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⁻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 for 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 $\mu\text{mol l}^{-1}$ and an increase in the copper concentration by 5 $\mu\text{mol l}^{-1}$ 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 greatest 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 postmature birth, and small-for-gestational-age infants in women (Hambidge *et al.*, 1975; Jameson, 1976; Lazebnik *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 Shrader, 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 Picanso, 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

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

Herd number (Number milked) [Number sampled]	Number aborted/ number not aborted	Days in gestation at abortion
1 (757) [110]	9/101 (8.2%) ^a	122 ^b (145) ^c 77–150 ^d
3 (1188) [112]	13/99 (11.6%)	135 (125) 52–236
4 (631) [102]	14/88 (13.7%)	117 (116.5) 71–183
2 Zn methionine (97) [118]	2/116 (1.7%)	113 (113) 107–119
2 Methionine (104) [128]	5/123 (3.9%)	89 (84) 71–116
1, 3, 4 Untreated (2576) [324]	36/288 (11.1%)	125 (122) 52–236

^aPercentage of cows that aborted [aborted/(nonaborted + aborted) × 100].

^bMean number of days in gestation at abortion.

^cMedian number of days in gestation at abortion.

^dMinimum and maximum.

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).

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 bovis*, infectious bovine rhinotracheitis virus (IBRV), bovine virus diarrhoea virus (BVDV), bovine parainfluenza-3 and antigens to five *Leptospira* serovars

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®, 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 g Zn week⁻¹; Zinpro Corporation, Bloomington, MN) or an equivalent amount of methionine and NaSO₄ (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 kg BW $\times$ 7 days $\times$ 40 mg Zn kg⁻¹ dietary dry matter = 7350 mg week⁻¹).

Blood was collected once a month in plastic syringes with ammonium heparin (Sarstedt Laboratories Inc., Newton, NC) and centrifuged at 4°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°C prior to determination of Zn and Cu, or at -70°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 (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) (Ingman Laboratories, Minneapolis, MN 55415). Dietary analysis of feedstuffs was completed for herd 2 only (range of monthly feed samples: 27–54 mg Zn; 13–19 mg Cu; 43–88 mg Mn; and 165–421 mg Fe kg⁻¹ diet). The concentration of metals in the supplements was determined by inductively coupled plasmapheresis (Zn methionine supplement, 7.33 g Zn per cow week⁻¹; methionine supplement, 0.001 g Zn per cow week⁻¹) (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 (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 ml 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 (Keen and Feldman, 1987). Samples were wet ashed with nitric acid, as described by Clegg *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 (RJ-24) (J. S. Garvey), as reported by Keen *et al.* (1993b). Crossreaction with bovine metallothionein isoforms has been documented (Winge *et al.*, 1986) and verified by western blot.

Statistical analyses

Preliminary descriptive data provided the mean, SEM 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 (Sigmaplot®, Jandel Scientific, Corte Madera, CA). Differences between aborting and nonaborting cows for each metabolite were determined by Student's *t* test (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 (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 (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 aborting were estimated along with their approximate 95% confidence interval (95% CI) as:

$$\text{ROR} = e^{\alpha + \beta_1 + \beta_2 \dots \beta_i} / e^{\alpha + \beta_1 + \beta_2 \dots \beta_i} = e^{\beta_i X_i},$$

and

$$95\% \text{ CI} = e^{\beta_i X_i \pm 2(\text{SE of } \beta_i X_i)},$$

where $\beta_i X_i$ 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 (BMDP 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:

$$\lambda(t) = \lambda_0(t) e^{[(\beta_1 X_1 + \beta_2 X_2 \dots \beta_i X_i) + \beta(Zt)]}$$

The unknown baseline hazard was $\lambda_0(t)$, β_i and X_i were the non-time-dependent co-variables (supplement level, as described for logistic regression, number of days that the cows had lactated before conception and age at conception), and $\beta(Zt)$ 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-variables 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-variables 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 Hornung (1985). Relative risks (RR) for abortion were estimated along with their approximate 95% CI as follows:

$$\text{RR} = e^{[(\beta_1 X_1 + \beta_2 X_2 \dots \beta_i X_i) + \beta(Zt)]} / e^{[(\beta_1 X_1 + \beta_2 X_2 \dots \beta_i X_i) + \beta(Zt)]} = e^{\beta_i X_i},$$

and

$$95\% \text{ CI} = e^{\beta_i X_i \pm 2(\text{SE of } \beta_i X_i)},$$

where $\beta_i X_i$ 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 ($14.72 \mu\text{mol l}^{-1}$) than did nonaborting cows in herds 1 ($13.56 \mu\text{mol l}^{-1}$), 2 ($13.87 \mu\text{mol l}^{-1}$) and 4 ($13.98 \mu\text{mol l}^{-1}$) ($P < 0.001$). Nonaborting cows in herd 2 had higher serum Cu concentrations ($13.76 \mu\text{mol l}^{-1}$) than did nonaborting cows in herds 1 ($9.31 \mu\text{mol l}^{-1}$), 3 ($9.05 \mu\text{mol l}^{-1}$) and 4 ($9.19 \mu\text{mol l}^{-1}$) ($P < 0.001$). Nonaborting cows 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 , $13.79 \mu\text{mol l}^{-1}$), Cu (14.06 ,

13.48 $\mu\text{mol l}^{-1}$), 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 $\mu\text{mol l}^{-1}$, serum Cu concentration of 15 $\mu\text{mol l}^{-1}$ and a serum Cu:Zn ratio of 15:10 were compared with a reference cow with a serum Zn concentration of 20 $\mu\text{mol l}^{-1}$, a serum Cu concentration of 10 $\mu\text{mol l}^{-1}$ and a serum Cu:Zn ratio of 10:20. Zn and Cu concentrations $< 5 \mu\text{mol l}^{-1}$ (5 of 3155 and 8 of 3155 observations, respectively), Zn concentrations $> 25 \mu\text{mol l}^{-1}$ (28 of 3155), and Cu concentrations $> 20 \mu\text{mol l}^{-1}$ (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 preceded 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 $\mu\text{mol Zn l}^{-1}$ (i.e. from 20 to 10 $\mu\text{mol l}^{-1}$) and 5 $\mu\text{mol Cu l}^{-1}$ (i.e. from 10 to 15 $\mu\text{mol l}^{-1}$) 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 $\mu\text{mol l}^{-1}$ with a reference concentration of 20 $\mu\text{mol Zn l}^{-1}$). 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 $\mu\text{mol l}^{-1}$ with a reference concentration of 10 $\mu\text{mol Cu l}^{-1}$) 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-variables supplement level, maternal age at conception, and the time-dependent co-variables $\text{Zn} \times (\text{time})$, $\text{Cu} \times (\text{time})$, and $(\text{Cu:Zn}) \times (\text{time})$ 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 $\mu\text{mol l}^{-1}$ and an increase in the Cu concentration of 5 $\mu\text{mol l}^{-1}$ 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 (*Leptospira*) 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

Herd number	Zinc ($\mu\text{mol l}^{-1}$)		Copper ($\mu\text{mol l}^{-1}$)		Metallothionein (nmol l^{-1})		Copper:zinc ratio	
	Pregnant	Aborted	Pregnant	Aborted	Pregnant	Aborted	Pregnant	Aborted
1	13.56 $\pm$ 0.15 ^a 2.5–33.9 ^b (563) ^c	12.41 $\pm$ 0.45 ^d 8.6–17.7 (26)	9.31 $\pm$ 0.07 2.2–16.7 (565)	10.02 $\pm$ 0.24 ^e 7.5–12.0 (26)	0.18 $\pm$ 0.03 0–10.1 (565)	0.22 $\pm$ 0.04 0–0.6 (26)	0.73 $\pm$ 0.01 0.3–3.4 (563)	0.83 $\pm$ 0.04 ^d 0.6–1.3 (26)
3	14.72 $\pm$ 0.14 2.6–28.1 (543)	14.78 $\pm$ 0.49 9.1–22.9 (39)	9.05 $\pm$ 0.07 1.3–17.4 (546)	9.67 $\pm$ 0.22 ^d 7.4–13.0 (39)	2.84 $\pm$ 0.52 0–139.0 (546)	2.12 $\pm$ 0.83 0–19.3 (39)	0.64 $\pm$ 0.01 0.2–1.3 (542)	0.69 $\pm$ 0.04 0.4–1.4 (39)
4	13.98 $\pm$ 0.16 4.1–33.6 (478)	13.14 $\pm$ 0.69 5.3–29.5 (32)	9.19 $\pm$ 0.10 5.0–19.5 (480)	9.20 $\pm$ 0.27 6.9–12.1 (32)	0.14 $\pm$ 0.02 0–6.5 (480)	0.47 $\pm$ 0.25 0–7.0 (32)	0.69 $\pm$ 0.01 0.2–2.6 (478)	0.76 $\pm$ 0.05 0.3–1.7 (32)
2 Zn methionine	13.95 $\pm$ 0.10 4.1–26.3 (697)	14.11 $\pm$ 0.41 12.4–15.5 (8)	14.06 $\pm$ 0.11 8.2–28.6 (697)	17.06 $\pm$ 1.53 ^d 11.9–21.7 (8)	2.82 $\pm$ 0.42 0–122.6 (694)	0.32 $\pm$ 0.23 0–1.9 (8)	1.06 $\pm$ 0.02 0.5–4.18 (697)	1.23 $\pm$ 0.13 0.8–1.64 (8)
2 Methionine	13.79 $\pm$ 0.10 5.4–26.1 (743)	14.33 $\pm$ 0.59 7.2–18.0 (17)	13.48 $\pm$ 0.87 6.4–21.0 (743)	14.78 $\pm$ 0.87 6.4–21.0 (17)	3.98 $\pm$ 0.61 0–140.3 (738)	7.40 $\pm$ 5.13 0–79.4 (17)	1.02 $\pm$ 0.01 0.23–3.4 (743)	1.04 $\pm$ 0.06 0.7–1.5 (17)
1, 3, 4	14.08 $\pm$ 0.09 2.5–33.9 (1584)	13.60 $\pm$ 0.34 5.3–29.5 (97)	9.19 $\pm$ 0.05 1.3–19.5 (1591)	9.61 $\pm$ 0.14 ^e 6.9–13.0 (96)	1.08 $\pm$ 0.18 0–139.0 (1593)	1.06 $\pm$ 0.35 0–19.3 (97)	0.69 $\pm$ 0.01 0.2–3.3 (1583)	0.75 $\pm$ 0.02 ^e 0.3–1.7 (97)

All cows were pregnant when blood was collected, except for one additional blood collection after abortion.
Values given are: ^ameans $\pm$ SEM; ^brange and ^cnumber of observations.
^dAborted cows significantly differ from pregnant cows ($P < 0.05$).
^eAborted cows significantly differ from pregnant cows ($P < 0.01$).

Table 3. Logistic regression models estimating the adjusted odds of a cow aborting within a given herd and age at conception [serum zinc (Zn), copper (Cu) and metallothionein concentrations and ratios of copper:zinc or adjusted copper:zinc ratios were included for their main effects, and gestation time (days) was divided into intervals of 30 days]

Time interval	Number aborted/ number not aborted	Regression coefficient (SEM)	Z value	P value
Days 31–60	30/301			
Constant		2.125 (1.33)	– 1.60	0.1034
Supplement level				
D1		– 0.9155 (0.862)	– 1.06	
D2		– 1.379 (0.828)	– 1.67	
D3		– 1.599 (0.835)	– 1.92	
D4		– 1.777 (0.824)	– 2.16	0.0130
Age at conception		– 0.0123 (0.009)	– 1.37	0.1831
Zinc		0.1547 (0.0752)	2.06	0.0285
Days 61–90	41/409			
Constant		7.242 (1.59)	4.56	< 0.00001
Supplement level				
D1		– 1.114 (0.869)	– 1.28	
D2		– 2.179 (0.954)	– 2.28	
D3		– 2.556 (0.966)	– 2.65	
D4		– 2.786 (0.934)	– 2.98	0.0130
Age at conception		– 0.0231 (0.0077)	– 3.00	0.0035
Copper		– 0.1451 (0.0798)	– 1.82	0.0749
Days 91–120	32/394			
Constant		7.988 (1.84)	4.33	< 0.00001
Supplement level				
D1		– 0.8032 (1.25)	– 0.643	
D2		– 2.875 (1.21)	– 2.37	
D3		– 3.212 (1.21)	– 2.66	
D4		3.264 (1.20)	– 2.71	0.0037
Age at conception		– 0.021 (0.0089)	– 2.36	0.0219
Copper		– 0.1635 (0.0859)	– 1.90	0.0648

Gestation days 0–30, 121–150, 151–180, 181–210, 211–240 and 241–260 were not reported because of sparse data or because estimation of their coefficients failed to converge (PCONV > 0.001; LCONV > 0.0001) or did not meet tolerance criteria (0.001). Supplement level was coded as follows:

- (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 1, 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.

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 $10 \mu\text{mol l}^{-1}$ and an increase in the concentration of Cu of $5 \mu\text{mol l}^{-1}$, 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-variables serum zinc, copper and metallothionein concentrations, and ratios of copper:zinc

Variable	Regression coefficient (SE)	Z value	P value	Relative risk (95% confidence intervals)
Univariate estimate ^a				
Supplement level				
D1	1.0566 (0.5936)	1.7802	0.0750	
D2	3.2686 (0.6682)	4.8918	< 0.0001	
D3	3.6906 (0.6660)	5.5411	< 0.0001	
D4	3.7264 (0.6409)	5.8145	< 0.0001	
(1)				1.0 ^b
(2)				2.98 (0.35, 23.97)
(3)				26.27 (2.31, 299.38)
(4)				40.87 (3.50, 458.43)
(5)				41.53 (3.77, 457.02)
Age at conception	0.0218 (0.0048)	4.5135	< 0.0001	1.92 ^c (1.44, 2.58)
Time-dependent zinc concentration	- 0.6574 (0.1686)	- 3.8981	< 0.0001	716.23 ^d (24.58, 20868)
Time-dependent copper concentration	1.0065 (0.2353)	4.2778	< 0.0001	153.31 ^e (1.71, 1612)
Time-dependent copper:zinc	- 9.2763 (2.8194)	- 3.2902	0.0010	< 0.00 ^f (0.00, 0.026)
Attributable risk for the combined time-dependent covariates = 90.27%				10.28 ^g (4.69, 22.5)

^aTime-dependent co-variables were based on the intercept and slope $\times$ (time) of changes in Zn, Cu or metallothionein concentrations, and ratios of Cu:Zn throughout gestation.

^bReference level.

^cComparing 60 months with a reference age at conception of 30 months.

^dComparing 10 $\mu\text{mol Zn l}^{-1}$ with a reference concentration of 20 $\mu\text{mol Zn l}^{-1}$ (mid-range of normal plasma Zn concentration).

^eComparing 15 $\mu\text{mol Cu l}^{-1}$ with a reference concentration of 10 $\mu\text{mol Cu l}^{-1}$ (mid-range of plasma Cu in this study).

^fComparing the ratio of 15 $\mu\text{mol Cu l}^{-1}$:10 $\mu\text{mol Zn l}^{-1}$ with a reference value of 10 $\mu\text{mol Cu l}^{-1}$:20 $\mu\text{mol Zn l}^{-1}$.

^gCombined relative risk, comparing 10 $\mu\text{mol Zn l}^{-1}$ with a reference concentration of 20 $\mu\text{mol Zn l}^{-1}$, 15 $\mu\text{mol Cu l}^{-1}$ with a reference of 10 $\mu\text{mol Cu l}^{-1}$, and a ratio of 15 $\mu\text{mol Cu l}^{-1}$:10 $\mu\text{mol Zn l}^{-1}$ with a reference of 10 $\mu\text{mol Cu l}^{-1}$:20 $\mu\text{mol Zn l}^{-1}$. 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 1, 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 $\mu\text{mol l}^{-1}$; Graham, 1991). Marginal 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

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 ($\alpha = 0.05$) and to identify correctly a treatment effect when treatment truly reduces the rate of abortion ($\beta = 0.95$) 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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References

- Anderson GB (1991) Fertilization, early development, and embryo transfer. In *Reproduction in Domestic Animals* (4th Edn) pp 279–313 Ed. PT Cupps. Academic Press, San Diego
- Anderson ML, Blanchard PC, Barr BC and Hoffman RL (1991) A survey of causes of bovine abortion occurring in the San Joaquin Valley, California *Journal of Veterinary Diagnostic Investigation* **2** 283–287
- Apgar J (1992) Zinc and reproduction: an update *Journal of Nutritional Biochemistry* **3** 266–278
- Association of Official Analytical Chemists (1980) *Official Methods of Analysis of the Association of Official Analytical Chemists* (13th Edn) pp 125–161 Ed. W Horowitz. Association of Official Analytical Chemists, Washington DC
- Austen J, Mahomed K, James D and Hathaway M (1989) Maternal leucocyte zinc levels and spontaneous first trimester abortion *Early Human Development* **20** 77–78
- Barber EF and Cousins RJ (1988) Interleukin-1-stimulated induction of ceruloplasmin synthesis in normal and copper-deficient rats *Journal of Nutrition* **118** 375–381
- Bell LT, Branstrator M, Roux C and Hurley LS (1975) Chromosomal abnormalities in maternal and fetal tissues of magnesium- or zinc-deficient rats *Teratology* **12** 221–226
- Breskin MW, Worthington-Roberts BS, Knopp RH, Brown Z, Plovie B, Mottot NK and Mills JL (1983) First trimester serum zinc concentrations in human pregnancy *American Journal of Clinical Nutrition* **38** 943–953
- Cherry FF, Sandstead HH, Rojas P, Johnson LK, Batson HK and Wang XB (1989) Adolescent pregnancy: associations among body weight, zinc nutriture, and pregnancy outcome *American Journal of Clinical Nutrition* **50** 945–954
- Clegg MS, Keen CL, Lonnerdal B and Hurley LS (1981) Influence of ashing techniques on the analysis of trace elements in animal tissue. I. Wet ashing *Biological Trace Element Research* **3** 107–115
- Cox DR and Oakes D (1984) *Analysis of Survival Data*. Chapman and Hall, London
- Dinarello CA (1989) Interleukin-1 and its biologically related cytokines *Advances in Immunology* **44** 153–205
- Dixon WJ, Sampson P and Mundt P (1990) One- and two-way analysis of variance with data screening. In *BMDP Statistical Software Manual* Vol. 1 pp 189–212 Ed. WJ Dixon. University of California Press, Berkeley
- Egan AR (1972) Reproductive responses to supplemental zinc and manganese in grazing Dorset Horn ewes *Australian Journal of Experimental Agriculture and Animal Husbandry* **12** 131–135
- Engelman L (1990) Stepwise logistic regression. In *BMDP Statistical Software Manual* Vol. 2 pp 1013–1046 Ed. WJ Dixon. University of California Press, Berkeley
- Fleiss JL (1972) *Statistical Methods for Rates and Proportion* p 176. John Wiley and Sons, New York
- Garvey JS (1991) Antibodies to metallothionein *Methods in Enzymology* **205** 141–174
- Ghosh A, Fong LYY, Wan CW, Liang ST, Woo JSK and Wong V (1985) Zinc is not a cause for abortion, congenital abnormality and small-for-gestational age infant in Chinese women *British Journal of Obstetrics and Gynaecology* **92** 886–891
- Giri SN, Emau P, Cullor JS, Stabenfeldt GH, Bruss ML, BonDurant RH and Osburn BI (1990) Effects of endotoxin infusion on circulating levels of eicosanoids, progesterone, cortisol, glucose and lactic acid and abortion in pregnant cows *Veterinary Microbiology* **21** 211–231
- Giri SN, Stabenfeldt GH, Moseley TA, Graham TW, Bruss ML, BonDurant RH, Cullor JS and Osburn BI (1991) Role of eicosanoids in abortion and its prevention by treatment with flunixin meglumine in cows during their first trimester of pregnancy *Journal of Veterinary Medicine A* **38** 445–459
- Goldhaber MK and Fireman BH (1991) The life table revisited: spontaneous abortion rates in three Kaiser Permanente cohorts *Epidemiology* **2** 33–39
- Graham TW (1991) Trace element deficiencies in cattle *Veterinary Clinics of North America Food Animal Practice* **7** 153–215
- Grider A, Bailey LB and Cousins RJ (1990) Erythrocyte metallothionein as an index of zinc status in humans *Proceedings of the National Academy of Sciences USA* **86** 1259–1262
- Hambidge KM, Neldner KH and Walravens PA (1975) Zinc, acrodermatitis enteropathica and congenital malformations *Lancet* **i** 577–578
- Hertz-Picciotto I, Swan SH, Neutra RR and Samuels SJ (1989) Spontaneous abortions in relation to consumption of tap water: an application of methods from survival analysis to a pregnancy follow-up study *American Journal of Epidemiology* **130** 79–93
- Higashi A, Tajiri A, Matsukura M and Matsuda I (1988) A prospective survey of serial maternal serum zinc levels and pregnancy outcome *Journal of Pediatric Gastroenterology and Nutrition* **7** 430–433
- Hinks LJ, Ogilvy-Stuart A, Hambidge KM and Walker V (1989) Maternal zinc and selenium status in pregnancies with a neural tube defect or elevated plasma alpha-fetoprotein *British Journal of Obstetrics and Gynaecology* **96** 61–66
- Hopkins A (1990) Survival analysis with covariates. In *BMDP Statistical Software Manual* Vol. 2 pp 769–806 Ed. WJ Dixon. University of California Press, Berkeley

- Hopkins A and Hornung R (1985) New 2L features with illustrative examples *BMDP Technical Report No. 80* pp 1–21. University of California Press, Berkeley
- Hunt IF, Murphy NJ, Cleaver AE, Faraji B, Swendseid ME, Coulson AH, Clark VA, Browdy BL, Cabalum MT and Smith JC (1984) Zinc supplementation during pregnancy: effects on selected blood constituents and on progress and outcome of pregnancy in low-income teenagers of Mexican descent *American Journal of Clinical Nutrition* **40** 508–521
- Hunt IF, Murphy NJ, Cleaver AE, Faraji B, Swendseid ME, Browdy BL, Coulson AH, Clark VA, Settlege RH and Smith JC (1985) Zinc supplementation during pregnancy in low-income teenagers of Mexican descent: effects on selected blood constituents and on progress and outcome of pregnancy *American Journal of Clinical Nutrition* **42** 815–828
- Hurley LS and Shraider RE (1975) Abnormal development of preimplantation rat eggs after three days of maternal zinc deficiency *Nature* **254** 427–429
- James WH (1983) Birth order, maternal age and spontaneous abortion *Early Human Development* **8** 179–180
- Jameson S (1976) Effects of zinc deficiency in human reproduction *Acta Medica Scandinavica Supplementum* **593** 1–89
- Kahn HA and Semplos CT (1989) *Statistical Methods in Epidemiology* pp 72–83. Oxford University Press, New York
- Kalbfleisch JD and Prentice RL (1980) *The Statistical Analysis of Failure Time Data*. John Wiley and Sons, New York
- Keen CL and Feldman BF (1987) Measurement of zinc and copper in plasma: potential negative effects of anticoagulant choice on analyzed values *Agri-Practice* **8** 4–5
- Keen CL and Gershwin ME (1990) Zinc deficiency and immune function *Annual Review of Nutrition* **10** 415–431
- Keen CL and Hurley LS (1989) Zinc and reproduction: effects of deficiency on foetal and postnatal development. In *Zinc in Human Biology* pp 183–220 Ed. CF Mills. Springer-Verlag, Berlin
- Keen CL, Taubeneck MW, Daston GP, Rogers JM and Gershwin ME (1993a) Primary and secondary zinc deficiency as factors underlying abnormal CNS development *Annals of the New York Academy of Science* **678** 37–47
- Keen CL, Golub MS, Lonnerdal B, Olin KL, Graham TW, Uriu-Hare J and Gershwin ME (1993b) Influence of dietary zinc intake on pregnancy outcome in Rhesus monkeys *Pediatric Research* **33** 141–174
- Kirkbride CA (1992a) Etiologic agents detected in a 10-year study of bovine abortions and stillbirths *Journal of Veterinary Diagnostic Investigation* **4** 175–180
- Kirkbride CA (1992b) Viral agents and associated lesions detected in a 10-year study of bovine abortions and stillbirth *Journal of Veterinary Diagnostic Investigation* **4** 374–379
- Kirkbride CA (1993) Bacterial agents detected in a 10-year study of bovine abortions and stillbirths *Journal of Veterinary Diagnostic Investigation* **5** 64–68
- Kline J, Stein Z, Susser M and Warburton D (1985) Fever during pregnancy and spontaneous abortion *American Journal of Epidemiology* **121** 832–842
- Kline J, Stein Z and Susser M (1989) *Conception to Birth: Epidemiology of Prenatal Development*. Oxford University Press, New York
- Knudtson WU and Kirkbride CA (1992) Fungi associated with bovine abortion in the northern plains states (USA) *Journal of Veterinary Diagnostic Investigation* **4** 181–185
- Lazebnik N, Kuhnert BR, Kuhnert PM and Thompson KL (1988) Zinc status, pregnancy complications, and labor abnormalities *American Journal of Obstetrics and Gynecology* **158** 161–166
- Lohuis JACM, Verheijden JHM, Burvenich C and van Miert ASJPAM (1988a) Pathophysiological effects of endotoxins in ruminants 1. Changes in body temperature and reticulo-rumen motility, and the effect of repeated administration *Veterinary Quarterly* **10** 109–116
- Lohuis JACM, Verheijden JHM, Burvenich C and van Miert ASJPAM (1988b) Pathophysiological effects of endotoxins in ruminants 2. Metabolic aspects *Veterinary Quarterly* **10** 117–125
- McMichael AJ, Dreosti IE and Gibson GT (1982) Maternal zinc status and pregnancy outcome: a prospective study. In *Clinical Applications of Recent Advances in Zinc Metabolism* pp 53–66 Eds AS Prasad, IE Dreosti, BS Hetzel. Alan R. Liss, Inc., New York
- Mahomed K, James DK, Golding J and McCabe R (1989) Zinc supplementation during pregnancy: a double blind randomised controlled trial *British Medical Journal* **299** 826–830
- Masters DG and Fels HE (1980) Effect of zinc supplementation on the reproductive performance of grazing Merino ewes *Biological Trace Element Research* **2** 281–290
- Masters DG and Moir RJ (1983) Effect of zinc deficiency on the pregnant ewe and developing foetus *British Journal of Nutrition* **49** 365–372
- Meadows NJ, Ruse W, Smith MF, Day J, Keeling PWN, Scopes JW and Thompson RPH (1981) Zinc and small babies *Lancet* **ii** 1135–1137
- Mukherjee MD, Sandstead HH, Ratnaparkhi MV, Johnson LK, Milne DB and Stelling HP (1984) Maternal zinc, iron, folic acid, and protein nutriture and outcome of human pregnancy *American Journal of Clinical Nutrition* **40** 496–507
- National Research Council (US) Subcommittee on Dairy Cattle Nutrition (1988) *Nutrient Requirements of Dairy Cattle* (6th Edn). National Academy Press, Washington DC
- Naylor AF and Warburton D (1979) Sequential analysis of spontaneous abortion: II. Collaborative study data show that gravidity determines a very substantial rise in risk *Fertility and Sterility* **31** 282–286
- Peters JM, Wiley LM, Zidenberg-Cherr S and Keen CL (1991) Influence of short-term maternal zinc deficiency on the *in vitro* development of pre-implantation mouse embryos *Proceedings of the Society for Experimental Biology and Medicine* **198** 561–568
- Sato M, Mehra RK and Bremner I (1984) Measurement of plasma metallothionein in the assessment of the zinc status of zinc-deficient and stressed rats *Journal of Nutrition* **114** 1683–1689
- Shuster DE, Kehrl ME and Stevens MG (1993) Cytokine production during endotoxin-induced mastitis in lactating dairy cows *American Journal of Veterinary Research* **54** 80–85
- Simmer K, Lort-Phillips L, James C and Thompson RPH (1990) A double-blind trial of zinc supplementation in pregnancy *European Journal of Clinical Nutrition* **45** 139–144
- Sookne D and Forsythe A (1990) t tests. In *BMDP Statistical Software Manual* Vol. 1 pp 145–162 Ed. W J Dixon. University of California Press, Berkeley
- Stabel JR and Spears JW (1989) Effect of copper on immune function and disease resistance *Advances in Experimental Medicine and Biology* **258** 243–252
- Thomas EA, Bailey LB, Kauwell GA, Lee D-Y and Cousins RJ (1992) Erythrocyte metallothionein response to dietary zinc in humans *Journal of Nutrition* **122** 2408–2414
- Thurmond MC and Picanso JP (1990) A surveillance system for bovine abortion *Preventive Veterinary Medicine* **8** 41–53
- Thurmond MC, Picanso JP and Jameson CM (1990a) Considerations for use of descriptive epidemiology to investigate fetal loss in dairy cows *Journal of the American Veterinary Medical Association* **197** 1305–1312
- Thurmond MC, Picanso JP and Hietala S (1990b) Prospective serology and analysis in diagnosis of dairy cow abortion *Journal of Veterinary Diagnostic Investigation* **2** 274–282
- Wells JL, James DK, Luxton R and Pennock CA (1987) Maternal leucocyte zinc deficiency at start of third trimester as a predictor of fetal growth retardation *British Medical Journal* **294** 1054–1056 (correction on page 1258)
- Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG and Nisula BC (1988) Incidence of early loss of pregnancy *New England Journal of Medicine* **319** 189–194
- Winge DR, Gray WR, Zelazowski A and Garvey JS (1986) Sequence and antigenicity of calf metallothionein II *Archives of Biochemistry and Biophysics* **245** 254–262