Serum zinc and copper concentrations in relation to spontaneous abortion in cows: implications for human fetal loss

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The purpose of this study was to investigate the effect of the status of dietary zinc and serum zinc and copper concentrations on the risk of fetal loss in 570 cows. Three herds received no supplements (herds 1, 3, 4), while cows in herd 2 received supplements of either 7 g zinc week\textsuperscript{-1} (n = 118), as zinc methionine, or a control diet containing methionine (n = 128). Serum zinc, copper and metallothionein concentrations were determined once a month throughout gestation. Logistic regression and survival analysis were used to examine associations between risk of fetal loss and serum zinc, copper, copper:zinc, or metallothionein concentrations, supplement level, and maternal age at conception. The risk of fetal loss increased when both serum zinc decreased and copper concentrations increased (P < 0.0001; relative risk = 10.28, 95% confidence intervals = 4.69, 22.5). The attributable risk, for a decline in the zinc concentration by 10 μmol l\textsuperscript{-1} and an increase in the copper concentration by 5 μmol l\textsuperscript{-1} was 90.27%. Methionine-supplemented cows had a higher risk of fetal loss compared with zinc-methionine-supplemented cows (one-tailed P = 0.0375; relative risk = 2.98). Cows in herds 1, 3 and 4 had a higher risk for abortion than did zinc-methionine-supplemented cows in herd 2 (relative risk = 26.27, 95% confidence intervals = 2.31, 299.38; relative risk = 40.87, 95% confidence intervals = 3.50, 458.43; relative risk = 41.53, 95% confidence intervals = 3.77, 457.02, respectively). Our results suggest that inflammation and zinc nutriture may play an important role in fetal loss in dairy cows.

Introduction
Patterns of fetal loss in dairy cows and women are similar: both experience spontaneous abortion at comparable frequencies (10% versus 12–15%, respectively; Kline et al., 1989; Thurmond et al., 1990a, b; Thurmond and Picanso, 1990) and the duration of gestation for cattle (284 days) and women (270 days) is comparable (Anderson, 1991). Spontaneous bovine fetal loss is defined as fetal death between gestational day 42 (clinical recognition of pregnancy per rectum) and gestational day 260 (Thurmond and Picanso, 1990). Spontaneous human fetal loss is defined as fetal death before the 28th week of pregnancy (gestational day 196). It should be noted that many human epidemiological studies delimit the period at risk for fetal loss to the period after clinical recognition of pregnancy (5–7 weeks after the last menstrual period) until the 28th week (Kline et al., 1989). In cattle, the distribution and period of risk for fetal loss, after clinically recognized pregnancy, is similar (peak incidence at 6–18 weeks; Thurmond et al., 1990a, b; Thurmond and Picanso, 1990) to that described for women (peak incidence at 7–19 weeks; Wilcox et al., 1988; Kline et al., 1989; Hertz-Picciotto et al., 1989; Goldhaber and Fireman, 1991).

Karyotypically abnormal fetuses account for approximately a third of spontaneously aborted fetuses in women (22–61%), but the cause of these chromosomal aberrations is rarely established (Kline et al., 1989). In the remaining two-thirds of karyotypically normal aborted human fetuses, fever and infection are significant risk factors for fetal loss (Kline et al., 1985). Similarly, many infectious agents have been associated with approximately 30% of bovine abortions (Anderson et al., 1991; Kirkbride, 1992a, b, 1993; Knudtson and Kirkbride, 1992), but most cases lack an aetiological diagnosis. However, the reported fetal age is consistently 2–3 months older than the period of greatest risk for abortion (Thurmond and Picanso, 1990; Anderson et al., 1991; Kirkbride, 1992a, b, 1993; Knudtson and Kirkbride, 1992); these bovine fetuses may not be representative of fetuses lost during the peak incidence of abortion. As in the case of early fetal loss in women, factors leading to chromosomal aberrations in cattle may be important causes of abortion in cattle.

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Primary or secondary zinc (Zn) deficiency has been suggested to be a causal factor in fetal loss, malformations, premature and postmaternal birth, and small-for-gestational-age infants in women (Hambidge et al., 1975; Jameson, 1976; LaZebrink et al., 1988; Cherry et al., 1989; Hinks et al., 1989; Keen and Hurley, 1989; Apgar, 1992; Keen et al., 1993a, b), nonhuman primates (Keen et al., 1993b), rodents (Keen and Hurley, 1989; Apgar, 1992) and sheep (Egan, 1972; Masters and Fels, 1980; Masters and Moir, 1983; Apgar, 1992). In addition, periconceptional Zn deficiency has been associated with abnormalities in early embryonic growth in rats and mice (Hurley and Shramer, 1975; Peters et al., 1991) and karyotypic changes in severely Zn-deficient rats (Bell et al., 1975). Zn supplementation has been suggested to reduce pregnancy complications and improve fetal growth and pregnancy outcome in women (Jameson, 1976; McMichael et al., 1982; Cherry et al., 1989; Simmer et al., 1990) and typically managed sheep (Egan, 1972; Masters and Fels, 1980; Masters and Moir, 1983). Low concentrations of Zn (serum and/or leukocyte Zn concentrations) in women have been associated with a higher incidence of fetal loss and abnormalities compared with controls (Hambidge et al., 1975; Jameson, 1976; Meadows et al., 1981; Breskin et al., 1983; Mukherjee et al., 1984; Wells et al., 1987; Higashi et al., 1988; Hinks et al., 1989; Cherry et al., 1989; Keen and Hurley, 1989; Apgar, 1992; Keen et al., 1993b), although others have reported that the Zn status may not affect pregnancy outcome (Hunt et al., 1984; Ghosh et al., 1985; Hunt et al., 1985; Austen et al., 1989; Mahomed et al., 1989; Apgar, 1992).

The purpose of the present study was to examine relationships between fetal loss and monthly serum concentrations of Zn, Cu and metallothionein supplementation in 570 commercial dairy cows, while adjusting for herd of origin and age at conception.

### Materials and Methods

#### Animals

To increase the power of our test and to decrease the chance that an unrepresentative herd was selected, we examined the relationship between abortion and maternal and environmental factors in four commercial herds of Holstein dairy cows. The infrequency of fetal loss (10%; Thurmond and Piceno, 1990) means that many pregnancies must be analysed to detect differences between presumed causally related factors and fetal loss in aborting and nonaborting cows. If a factor (i.e. increasing maternal age, higher serum copper concentration, lower serum zinc concentration) has the same magnitude and direction of change in aborting compared with nonaborting cows in several herds, then the likelihood that those observed changes are causally associated with abortion is increased (Kline et al., 1989). The Animal Use and Care Administrative Advisory Committee approved all procedures and methods of animal handling. Demographical information for all herds included age, number of previously completed pregnancies (parity), date of supplementation, date of conception, date of abortion, and date of blood collection. After generation and verification of all laboratory data, demographical information, treatment group and data for identification of pregnancy outcome were collated for each dam. Demographical information for each herd included the total number of adult cows in the herd, the number of cows in the study that aborted, the number of cows in the study that did not abort, the percentage of cows in the study that aborted, the mean number of days to abortion, the median number of days to abortion, and the minimum and maximum number of days to abortion (Table 1).

<table>
<thead>
<tr>
<th>Herd number</th>
<th>Number milked</th>
<th>Number aborted/ number not aborted</th>
<th>Days in gestation at abortion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/101</td>
<td>(8.2%)</td>
<td>122^b</td>
</tr>
<tr>
<td></td>
<td>(757)</td>
<td></td>
<td>(145)^c</td>
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<tr>
<td>3</td>
<td>13/99</td>
<td>(11.6%)</td>
<td>135</td>
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<tr>
<td></td>
<td>(1188)</td>
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<td>(125)</td>
</tr>
<tr>
<td>4</td>
<td>14/88</td>
<td>(13.7%)</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>(631)</td>
<td></td>
<td>(116.5)</td>
</tr>
<tr>
<td>2</td>
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<td>(1.7%)</td>
<td>113</td>
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<td></td>
<td>(118)</td>
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<td>(113)</td>
</tr>
<tr>
<td>1, 3, 4</td>
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<td>(11.1%)</td>
<td>125</td>
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<td>(122)</td>
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<tr>
<td></td>
<td>(2576)</td>
<td></td>
<td>52–236</td>
</tr>
</tbody>
</table>

*Percentage of cows that aborted [aborted/(nonaborted + aborted) × 100].
^Mean number of days in gestation at abortion.
^Minimum number of days in gestation at abortion.
^Minimum and maximum.

Table 1. Demographic information, rates of abortion and times to abortion in Holstein dairy cows

Three dairy herds (herds 1, 3 and 4) were monitored for risk of fetal loss; demographical, health, vaccination and management information, and methods used to assess prospectively the effects of infectious agents on abortion in these cows have been reported (Thurmond et al., 1990b). Briefly, pregnant cows were enrolled at diagnosis of pregnancy and blood was collected monthly until death, removal from the herd (2–3 months after abortion) or until cessation of lactation. Serum was removed from the red cell mass and stored at −70°C in plastic microfuge tubes identified by accession number. Cows in herds 1, 3 and 4 were considered to be typically fed controls and were used for comparison with the Zn-supplemented cows in herd 2.

Cows in herd 2 were administered vaccines for the common abortifacient agents Brucella abortus, infectious bovine rhinotracheitis virus (IBRV), bovine virus diarrhoea virus (BVDV), bovine parainfluenza-3 and antigens to five Leptospira serovars.

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between 4 and 6 months of age. This vaccination protocol was similar to that in herds 1, 3 and 4. With the exception of the vaccine for brucellosis; this was repeated at 14–16 months of age and after each full gestation. Cows in herd 2 had a rolling average milk production of 10 634.5 kg milk and 367 kg fat per cow year \(^{-1}\). The average time from calving to conception was 121 days and the intercalving interval was 13.52 months.

### Dietary supplementation

A prospective double-masked Zn supplementation trial was conducted on herd 2 between 3 June 1990 and 30 June 1991. A random numbers generator (Statgraphics\textsuperscript{®}, STSC Inc., Rockville, MD) was used to assign cows in herd 2 to either a Zn-methionine-supplemented or a methionine-supplemented group. Cows were given a colour-coded paste supplement once a week \((n = 118)\), which contained a hydrogenated vegetable oil carrier with either Zn methionine \((7 \text{ g Zn week} \cdot 1^{-1}; \text{ Zinpro Corporation, Bloomington, MN})\) or an equivalent amount of methionine and \(\text{NaSO}_{4}{\text{ }}\) (control cows \(n = 128\)). The amount of Zn supplementation was intended to double the National Research Council’s \(1988\) weekly dietary intake of Zn at the time that a cow would become pregnant (maximum dietary dry matter intake of 3.5\% for a cow’s bodyweight (BW) \(\times 750 \text{ kg BW} \times 7 \text{ days} \times 40 \text{ mg Zn kg}^{-1} \text{ dietary dry matter} = 7350 \text{ mg week}^{-1}\)).

Blood was collected once a month in plastic syringes with ammonium heparin \((\text{Sarstedt Laboratories Inc., Newton, NC})\) and centrifuged at \(4^\circ\text{C}\) and \(1200\ g\) for 20 min. The plasma was removed and allocated separately into plastic microfuge tubes previously identified with an accession number to maintain a masked trial. Plasma was frozen in liquid nitrogen, returned to the laboratory and stored at \(-20^\circ\text{C}\) prior to determination of Zn and Cu, or at \(-70^\circ\text{C}\) before determination of metallothionein. Reproductive examinations were performed per rectum twice a month, and pregnancy was confirmed at approximately gestational day 40, again at day 150 and at cessation of lactation (gestational day 220). Date of abortion was estimated as described by Thurmond and Picanso \(1990\). Period of risk for abortion was defined as gestational days 42–260 \((\text{Thurmond and Picanso, } 1990)\).

### Feed and supplement analysis

Feed concentrations of Cu, Fe, Mn and Zn were determined following the methods of the Association of Official Analytical Chemists \(1980\) \((\text{Ingman Laboratories, Minneapolis, MN } 55415)\). Dietary analysis of feedstuffs was completed for herd 2 only \((\text{range of monthly feed samples: } 27–54 \text{ mg Zn;} 13–19 \text{ mg Cu; } 43–88 \text{ mg Mn;} \text{ and } 165–421 \text{ mg Fe} \text{ kg}^{-1} \text{ diet})\). The concentration of metals in the supplements was determined by inductively coupled plasmapheresis \((\text{Zn methionine supplement, } 7.33 \text{ g Zn per cow week} \cdot 1^{-1}; \text{ methionine supplement, } 0.001 \text{ g Zn per cow week} \cdot 1^{-1})(\text{California Veterinary Diagnostic Laboratory System, Davis, CA})\). Chemical analysis of the diet in herds 1, 3 and 4 was not available. However, estimated intakes of Zn, Cu, Mn and Fe, based on analysis of individual dietary components, were as follows: 60–80 mg Zn; 15–20 mg Cu; 60–80 mg Mn; and 300–400 mg Fe kg\(^{-1}\) diet \((\text{Dairyman’s Creamery Cooperative Association, Tulare, CA})\).

### Determination of trace elements

Zn and Cu concentrations and the Cu:Zn ratio were determined in 0.4–1 m\(\text{l}\) aliquots of serum or plasma. We have previously shown that bovine Zn or Cu concentrations are similar when either plasma or serum Zn and Cu concentrations are determined, both plasma and serum samples are therefore termed serum \((\text{Keen and Feldman, } 1987)\). Samples were wet ashed with nitric acid, as described by Clegg \textit{et al.} \(1981\). One blank was analysed for every ten samples and two external controls were analysed with every 25 samples. Each sample was assayed at least twice, and results of external controls were used to calculate intra-assay and interassay coefficients of variation.

### Determination of metallothionein

Serum metallothionein concentrations were determined by the methods of Garvey \(1991\), using rabbit anti-rat metallothionein \((\text{RJ}-24)(\text{J. S. Garvey, as reported by Keen et al. } 1993b)\). Crossreaction with bovine metallothionein isoforms has been documented \((\text{Winge et al., } 1986)\) and verified by western blot.

### Statistical analyses

Preliminary descriptive data provided the mean, SD and the range of the serum Zn, Cu and metallothionein concentrations, and the Cu:Zn ratio. Plots of Zn, Cu and metallothionein concentrations for each cow throughout gestation were generated \((\text{SigmaPlot®}, \text{Jandel Scientific, Corte Madera, CA})\). Differences between aborting and nonaborting cows for each metabolite were determined by Student’s \(t\) test \((\text{BMDP 3D; Sookne and Forsythe, } 1990)\). Analysis of variance was used to examine for differences in the serum concentrations of Zn, Cu and metallothionein and the Cu:Zn ratio \((\text{BMDP 7D; Dixon et al., } 1990)\), as a means of screening for potentially confounding or explanatory variables. Differences noted between herds or treatment groups indicated possible effect modification or confounding to be considered in logistic and survival analysis models.

Logistic regression was used to obtain one estimate for risk of abortion based on herd of origin, supplementation status, age at conception, mean concentrations of Zn, Cu and metallothionein, and the Cu:Zn ratio \((\text{BMDP LR; Engelman, } 1990)\). Abortion was coded as 0 and nonabortion as 1. Positively directed coefficients in the final model were interpreted as having a lower probability of abortion than negatively directed coefficients. The final model had the general form: abortion = constant + supplement level + age at conception + time of sample collection + Zn + Cu + metallothionein + Cu:Zn. Five herd and treatment categories were created for use in logistic regression to form a single categorical variable: supplement level. Supplement level was classified as Zn-supplemented cows in herd 2 (presumed reference level), methionine-supplemented cows in herd 2, and
nonsupplemented cows (in herds 1, 3 or 4). Supplement level was included as a partial design variable (Engelman, 1990), and was forced into the model with the presumption that it was a confounding or explanatory variable. Age at conception was also forced into the model as an interval-scaled continuous variable that was thought to be a proxy measure for gravidity and parity, which are known risk factors for abortion (Thurmond et al., 1990a). Time of sample collection was divided into intervals of 30 days of gestation (e.g. 0–30, 31–60). Adjusted risk odds ratios (ROR) for abortion were estimated along with their approximate 95% confidence interval (95% CI) as:

\[
ROR = e^{\beta_1 + \beta_2 \ldots + \beta_k + \beta} = e^{\beta X_i},
\]

and

\[
95\% \text{ CI} = e^{\beta X_i \pm 2SE \text{ of } \beta X_i},
\]

where \( \beta X_i \) is the difference between the coefficient of interest (\( x \)) and that of the reference level (\( y \)).

Survival analysis was used as a second method for comparing relationships among nutritional, maternal and environmental factors and abortion. The Cox proportional hazard model (BMIDP 2L; Hopkins, 1990) was used to determine the instantaneous risk of abortion (hazard) and relative risk of pregnancy failure when accounting for daily variations in Zn, Cu and metallothionein concentrations, and the Cu:Zn ratio. Pregnancy failure was defined as gestational day of fetal expulsion. Censoring occurred on the gestational day that a nonaborting cow was removed from the herd or the end of the risk period (260 days). The model had the general form:

\[
\lambda(t) = \lambda_o(t) e^{\beta X_1 + \beta X_2 \ldots + \beta X_k + \beta(Z_t)}.
\]

The unknown baseline hazard was \( \lambda_o(t) \), \( \beta \), and \( X_i \) were the non-time-dependent co-variates (supplement level, as described for logistic regression, number of days that the cows had lactated before conception and age at conception), and \( \beta(Z_t) \) was the time-dependent function for Zn, Cu and metallothionein concentrations and the Cu:Zn ratio. The concentrations of Zn, Cu and metallothionein and the Cu:Zn ratio were included in the model as time-dependent co-variates because their values changed with the risk of abortion; a change in their values may be important predictors of pregnancy failure along with other explanatory variables (Cox and Oakes, 1984). To examine the time-dependent functions of the variables metallothionein, Zn and Cu concentration and the Cu:Zn ratio on the risk of abortion, a univariate linear least-squares estimate was determined. For each cow, an estimate of the intercept (time = 0) and slope for each metabolite was made, where the metallothionein, Zn and Cu concentrations and the Cu:Zn ratio were dependent variables and gestational age (time) was the independent variable. A coefficient was interpreted to be positively associated with hazard for abortion when positive.

Validity of the assumptions of proportionality for non-time-dependent co-variates and the log-linear nature of the model were tested as suggested by Kalbfleisch and Prentice (1980). Time-dependent strata were created so that an individual’s time of entry into the model was adjusted to the day the cow was first bled (time of first recognition of pregnancy and inclusion in the trial). Use of time-dependent strata adjusts the time that a cow enters the model, preventing inclusion of cows that were enrolled in the study after a given failure time, following the recommendations of Hopkins and Hormung (1985). Relative risks (RR) for abortion were estimated along with their approximate 95% CI as follows:

\[
RR = e^{(\beta X_1 + \beta X_2 \ldots + \beta X_k + \beta(Z_t))} / e^{(\beta X_1 + \beta X_2 \ldots + \beta X_k + \beta(Z_t))} = e^{\beta X_i},
\]

and

\[
95\% \text{ CI} = e^{\beta X_i \pm 2SE \text{ of } \beta X_i},
\]

where \( \beta X_i \) is the difference between the coefficient of interest (\( x \)) and that of the reference level (\( y \)). Variance for multiple coefficients (i.e. supplement level) was estimated as suggested by Kalbfleisch and Prentice (1980). Risk for fetal loss in the Zn-methionine-supplemented and methionine-supplemented cows was a priori assumed to be lower in the group given Zn methionine; comparison of risk of fetal loss was therefore one-tailed (\( P < 0.05 \)). In all other comparisons a direction of change was not assumed; comparisons were therefore two-tailed (\( P < 0.05 \)). A coefficient was considered to be significant when its Z value was > 1.96 (Kalbfleisch and Prentice, 1980). Attributable risk was estimated as: (relative risk – 1)/relative risk (Kahn and Sempos, 1989).

### Results

#### Assay variation

There were 246 cows in herd 2 and 324 cows in herds 1, 3 and 4 included for analysis (Table 1). Intra-assay coefficients of variation for determining serum Zn and Cu concentrations were 6.8% and 5.4%, respectively. Interassay coefficients of variation were 10.3% for Zn and 5.7% for Cu. Most samples (> 90%) were near or below the assay limit of detection for serum metallothionein. While duplicate samples had a coefficient of variation < 5%, the intra-assay variation at these low metallothionein concentrations was > 50%, and the interassay variation was > 100%.

#### Descriptive statistics

Demographical and descriptive results for aborting and nonaborting cows for each supplement level are presented in Tables 1 and 2. Supplementation was begun before conception in all cows that aborted. Nonaborting cows in herd 3 had higher serum Zn concentrations (14.72 µmol l⁻¹) than did nonaborting cows in herds 1 (13.56 µmol l⁻¹), 2 (13.87 µmol l⁻¹) and 4 (13.98 µmol l⁻¹) (P < 0.001). Nonaborting cows in herd 2 had higher serum Cu concentrations (13.76 µmol l⁻¹) than did nonaborting cows in herds 1 (9.31 µmol l⁻¹), 3 (9.05 µmol l⁻¹) and 4 (9.19 µmol l⁻¹) (P < 0.001). Nonaborting in herds 1 (0.18 nmol l⁻¹) and 4 (0.14 nmol l⁻¹) had lower serum metallothionein concentrations than did nonaborting cows in either herd 2 (3.42 nmol l⁻¹) or 3 (2.84 nmol l⁻¹) (P < 0.01). Nonaborting cows in herd 2 had a higher serum Cu:Zn ratio (1.04) than did nonaborting cows in herds 1 (0.73), 3 (0.64) or 4 (0.69) (P < 0.01); both herds 1 and 4 had a higher serum Cu:Zn ratio than did herd 3 (P < 0.05). Nonaborting Zn-methionine-treated cows and methionine-treated cows had similar serum Zn (13.95, 13.79 µmol l⁻¹), Cu (14.06, 13.86 µmol l⁻¹) and Zn (13.71, 13.51 µmol l⁻¹), respectively.
Zinc, copper and spontaneous abortion

13.48 μmol l⁻¹), and metallothionein (2.82, 3.98 nmol l⁻¹) concentrations, and serum Cu:Zn ratios (1.06, 1.02) (P > 0.05).

For logistic regression and survival analysis models, ROR and RR for abortion in a cow with a serum Zn concentration of 10 μmol l⁻¹, serum Cu concentration of 15 μmol l⁻¹ and a serum Cu:Zn ratio of 15:10 were compared with a reference cow with a serum Zn concentration of 20 μmol l⁻¹, a serum Cu concentration of 10 μmol l⁻¹ and a serum Cu:Zn ratio of 10:20. Zn and Cu concentrations < 5 μmol l⁻¹ (5 of 3155 and 8 of 3155 observations, respectively). Zn concentrations > 25 μmol l⁻¹ (28 of 3155), and Cu concentrations > 20 μmol l⁻¹ (33 of 3155) were rarely observed in the 3155 serum samples in this study. Individual plots of serum Zn and Cu concentrations during gestation indicated that an increase in the serum Cu:Zn ratio predicted expulsion of the fetus by up to 3 months for 22 of 42 abortions. Most cows displayed minor variations in serum Cu and Zn concentrations throughout gestation. However, a magnitude of change of 10 μmol Zn l⁻¹ (i.e. from 20 to 10 μmol l⁻¹) and 5 μmol Cu l⁻¹ (i.e. from 10 to 15 μmol l⁻¹) was observed in aborting and nonaborting cows. Estimation of RR and ROR was limited to these ranges.

Logistic regression

Results from logistic regression models suggested that supplement level (treatment and herd), age at conception, serum Zn and Cu concentrations, and the time interval in gestation during which a cow was bled were important predictive variables for estimating the odds of abortion (Table 3). Odds of abortion increased as maternal age at conception increased for each time interval examined (gestational days 31–60, 61–90 and 91–120). Odds of abortion decreased as serum Zn concentrations increased during days 31–60 of gestation (ROR = 0.21; 95% CI = 0.05, 0.96; comparing a concentration of 10 μmol l⁻¹ with a reference concentration of 20 μmol Zn l⁻¹). Odds of abortion increased as serum Cu concentrations increased during days 31–60 of gestation (ROR = 0.48; 95% CI = 0.21, 1.08; comparing a concentration of 15 μmol l⁻¹ with a reference concentration of 10 μmol Cu l⁻¹) and days 61–90 (ROR = 0.44; 95% CI = 0.19, 1.04). Because the time interval during gestation appeared to interact with serum Zn and Cu concentrations, survival analysis was used to account for the potential interaction of gestational age with each metabolite.

Survival analysis

The co-variates supplement level, maternal age at conception, and the time-dependent co-variates Zn x (time), Cu x (time), and (Cu-Zn) x (time) contributed significantly to risk (hazard) of abortion (Table 4). The RR of fetal loss was lower in Zn-methionine-treated cows than in methionine-treated cows (one-tailed P = 0.0375; RR = 2.98). Cows in herds 1, 3 and 4 had a higher risk of fetal loss than did Zn-methionine-supplemented cows in herd 2 (RR = 26.27, 95% CI = 2.31, 299.38; RR = 40.87, 95% CI = 3.50, 458.43; RR = 41.53, 95% CI = 3.77, 457.02, respectively). Similar to logistic regression models, at a fixed gestational time, as serum Zn concentrations decreased and serum Cu concentrations increased, the risk of abortion increased (RR = 10.28, 95% CI = 4.69, 22.5). The attributable risk, for a decline in the Zn concentration by 10 μmol l⁻¹ and an increase in the Cu concentration of 5 μmol l⁻¹ Cu was 90.27%.

Discussion

Zinc supplement level, maternal age at conception, serum Zn and Cu concentrations and serum Cu:Zn ratios were associated with risk of fetal loss. Supplementation with Zn methionine was associated with a lower risk of fetal loss than methionine supplementation (controls). Similarly, Zn methionine supplementation was associated with a lower risk of fetal loss than that in unsupplemented herds 1, 3 and 4. Although cows in all four herds were the same breed (Holstein) and were considered to be in excellent health and have good rates of production, comparisons between Zn-methionine-supplemented cows in herd 2 and cows in herds 1, 3 and 4 should be treated with caution, given differing management and environmental (including nutritional) factors among the herds. Maternal age at conception was an important risk factor of fetal loss in this study, as has been reported for women (Naylor and Warburton, 1979; James, 1983). For cows within a given herd, gestational stage and maternal age, low Zn and high Cu concentrations, and a high Cu:Zn ratio were associated with fetal loss.

Serum metallothionein concentrations were not associated with fetal loss in the present study. While concentrations of metallothionein in erythrocytes have been reported to be low in Zn-deficient rats (Sato et al., 1984) and humans (Grider et al., 1990; Thomas et al., 1992), pregnant Zn-deficient rhesus monkeys were reported to have high plasma metallothionein concentrations (Keen et al., 1993b). The increased metallothionein concentrations in the pregnant monkeys were attributed to inflammation that was secondary to their severe chronic Zn deficiency. Reasons for a lack of association between Zn supplement status, fetal loss and serum metallothionein in this study are most likely because cows in this study were considered to have adequate concentrations of Zn and did not have a chronic inflammatory disease (i.e. marked Zn deficiency). Results obtained in the current study do not support the routine use of the measurement of serum concentrations of metallothionein as a diagnostic tool for Zn status or abortion in cattle.

An association between lower Zn and higher serum Cu concentrations and fetal loss can most probably be attributed to inflammation (Graham, 1991). Endotoxin-induced inflammation has been documented to cause bovine abortion experimentally (Giri et al., 1990, 1991). Increased risk of fetal loss has previously been associated with changes in antibody titres to gram-negative organisms (Leptospora) and common viral pathogens (IBRV and BVDV) in herds 1, 3 and 4 (Thurmond et al., 1990b). Inflammation induced by microbial infection or microbial products (e.g. endotoxin) is associated with a decline in the plasma concentration of Zn, and a rise in the serum concentration of Cu (Barber and Cousins, 1988; Lohuis et al., 1988a, b; Dinarello, 1989; Shuster et al., 1993).
Table 2. Mean serum zinc, copper and metallothionein concentration and copper:zinc ratios during gestation in nonaborting and aborting Holstein dairy cows

<table>
<thead>
<tr>
<th>Herd number</th>
<th>Zinc (μmol l⁻¹)</th>
<th>Copper (μmol l⁻¹)</th>
<th>Metallothionein (nmol l⁻¹)</th>
<th>Copper/zinc ratio</th>
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<td>13.56 ± 0.15²</td>
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<tr>
<td>2</td>
<td>14.72 ± 0.14</td>
<td>14.78 ± 0.49</td>
<td>9.05 ± 0.07</td>
<td>9.67 ± 0.22²</td>
</tr>
<tr>
<td></td>
<td>(543)</td>
<td>(39)</td>
<td>(546)</td>
<td>(39)</td>
</tr>
<tr>
<td>3</td>
<td>13.98 ± 0.16</td>
<td>13.14 ± 0.69</td>
<td>9.19 ± 0.10</td>
<td>9.20 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>(4.1–33.6)</td>
<td>(32)</td>
<td>(480)</td>
<td>(32)</td>
</tr>
<tr>
<td>4</td>
<td>13.95 ± 0.10²</td>
<td>14.11 ± 0.41</td>
<td>14.06 ± 0.11</td>
<td>17.06 ± 1.53²</td>
</tr>
<tr>
<td></td>
<td>(697)</td>
<td>(8)</td>
<td>(697)</td>
<td>(8)</td>
</tr>
<tr>
<td>2</td>
<td>13.79 ± 0.10²</td>
<td>14.33 ± 0.59</td>
<td>13.48 ± 0.87</td>
<td>14.78 ± 0.87</td>
</tr>
<tr>
<td>Zn methionine</td>
<td>4.1–26.3</td>
<td>12.4–15.5</td>
<td>8.2–28.6</td>
<td>11.9–21.7</td>
</tr>
<tr>
<td></td>
<td>(697)</td>
<td>(8)</td>
<td>(697)</td>
<td>(8)</td>
</tr>
<tr>
<td>3</td>
<td>14.06 ± 0.09²</td>
<td>13.60 ± 0.34</td>
<td>9.19 ± 0.05</td>
<td>9.61 ± 0.14²</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.5–33.9</td>
<td>5.3–29.5</td>
<td>1.3–19.5</td>
<td>6.9–13.0</td>
</tr>
<tr>
<td></td>
<td>(1584)</td>
<td>(97)</td>
<td>(1591)</td>
<td>(96)</td>
</tr>
</tbody>
</table>

All cows were pregnant when blood was collected, except for one additional blood collection after abortion.

Values given are: "mean ± SEM. "range and "number of observations.

²Aborted cows significantly differ from pregnant cows (P < 0.05).

²Aborted cows significantly differ from pregnant cows (P < 0.01).
Individual plots of serum Zn and Cu concentrations during gestation suggest that a higher serum Cu: Zn ratio precedes expulsion of the fetus by up to 3 months for 22 of 42 abortions. This was supported by the results of logistic regression, in which lower serum Zn concentrations during the second month (31–60 days) and higher serum Cu concentrations during the third and fourth months of gestation (61–90 and 91–120 days) were associated with fetal loss. Results of survival analysis, which examined relationships between explanatory variables and pregnancy outcome at the time of fetal loss showed that lower Zn and higher serum Cu concentrations were highly correlated with fetal loss. Thus, lower serum Zn and higher serum Cu concentrations are associated with fetal loss before and at the time of fetal expulsion. Inflammatory or infectious changes, marked by fever, are associated with karyotypically normal fetal loss in women (Kline et al., 1985). Fever was noted to precede fetal expulsion by up to 62 days, but the odds of abortion were highest for fevers that occurred during the same month as fetal loss (Kline et al., 1985).

The present data suggest that by avoiding exposure to those factors that would cause a decrease in the plasma concentration of Zn of 10 µmol l⁻¹ and an increase in the concentration of Cu of 5 µmol l⁻¹, approximately nine of ten abortions in cows reared under similar conditions to those in this study might be prevented (attributable risk of 90.27%). In addition, these data suggest that approximately 90% of the abortions observed in this study are likely to be caused by inflammatory changes, rather than genetic aberrations. We suggest that future research efforts be directed at improving vaccines (e.g. endotoxin vaccines) or pharmacological intervention tactics (including nutrient intervention) that can attenuate inflammatory changes during pregnancy.
Table 4. Cox proportional hazards models describing relationships between risk of abortion and dietary treatment, herd, age at conception, the time-dependent co-variates serum zinc, copper and metallothionein concentrations, and ratios of copper:zinc

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient (se)</th>
<th>Z value</th>
<th>P value</th>
<th>Relative risk (95% confidence intervals)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Univariate estimate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Supplement level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>1.0566 (0.5936)</td>
<td>1.7802</td>
<td>0.0750</td>
<td>1.00</td>
</tr>
<tr>
<td>D2</td>
<td>3.2686 (0.6682)</td>
<td>4.8918</td>
<td>&lt;0.0001</td>
<td>2.98 (0.35, 23.97)</td>
</tr>
<tr>
<td>D3</td>
<td>3.6906 (0.6660)</td>
<td>5.5411</td>
<td>&lt;0.0001</td>
<td>26.27 (2.31, 299.36)</td>
</tr>
<tr>
<td>D4</td>
<td>3.7264 (0.6409)</td>
<td>5.8145</td>
<td>&lt;0.0001</td>
<td>40.87 (3.50, 458.43)</td>
</tr>
<tr>
<td>(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3)</td>
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<td></td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age at conception</strong></td>
<td>0.0218 (0.0048)</td>
<td>4.5135</td>
<td>&lt;0.0001</td>
<td>1.92 (1.44, 2.58)</td>
</tr>
<tr>
<td><strong>Time-dependent zinc concentration</strong></td>
<td>-0.0674 (0.1686)</td>
<td>-3.8981</td>
<td>&lt;0.0001</td>
<td>716.23 (24.58, 20868)</td>
</tr>
<tr>
<td><strong>Time-dependent copper concentration</strong></td>
<td>1.0065 (0.2353)</td>
<td>4.2778</td>
<td>&lt;0.0001</td>
<td>153.31 (1.71, 1612)</td>
</tr>
<tr>
<td><strong>Time-dependent copper:zinc</strong></td>
<td>-9.2763 (2.8194)</td>
<td>-3.2902</td>
<td>&lt;0.0010</td>
<td>&lt;0.0001 (0.00, 0.026)</td>
</tr>
<tr>
<td><strong>Attributable risk for the combined time-dependent covariates = 90.27%</strong></td>
<td></td>
<td></td>
<td></td>
<td>10.28 (4.69, 22.5)</td>
</tr>
</tbody>
</table>

*Time-dependent co-variates were based on the intercept and slope x (time) of changes in Zn, Cu or metallothionein concentrations, and ratios of Cu:Zn throughout gestation.

*Reference level.

*Comparing 60 months with a reference age at conception of 30 months.

*Comparing 10 µmol Zn l⁻¹ with a reference concentration of 20 µmol Zn l⁻¹ (mid-range of normal plasma Zn concentration).

*Comparing 15 µmol Cu l⁻¹ with a reference concentration of 10 µmol Cu l⁻¹ (mid-range of plasma Cu in this study).

*Comparing the ratio of 15 µmol Cu l⁻¹:10 µmol Zn l⁻¹ with a reference value of 10 µmol Cu l⁻¹:20 µmol Zn l⁻¹.

*Combined relative risk, comparing 10 µmol Zn l⁻¹ with a reference concentration of 20 µmol Zn l⁻¹, 15 µmol Cu l⁻¹ with a reference of 10 µmol Cu l⁻¹, and a ratio of 15 µmol Cu l⁻¹:10 µmol Zn l⁻¹ with a reference of 10 µmol Cu l⁻¹:20 µmol Zn l⁻¹.

Supplement level was coded as:

(1) Herd 2, Zn methionine: D1 = 0, D2 = 0, D3 = 0, D4 = 0.
(2) Herd 2, Methionine: D1 = 1, D2 = 0, D3 = 0, D4 = 0.
(3) Herd 3, Untreated: D1 = 0, D2 = 1, D3 = 0, D4 = 0.
(4) Herd 3, Untreated: D1 = 0, D2 = 0, D3 = 1, D4 = 0.
(5) Herd 4, Untreated: D1 = 0, D2 = 0, D3 = 0, D4 = 1.

Differences in periods examined for risk of fetal loss (i.e. weeks 0–12 and 12–28) can lead to bias in the interpretation of periods of greatest risk, as was previously suggested for women (Wilcox et al., 1988; Goldhaber and Fireman, 1991) and as suggested by our current data. In studies of associations between Zn concentration and fetal loss, patients should be enrolled before the period of greatest risk of fetal loss (i.e. <6–8 weeks). Similarly, the entire risk period should be monitored because of changing nutritional requirements (maternal and fetal) throughout gestation (Breskin et al., 1983; Keen et al., 1993a). In one study, in which maternal samples were collected 24 h after abortion or delivery, the authors concluded that the serum concentration of Zn was not associated with human fetal loss. However, changes in serum Zn concentration after expulsion are not likely to be causally related to the events that led to fetal expulsion. In contrast, our survival analysis model weighted the estimated metabolite concentration at the time of expulsion to the concentration of the samples that preceded fetal expulsion.

Serum Zn and Cu concentrations in herd 2 were equimolar and within the expected mid-range for both elements (Graham, 1991). Herds 1, 3 and 4 had adequate Zn concentrations, but serum Cu concentrations were at the low end of the expected range (7–25 µmol l⁻¹; Graham, 1991). Margin Cu status may be another factor that contributed to the higher incidence of abortion in herds 1, 3 and 4 compared with herd 2. Low Cu concentrations have been associated with increased susceptibility to infectious agents in cattle (Stabel and Spears, 1989). Relationships between Cu deficiency and abortion in ruminants have been suggested before (Graham, 1991), but appear to be confounded by multiple nutrient interactions. Similarly, Zn deficiency is associated with an increased incidence of opportunistic infections and fetal loss in many species (Keen and Gershwin, 1990; Graham, 1991; Apgar, 1992). However, because measurements of Zn and Cu status are considered unreliable (Graham, 1991; Thomas et al., 1992), supplementation trials are required to assess the effects of nutrient status on risk of fetal loss fully.

To the authors’ knowledge, periconceptional intervention trials using Zn alone have not been reported previously for cattle or women. In a recent Zn supplementation trial, 1 of 248 Zn-supplemented women spontaneously aborted, compared with 5 of 249 women given a placebo (Mahomed et al., 1989). However, enrolment in the study at <20 weeks of gestation
Zinc, copper and spontaneous abortion

appeared to be well after conception and the period of greatest risk of fetal loss. Supplementing typically managed ewes with Zn before pregnancy results in higher fertility (Egan, 1972; Masters and Fels, 1980; Masters and Moir, 1983). In the current trial, supplementation with Zn methionine reduced the incidence of abortion compared with methionine-supplemented control cows, and there was a strong association between low serum Zn concentrations and risk of abortion. It is apparent that Zn methionine supplementation can reduce fetal loss in some mammalian populations.

To distinguish the mechanism(s) of how Zn might reduce the incidence of fetal loss, supplementation trials coupled with monitoring of daily health are needed. In particular, better characterization of markers of inflammation and environmental, nutritional and microbiological factors that may contribute to, or protect from, risk of fetal loss are necessary. Similarly, serum Cu concentrations in the three herds (herds 1, 3 and 4) with the highest rates of abortion were low, suggesting that low Cu concentrations may be associated with spontaneous abortion. Thus, the efficacy of Cu supplementation on prevention of spontaneous abortion should also be determined. The required sample size to avoid finding a treatment effect when one does not exist (α = 0.05) and to identify correctly a treatment effect when treatment truly reduces the rate of abortion (β = 0.95) is approximately 800 per group if a reduction in the incidence of abortion from 10% to 5% is to be discerned (Fleiss, 1972).

In summary, low concentrations of Zn (serum or dietary) were associated in this study with an increased risk of fetal loss in four different environments. This study points to problems in methodology and approach for diagnosing nutrient status as a cause of fetal loss. Our data suggest that inflammation may be an important cause of fetal loss in California Holstein dairy cows. Whether a similar pattern of change in dietary and serum Zn or Cu concentrations may predict fetal loss in women needs further definition.

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