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CLINICAL STUDY

Biochemical profile of heifers with spontaneous humeral fractures suggest that protein-energy malnutrition could be an important factor in the pathology of this disease

A Wehrle-Martinez [a], KE Dittmer [a], PJ Back [a,b], CW Rogers [a,b] and K Lawrence [a]

[a]School of Veterinary Sciences, Massey University, Palmerston North, New Zealand; [b]School of Agriculture and Environmental Sciences, Massey University, Palmerston North, New Zealand

ABSTRACT

Case history: Serum and liver samples from 35, 2-year-old dairy heifers that had fractured one or both humeri post-calving between July and December 2019 were submitted to a diagnostic laboratory for analysis. Serum samples were analysed for albumin, β-hydroxybutyrate (BHB), creatinine, Ca, Mg, phosphate, non-esterified fatty acids (NEFA), and serum Cu concentration. Liver samples were analysed for liver Cu concentration. Data were compared to published reference intervals. Data values for heifers that prior to fracture had grazed fodder beet were also compared to values for those that had grazed pasture.

Clinical findings: Sixty-nine percent of heifers with humeral fracture had serum creatinine concentrations below the lower value of the reference range (55–130 μmol/L). In 3/32 (9%) heifers, serum NEFA concentrations were increased above the reference value indicating body fat mobilisation (≥1.2 mmol/L for peri-partum cows) and in 20/35 (57%) heifers BHB serum concentrations were above the reference value indicating subclinical ketosis (≥1.1 mmol/L for peri-partum cows). In 24/35 (69%) heifers, liver Cu concentration was low (≤ 44 μmol/kg) or marginal (45–94 μmol/kg). The concentration of Cu in serum was low (≤ 4.5 μmol/L) in 2/33 (6%) heifers and marginal (4.6–7.9 μmol/L) in 5/33 (15%) heifers. There was moderate positive correlation between the logged concentrations of Cu in paired liver and serum samples, $r_{(31)} = 0.43$; (95% CI = 0.1–0.79; p = 0.014). One heifer had a serum phosphate concentration below the lower limit of the reference range (< 1.10 mmol/L). For all heifers, the concentrations of albumin, Ca, and Mg in serum were within the reference intervals (23–38 g/L, 2.00–2.60 mmol/L, and 0.49–1.15 mmol/L respectively). Over winter, 15/35 (43%) heifers grazed predominantly pasture, 14/35 (40%) grazed fodder beet and 6/35 (17%) had a mixed diet.

Clinical relevance: In some of these heifers with humeral fractures, there was evidence for protein and/or energy malnutrition in the form of elevated NEFA and BHB concentrations and low creatinine concentrations in serum. Liver Cu concentrations were also reduced in most affected heifers. However, the absence of a control group means it is not possible to determine if these are risk factors for fracture or features common to all periparturient heifers. Clinical trials and molecular studies are needed to determine the true contribution of Cu and protein-energy metabolism to the pathogenesis of spontaneous humeral fractures in dairy heifers.

Abbreviations: BHB: ß-hydroxybutyrate; NEFA: Non-esterified fatty acids

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Introduction

Spontaneous fracture of the humerus of dairy heifers was first reported in New Zealand in 2008, although, anecdotally, cases had been observed since the 1970s (Weston 2008). The incidence appears to be increasing, affecting animal welfare, farmers and veterinarians, and resulting in major economic losses to the dairy industry (Dittmer *et al*. 2016; Gibson *et al*. 2021). A specific cause for the condition has not been established and outside New Zealand, only sporadic cases have been reported in Australia (Dittmer *et al*. 2016; Anonymous 2020; Gibson *et al*. 2021). The condition is described predominantly in heifers following their first calving but is occasionally reported in heifers prior to calving, and in cows in their second lactation (Dittmer *et al*. 2016; Gibson *et al*. 2021).

When investigating an outbreak of pathological bone fractures in ruminants, nutritional deficiencies, such as energy, protein, Ca, P, and vitamin D, should be considered as these can lead to abnormal bone growth or metabolic bone diseases such as osteoporosis and rickets/osteomalacia (Constable *et al*. 2016; Craig *et al*. 2016). In the first reported outbreak of spontaneous humeral fractures in dairy heifers, low liver and serum Cu concentrations were reported in many of the affected heifers and it was hypothesised that Cu deficiency in the months leading up to fracture

CONTACT A Wehrle-Martinez A.WehrleMartinez@massey.ac.nz
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had caused increased bone fragility resulting in fracture (Weston 2008). A recent investigation into humeral fractures in dairy heifers concluded that affected heifers had suffered from osteoporosis which was probably associated with periods of protein and or energy malnutrition, increased osteoclastic bone resorption to meet the Ca demands of lactation, and periods of Cu deficiency (Wehrle-Martinez *et al*. 2022). It was suggested that these factors decreased bone strength and led to fracture of the humerus (Wehrle-Martinez *et al*. 2022).

It is well known that dairy cows and heifers go through particular changes in their metabolism to supply adequate energy and minerals to the developing fetus, for parturition and to initiate and maintain lactation (Horst *et al*. 2005). Meeting the changing mineral and energy demands from pregnancy through to lactation is essential for successful transition through these periods and for avoiding peri and post-parturient metabolic diseases (Horst *et al*. 2005; McArt *et al*. 2013). When there is suboptimal nutrition, it is possible that alongside the more common presentations of metabolic diseases such as milk fever or acetonaemia, there can also be more gradual concurrent negative effects on bone mineralisation.

To date, the energy and mineral status of dairy heifers euthanised following a humeral fracture has not been reported. This report describes the concentrations of serum biochemistry analytes and concentrations of Cu in serum and liver in heifers that were euthanised due to spontaneous humeral fracture. In the absence of a control group, the concentrations in the fracture group were compared to published reference ranges, acknowledging that concentrations below the reference value are not synonymous with the development of clinical disease.

Case history

This was an observational study using liver and serum samples from 35, 2-year-old spring-calving heifers, on 17 different dairy farms, that fractured one or both humeri in the post-partum period from July to December 2019. Of the 17 different farms, 11 farms were located in Southland, nine in Canterbury, nine in Otago, one in Manawatū, two in the West Coast, two in Waikato, and one in the Tasman region of New Zealand. The heifers were sampled after the humeral fracture was reported by the farmers to their veterinarian. Blood samples were collected by veterinarians into 10 mL vacutainer tubes (BD, Plymouth, UK) with no additives as part of a diagnostic investigation into the cause of the fracture. A sample of liver was collected from all the affected heifers after euthanasia. The serum and liver samples were submitted to IDEXX Laboratories New Zealand Pty. Ltd. (Palmerston North, NZ). Serum samples from all 35 heifers were analysed for the concentration of the following biochemistry analytes as part of a bovine metabolic test profile: albumin, β-hydroxybutyrate (BHB), creatinine, Ca, Mg, and phosphate. The concentration of non-esterified fatty acids (NEFA) in serum was measured in 32 heifers and serum Cu concentration in 33 heifers. In liver samples, liver Cu concentration was measured.

Data on breed and the predominant diet offered in the winter months leading up to fracture occurrence were recorded by farmers and or veterinarians on the submission form for each case submitted. The feed category descriptors used within the submission form were pasture, fodder beet and other/mixed (described as a mix of feeds including kale, oats, swedes, maize silage), depending upon which one constituted the predominant feed on a dry matter intake basis.

Data analysis

Control samples were not available for comparison, and so the raw data (Supplementary Material, Table 1) were summarised as mean and SD and compared to reference intervals provided by IDEXX Laboratories (Table 1). The reference ranges for BHB and NEFA are, according to the reference laboratory, for adequately fed cattle (on a grass or total mixed ration diet) with variation in the average serum concentration according to the number of days peripartum. Because the exact time after calving for each heifer was not known, conservatively a serum NEFA concentration ≥ 1.2 mmol/L was interpreted as indicative of mobilisation of fat at the time of sampling. Similarly, BHB serum concentrations ≥ 1.1 mmol/L were considered high and indicative of negative energy balance.

Table 1. Mean (±SD) and range of concentrations of albumin, creatinine, phosphate, calcium, magnesium, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) in serum of dairy heifers with humeral fracture, and the proportion of heifers whose concentrations were low (L) or increased (I) compared to references ranges.

Analyte	n	Reference range	Mean (± SD)	Range	Proportion outside the reference range
Creatinine (μmol/L)	35	55–130	48.7 ± 10.52	36–73	24/35 (69%) L
BHB (mmol/L)	35	Increased: 1.1–10.0	1.1 ± 0.31	0.4–2.1	20/35 (57%) I
NEFA (mmol/L)	32[a]	Increased: 1.2–4.0	0.5 ± 0.36	0.2–1.4	3/32 (9%) I
Phosphate (mmol/L)	35	1.10–2.80	2.1 ± 0.48	0.78–2.93	1/35 (3%) L
Ca (mmol/L)	33[b]	2.00–2.60	2.4 ± 0.14	2.12–2.74	0/33
Mg (mmol/L)	33[b]	0.49–1.15	0.9 ± 0.20	0.5–1.32	0/33
Albumin (g/L)	35	23–38	32.4 ± 2.6	28–38	0/35

[a]Serum samples for NEFA concentration was only available for 33 heifers.
[b]In two cases, the concentration was reported as indeterminate by the reference laboratory.

The proportion of samples with analyte concentrations outside the reference ranges was also reported. Pearson's correlation coefficient was used to assess the strength of the linear relationship between the concentration of Cu in the liver and serum. Values from these parameters were first log-transformed to achieve a normal distribution. For the data summarising feeding management before fracture occurrence, simple proportions without 95% CI or comparisons are reported, as the data are a non-random sample from a population of heifers with fractures.

Statistical analysis was performed using SPSS statistics software, version 27 (SPSS Inc., Chicago, IL, USA).

For analysis of Cu data, serum Cu concentrations were categorised as low (0–4.5 µmol/L), marginal (4.6–7.9 µmol/L), adequate (8.0–20.0 µmol/L), or high (20.0–50.0 µmol/L) and liver Cu was categorised as low (0–44 µmol/kg), marginal (45–94 µmol/kg), adequate (95–2000 µmol/kg) or high (2000–50000 µmol/kg).

Clinical findings

Serum and liver samples were available from 35 heifers with humeral fractures collected from 17 farms (Supplementary Material, Table 1). The mean and SD for the biochemistry analytes are presented in Table 1. In 24/35 (69%) heifers with humeral fractures, the concentration of creatinine in serum was below the lower value of the reference range (55–130 µmol/L). In 3/32 (9%) heifers, serum NEFA concentration was greater than the reference value indicative of mobilisation of body fat (≥1.2 mmol/L for periparturient cows) and in 20/35 (57%) heifers BHB concentrations in serum were above the reference value indicating subclinical ketosis (≥1.1 mmol/L for periparturient cows). Three heifers with serum NEFA concentrations ≥ 1.2 mmol/L also had high serum BHB concentrations ≥ 1.1 mmol/L.

The concentration of phosphate in the serum of one heifer was below the lower limit of the reference range (<1.10 mmol/L). In two heifers, serum concentrations of Ca and Mg were reported as indeterminate by the referring laboratory. For the remaining heifers, the serum concentration of albumin, Ca, and Mg were within the reference intervals (23–38 g/L, 2.00–2.60 mmol/L, and 0.49–1.15 mmol/L respectively).

Case distribution according to breed showed that 15/34 (44%) heifers were Holstein-Friesian Jersey crossbreed and 19/34 (56%) were Holstein-Friesian. In the case of farms, 9/17 (53%) of farms had Holstein-Friesian Jersey crossbreed cows and 7/17 (41%) had Holstein-Friesian cows, with one submission not reporting breed data.

Over winter, 15/35 (43%) heifers grazed predominantly pasture on 9/17 (53%) farms, 14/35 heifers (40%) grazed fodder beet on 3/17 farms (18%) and 6/35 heifers had a mixed diet on 5/17 farms (29%). Creatinine concentration in serum was below the reference range in 13/15 (87%) heifers grazing pasture, 7/14 (50%) heifers grazing fodder beet, and 4/6 (67%) heifers receiving a mixed diet. The concentration of BHB in serum was above the reference range in 5/15 (33%) heifers grazing pasture, 9/14 (64%) heifers grazing fodder beet, and 6/6 (100%) heifers receiving a mixed diet.

Most heifers (24/35, 69%) had low or marginal liver Cu concentration (< 95 µmol/kg), but only 7/33 (21%) heifers had low or marginal serum Cu concentration (< 8 µmol/L) (Table 2). Of the heifers with low or marginal liver Cu concentration, 11/15 (73%) heifers grazed pasture, 9/14 (64%) heifers grazed fodder beet and 4/6 (67%) heifers received a mixed diet. For low or marginal serum Cu concentrations, 3/13 (23%) heifers grazed pasture, 4/14 (29%) heifers grazed fodder beet and 0/6 (0%) heifers received a mixed diet.

Paired samples (n = 33) showed that all seven heifers with low or marginal concentrations of Cu in serum had low or marginal concentrations of Cu in the liver. Of the remaining heifers, 16/33 (49%) had adequate or high serum Cu concentrations but low or marginal liver Cu concentrations and 10/33 (30%) had adequate Cu concentrations in both serum and liver. There was a moderate positive correlation between the logarithm of the concentration of Cu in liver and the logarithm of the concentration of Cu in serum, $r_{(31)} = 0.43$; (95% CI = 0.1–0.79; p = 0.014).

Table 2. Mean (±SD) and range of concentrations of copper (Cu) in serum and liver of dairy heifers with humeral fractures, and the proportion of heifers whose concentrations were within reference ranges categorised as low, marginal, adequate, or high.

Reference ranges for Cu concentration in tissues	N	Proportion	Mean (±SD)	Range
Serum	33[a]		14.8 ± 7.34	3.2–31.4
Low 0–4.5 µmol/L		2/33 (6%)		
Marginal 4.6–7.9 µmol/L		5/33 (15%)		
Adequate 8.0–20.0 µmol/L		18/33 (55%)		
High 20.0–50.0 µmol/L		8/33 (24%)		
Liver	35		187 ± 342	15–1540
Low 0–44 µmol/kg		21/35 (60%)		
Marginal 45–94 µmol/kg		3/35 (9%)		
Adequate 95–2,000 µmol/kg		11/35 (31%)		
High 2,000–50,000 µmol/kg		0/35 (0%)		

[a]Serum samples were only available for 33 heifers.

Discussion

Negative energy balance was common in heifers with humeral fracture, with 57% of heifers showing high BHB concentration and 69% of heifers having creatinine concentrations below the reference range. Furthermore, low or marginal liver copper concentration was a common finding in heifers with humeral fractures in New Zealand.

Although 57% of heifers had increased serum BHB concentration, the increases were moderate, with the mean for all heifers within the adequate range and the maximum value of 2.1 mmol/L recorded. Although there are several different cut points for BHB in establishing negative energy balance (Compton *et al*. 2014; Brunner *et al*. 2019), the prevalence of increased serum BHB concentration in our study was similar to these reports and indicates that the levels of negative energy balance present in these heifers were typical for dairy heifers in New Zealand. Thus, there is no evidence from our study to suggest a role for subclinical ketosis as a risk factor for humeral fractures in these dairy heifers.

Given the nature of this study and the uncertainty around interpreting point values for serum creatinine in ruminants, caution needs to be exercised when commenting on the 69% reported prevalence for low serum creatinine in the heifers in this study. Increased concentrations of serum creatinine are commonly associated with impaired renal function but in normally hydrated animals with normal renal function, serum creatinine concentration is weakly positively associated with muscle thickness and positively associated with body condition score (Agenäs *et al*. 2006; Russell and Roussel 2007; Megahed *et al*. 2019). Higher plasma creatinine concentrations have also been reported by Cozzi *et al*. (2011) as normal in primiparous (67 μmol/L) *vs.* multiparous cows (64 μmol/L) while the reference range used by the laboratory refers to adult cows.

While low serum creatinine concentration can be secondary to utilisation of muscle protein for energy production, in this study the impact of humeral fracture on energy and water intake may have confounded this effect. Nevertheless, this potential lower muscle mass in affected heifers is an intriguing factor not previously described. Humeral fractures are uncommon in ruminants, and this is thought to be because of the larger muscles around the humerus (Rakestraw 1996). Hence, lower muscle mass (either due to decreased formation and/or increased utilisation) should be further investigated as a risk factor for bone fractures in heifers.

Bone strength is dependent on the formation of collagen and elastin crosslinks, which requires the Cu-dependent enzyme lysyl oxidase (National Research Council 2001). Previous work has suggested an association between humeral fractures and low liver and serum Cu concentrations (Weston *et al*. 2012; Dittmer *et al*. 2016; Wehrle-Martinez *et al*. 2022). In this study, liver Cu concentrations were low or marginal in 69% of heifers, indicating deficient Cu intake of at least one month of duration (Ensley 2020). However, the impact of this on bone strength is unclear, as for 79% of the heifers, serum Cu was above marginal levels at euthanasia indicating that at the tissue level, Cu supplies were sufficient to maintain Cu-dependent enzymes such as lysyl oxidase.

Variability in the serum and liver Cu concentrations is a feature of previous reports of the clinical signs – including bone fracture – of Cu deficiency (Mills *et al*. 1976; Suttle and Angus 1978; Graham 1991). Further work on the role of Cu deficiency in the pathogenesis of humeral fractures is needed to determine its effect on the formation of collagen crosslinks in the bone.

In this study, for some of the heifers with humeral fractures, there was evidence for protein and/or energy malnutrition in the form of decreased creatinine, increased NEFA and BHB concentrations, and low Cu concentrations in the liver. However, the absence of a control group means it is not possible to determine if these are risk factors for fracture or features common to all peri-parturient heifers. Clinical trials and molecular studies are needed to determine the true contribution of Cu and energy/protein metabolism to the pathogenesis of spontaneous humeral fractures in heifers.

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ORCID

A Wehrle-Martinez http://orcid.org/0000-0002-3515-9164
K E Dittmer http://orcid.org/0000-0002-1813-2197
P J Back http://orcid.org/0000-0002-8939-686X
C W Rogers http://orcid.org/0000-0002-4253-1825
K Lawrence http://orcid.org/0000-0002-2453-1485

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Wehrle-Martinez, A

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