | 0.81  | **-0.52**           | 0.04                      | 0.11                      | 0.07       | -0.59          | -0.57| 0.01             | -0.37    | -0.03                  |
| STc⁻¹            |      | 1     | **-0.59**           | 0.15                      | 0.10                      | -0.11      | -0.45**        | -0.42**| 0.14             | -0.26    | 0.11                  |
| Heart rate interval |      |       | 1                   | -0.45                     | 0.41                      | **0.41**   | **0.41**       | **0.38**| -0.22            | 0.22     | -0.2                   |
| ECG recording time of day |      |       |                     | 1                         | **-0.60**                 | **-0.37**  |               |               | 0.32             | -0.24    | 0.13                  |
| Times from milking to ECG |      |       |                     |                           |                           |            |                |               |                 |          |                        |
| Milk yield       |      |       |                     |                           |                           | 1          | 0.18           | 0.19 | -0.19            | 0.12     | 0.02                  |
| Calving number   |      |       |                     |                           |                           |            | 1              | **0.95**| -0.19            | 0.19     | -0.25                 |
| Age              |      |       |                     |                           |                           |            |                | 1               |                 | -0.20    | 0.26                  |
| Body temperature |      |       |                     |                           |                           |            |                |                  |                 |          |                        |
| Standing         |      |       |                     |                           |                           |            |                |                  |                 | 1        | -0.18                 |
| Foraging or rumination |      |       |                     |                           |                           |            |                |                  |                 |          |                        |

*: |r|=0.50-0.70 (moderate correlation) ***: |r|=0.70-0.90 (high correlation) ****: |r|=0.90-1.00 (very high correlation) 

* p < 0.05 ** p < 0.01

tCa: Serum total calcium concentration
STc⁻¹: The inverse of ST peak intervals corrected for heart rate by Bazett’s formula
Milk yield: Milk yield immediately before