17 β -Estradiol and Estrone Concentrations in Plasma and Milk During Bovine Pregnancy

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ABSTRACT

Estrone (E_1) and 17 β -estradiol (E_2) are present in milk, but the mechanism(s) that regulate their appearance in milk are not known. The objectives of this study were to determine the impact of stage of pregnancy on the concentrations of E_1 and E_2 in plasma and milk and to determine the correlations between plasma and milk E_1 and E_2 and with milk components throughout pregnancy. Blood and milk samples were collected from 13 cows every 28 d throughout pregnancy. The E_1 and E_2 were quantified in plasma and milk using RIA after organic solvent extractions and Sephadex LH-20 column chromatography. Plasma E_1 concentrations averaged 0.8, 16.9, and 41.8 pg/mL in trimesters 1, 2, and 3, respectively. The respective E_1 concentrations in milk averaged 0.6, 7.9, and 27.1 pg/mL. The E_2 concentrations in plasma averaged 0.5, 0.9, and 2.0 pg/mL; milk E_2 averaged 0.3, 0.9, and 5.0 pg/mL. Plasma and milk E_2 concentrations were greater in trimester 3 compared with trimesters 1 and 2. The E_1 concentrations in milk were significantly correlated with plasma E_1 concentrations ($r = 0.77$), percentage of milk fat ($r = 0.50$), and milk yield ($r = -0.43$). The E_2 concentrations in milk were significantly correlated with plasma E_2 concentrations ($r = 0.93$), percentage of milk protein ($r = 0.63$), and milk yield ($r = -0.57$). The milk-to-plasma ratio of E_2 increased from 0.4 during trimester 1 to 2.2 in trimester 3, which suggested that the mechanism(s) regulating the appearance of E_2 in milk may change over the course of pregnancy.

Key words: estrone, 17 β -estradiol, plasma, whole milk

INTRODUCTION

Recent epidemiological and case-controlled studies have associated dairy product consumption and the estrogens therein with a number of adverse health consequences. Milk-derived estrogens have been suggested

to contribute to male reproductive disorders (Sharpe and Skakkebaek, 1993; Ganmaa et al., 2001); increased risks for breast, uterine, and ovarian cancers (Cho et al., 2003; Li et al., 2003; Ganmaa and Sato, 2005); and adolescent weight gain (Berkey et al., 2005). Although some suggest that the concentrations of estrogens in milk are low in relation to human production rates (Erb et al., 1977; Hartmann et al., 1998; Pape-Zambito et al., 2007), others argue that milk contains considerable quantities of estrogens (Remesar et al., 1999; Ganmaa et al., 2004; Qin et al., 2004). Ganmaa and Sato (2005) have hypothesized that milk produced by modern pregnant dairy cows in which a greater proportion of cows are pregnant compared with cows 100 yr ago contains large amounts of female sex hormones that promote the development of breast, ovarian, and uterine cancers. Therefore, it is important to reevaluate estrogen concentrations, particularly the more biologically active free estrogens in milk from pregnant dairy cows to assess human health risks.

Estrone (E_1) and 17 β -estradiol (E_2) have been quantified in the milk of pregnant cows (Monk et al., 1975; Malekinejad et al., 2006). Monk et al. (1975) reported mean milk E_1 concentrations of 57, 35, and 97 pg/mL and E_2 concentrations of 85, 52, and 49 pg/mL in trimesters 1, 2, and 3, respectively. Malekinejad et al. (2006) recently used liquid chromatography tandem mass spectrometry to detect E_1 and E_2 in milk from 5 pregnant cows. The E_1 concentrations averaged 9, 57, and 118 pg/mL, and the E_2 concentrations averaged 10, 20, and 21 pg/mL in trimesters 1, 2, and 3, respectively. Both Monk et al. (1975) and Malekinejad et al. (2006) sampled cows from trimesters 1, 2, and 3 of pregnancy; however, different cows were included from each trimester. Because the same cows were not sampled throughout pregnancy, one cannot distinguish animal variation from pregnancy-specific effects. Our lab reported greater quantities of E_2 in milk from cows 141 to 210 d pregnant compared with cows < 141 d pregnant or nonpregnant cows (Pape-Zambito et al., 2007).

Batra et al. (1980) and Abeyawardene et al. (1984) reported that milk E_2 concentrations were positively correlated with plasma E_2 and suggested that plasma

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E₂ concentrations were a predictor of milk E₂ concentrations. Other reports have clearly shown an increase in plasma estrogens as pregnancy advances. Hoffmann et al. (1997) reported that plasma estrogen concentrations began to increase around 120 d of pregnancy in the cow; estrogen concentrations continued to increase as gestation progressed. Patel et al. (1999) used organic solvent extraction, liquid chromatography, and RIA to quantify free (as opposed to conjugated) plasma E₁ and E₂ concentrations during pregnancy and found significant increases in E₁ and E₂ concentrations in plasma during each trimester. The concentrations of estrogens in milk were not determined in these studies.

Although estrogen concentrations in bovine plasma and milk during pregnancy have been quantified separately by individual researchers, the relationship between plasma and milk E₁ and E₂ concentrations throughout pregnancy has not been critically evaluated in the dairy cow. The objectives of this study were to collect plasma and milk samples from the same cows every 28 d throughout pregnancy to quantify E₁ and E₂. Also, because some have argued that milk from pregnant cows has unsafe amounts of estrogens, we wanted to determine if stage of pregnancy (trimester) significantly affected E₁ and E₂ concentrations in milk. Our final objective was to determine correlations between estrogen measurements and milk components to try to gain insight into the mechanism(s) that allow estrogens to accumulate in milk.

MATERIALS AND METHODS

Animal and Sample Collection

Fourteen pregnant Holstein cows (n = 7 parity 1; n = 6 parity 2; n = 1 parity 3) were selected from the Pennsylvania State University dairy herd for inclusion in the study. Herd health records were used to eliminate cows with previous reproductive and health problems including history of abortion, cystic follicles, difficulty conceiving (>5 services per conception), displaced abomasum, and chronic mastitis. Herd reproduction was conventionally managed with a 60-d voluntary waiting period before breeding was attempted. Cows entered the study after they were initially confirmed pregnant by ultrasonography followed by subsequent rectal palpation at an average of 52 d of pregnancy.

Blood and milk samples were collected an average of 11 d after confirmation of pregnancy by rectal palpation and thereafter collected every 28 d up to 250 d of pregnancy. All sampling procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee (authorization no. 16216). On the designated sampling days, blood (10 mL) was aseptically collected from the coccygeal vessels into heparinized syringes from 0930 to 1100 h. Samples were placed on ice immediately and transported to the laboratory. Hematocrit and plasma proteins were analyzed to assess health of animals. Hematocrit was determined using capillary tubes and centrifugation. Plasma protein concentrations were determined using a refractometer after the blood had been centrifuged for 30 min at 1,660 × g at 4°C. The remaining plasma was frozen at -20°C until analyzed for E₁ and E₂.

Milk samples (100 mL) were collected on the designated sampling days during the p.m. milking (1700 h). Duplicate samples were sent to the DHIA laboratory for fat, protein, lactose, and SCC analyses. The remaining milk was preserved with 0.02% sodium azide and homogenized for 15 s using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY) with a PTA probe on setting 5.5. These homogenized samples were stored at -20°C until analyzed for E₁ and E₂. Body and milk weights for each cow on each sampling day were obtained from herd records.

Blood and milk samples from the cows were designated as collected during trimesters 1 (50 to 93 d pregnant, n = 22), 2 (94 to 186 d pregnant, n = 47), or 3 (187 to 246 d pregnant, n = 29). Cows on the study were intentionally allowed only a 30-d dry period to continue blood and milk sampling to 250 d of pregnancy. One cow aborted its calf during the study; therefore, values for only 13 cows were used for data analyses.

E₁ and E₂ Extraction from Whole Milk and Plasma

The procedure for extraction of estrogens from milk was adapted from Monk et al. (1975) and Wolford and Argoudelis (1979) and is similar to that reported by Pape-Zambito et al. (2007). Homogenized milk samples were thawed in a warm water bath (37°C) and vortexed for 10 s before aliquotting 3.0 mL into each of four 50-mL screw-cap glass extraction tubes. Milk in each tube was extracted with 9.0 mL of ethyl acetate:hexanes (1:1, vol/vol; ethyl acetate - HPLC grade, catalog no. JT9280-33, J. T. Baker, Phillipsburg, NJ; hexanes - Am. Chem. Soc. grade, catalog no. 293253, Sigma, St. Louis, MO). The mixture was vortexed for 30 s then placed on an orbital shaker for 15 min. The vortex and shaking steps were repeated before incubation at -20°C for 2 h. The liquid organic layer from each individual sample was transferred to a glass tube and dried under N₂ at 55°C. The ethyl acetate:hexanes extraction was then repeated on the remaining aqueous phase. After freezing, the organic layer from the second ethyl acetate:hexanes extraction was transferred to the corresponding sample tube and dried. To remove triglycerides from the extract, 70% methanol (HPLC grade - catalog no. BJAH230-4, Burdick and Jackson, Muskegon, MI) was

warmed to 55°C, and 1.5 mL was added to the extract. This mixture was incubated at 55°C for 1 h with thorough mixing at 0, 15, 30, and 45 min. The mixture was subsequently incubated at -20°C for 2 h then centrifuged for 30 min at $1,370 \times g$ and 2°C to precipitate triglycerides. The supernatant solutions from the 4 replicates were pooled in a clean glass test tube and represented 12 mL of the original milk sample. This supernatant solution, which contained the steroid hormone fraction, was dried under N₂ at 80 to 100°C.

The estrogen-containing extract was reconstituted in 100 µL of benzene:methanol (9:1, vol/vol; benzene - Am. Chem. Soc. grade, catalog no. 319953, Sigma) for transfer onto the chromatographic column used to separate cholesterol, E₁, E₂, and other residual steroids (Mikhail et al., 1970). Sephadex LH-20 (catalog no. 17-0090-10, GE Healthcare, Piscataway, NJ) was swelled with benzene:methanol (9:1, vol/vol) and packed to 2.5 cm in glass columns (1-cm diameter - catalog no. 737-1006, BioRad, Hercules, CA). Steroids were eluted with benzene:methanol (9:1, vol/vol) as described by Kensinger et al. (1986) and Pape-Zambito et al. (2007). The E₁ and E₂ elution patterns were verified with 2, 4, 6, 7-³H-E₁ (catalog no. TRK321, GE Healthcare) and 2, 4, 6, 7-³H-E₂ (catalog no. TRK322, GE Healthcare). Other experiments in our lab confirmed the absence of cholesterol in the E₁ and E₂ fractions using this methodology. The E₁ and E₂ fractions were dried under N₂ at 55°C.

Plasma samples (3 mL) were processed similarly to the milk samples, except the triglyceride precipitation step was eliminated and 1 replicate rather than 4 replicates was extracted. In addition, a dry ice-ethanol bath was used to freeze the aqueous plasma layer before decanting the organic layer into a clean glass tube.

E₁ and E₂ Quantification by RIA

Dried E₁ and E₂ fractions from each plasma and milk sample were reconstituted in 125 µL of wether plasma before quantification using an RIA specific for E₁ (catalog no. DSL-8700, Diagnostic Systems Laboratory, Webster, TX) or E₂ (catalog no. 07-138106, MP Biomedicals, Irvine, CA). Each sample was run in duplicate in accordance with manufacturer instructions. Preliminary studies evaluated parallelism, recovery of a standard mass of E₁ and E₂ added to samples, as well as recoveries of ³H-E₁ and ³H-E₂ added to milk samples (Pape-Zambito et al., 2007).

Tritiated E₁ or E₂ were run as internal standards with each set of samples extracted to estimate E₁ and E₂ recovery from plasma and whole milk. Tritiated E₁ or E₂ was added to pooled homogenized whole milk and plasma samples. After the final reconstitution step in wether plasma, the internal standards (125 µL) were

pipetted into 7.0-mL scintillation vials with 5.0 mL of Ecolite scintillation fluid (ICN, Costa Mesa, CA) and counted on a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Control pooled milk and plasma samples were also run in the RIA with each set of unknown samples to determine intra- and interassay variation. Intra- and interassay CV averaged 2.0 and 9.5% for E₁ and 8.5 and 9.6% for E₂, respectively.

Reported E₁ and E₂ concentrations were corrected for recoveries of E₁ and E₂, respectively, as well as the volume of plasma or milk represented in the assay. Some plasma and milk samples had E₂ concentrations that were below the limit of quantification (**LOQ**) of the assay. Any plasma or milk sample that predicted below the LOQ was assigned the LOQ value of 0.53 pg of E₂/mL of plasma or 0.14 pg of E₂/mL of milk. The limits of detection for E₂ (provided by MP Biomedicals; adjusted appropriately for sample recoveries and volumes) were 0.40 pg/mL for plasma and 0.10 pg/mL for milk. All plasma and milk samples were within the LOQ for E₁ (0.64 pg/mL for plasma and 0.16 pg/mL for milk). The limits of detection for E₁ (provided by Diagnostic Systems Laboratory; adjusted appropriately for sample recoveries and volumes) were 0.05 pg/mL for plasma and 0.01 pg/mL for milk. Milk E₁ and E₂ mass was calculated by multiplying E₁ or E₂ concentration by the volume of milk produced at that milking. Milk volume (mL) was calculated using the following conversion factors beginning with kilograms of milk: 1 L of milk/1.032 kg of milk, 1,000 mL of milk/1 L of milk.

Statistical Analyses

For statistical analysis, SAS version 9.0 (SAS Institute, Cary, NC) was used. To determine if concentrations of E₁ and E₂ in plasma and milk changed throughout pregnancy, PROC MIXED was used. Cow (random), parity (fixed), and trimester (fixed) were class variables with the following model statements: dependent variable (plasma E₁, plasma E₂, milk E₁, milk E₂, E₁ mass, E₂ mass, E₁ milk:plasma, E₂ milk:plasma, hematocrit, plasma protein, percentage of milk fat, percentage of milk protein, percentage of milk lactose, log SCC, milk yield) = parity trimester. The repeated statement included trimester with subject = cow. The compound symmetry covariance structure produced the best-fit statistics for all analyses. The LSM option was used to determine differences between trimesters. To determine the correlations among variables of interest, PROC CORR was used.

RESULTS

Blood and milk samples were collected every 28 d from 13 confirmed pregnant cows ranging from 50 to

Table 1. Health indices, milk composition, yield, and mass of estrone (E_1) and 17 β -estradiol (E_2) accumulated in milk during pregnancy

Analyte	Trimester ¹			SEM
	1	2	3	
BW, kg	635.0 ^c	688.6 ^b	741.6 ^a	18.9
Hematocrit, %	26.9 ^b	27.9 ^a	27.9 ^a	0.4
Plasma protein, g/dL	6.8 ^a	6.8 ^a	7.0 ^a	0.1
Milk fat, %	3.5 ^b	3.6 ^{ab}	3.9 ^a	0.1
Milk protein, %	3.2 ^b	3.3 ^b	3.4 ^a	0.1
Milk lactose, %	4.9 ^a	4.8 ^{ab}	4.6 ^b	0.1
Log SCC	4.6 ^c	4.8 ^b	5.1 ^a	0.1
Milk yield (p.m.), kg	21.2 ^a	19.8 ^a	10.3 ^b	1.2
Milk E_1 mass, ² ng	14.2 ^c	153.5 ^b	293.0 ^a	33.1
Milk E_2 mass, ² ng	5.4 ^c	16.2 ^b	39.4 ^a	5.0

^{a-c}Means within rows with different superscripts differ ($P < 0.05$).

¹Trimester: 1 = 50 to 93 d pregnant ($n = 18$ to 22 samples); 2 = 94 to 186 d pregnant ($n = 42$ to 47 samples); 3 = 187 to 246 d pregnant ($n = 26$ to 29 samples).

²Milk E_1 and E_2 mass = E_1 or E_2 concentration (pg/mL) $\times$ p.m. milk yield (mL). Milk volume (mL) was calculated using the following conversion factors beginning with kilograms of milk: 1 L of milk/1.032 kg of milk, 1,000 mL of milk/1 L of milk.

246 d of pregnancy for a total of 98 blood and 98 milk samples. The final blood and milk samples were collected from cows at a mean of 237 ± 7 ($\pm$ SD) days of pregnancy. Due to the designation of trimesters and timing of sample collection, 22% ($n = 22$) of the samples were taken during trimester 1 and 48% ($n = 47$) were collected during trimester 2, with the remaining 30% ($n = 29$) of samples being collected in trimester 3. Cows had an average gestation length of 274 ± 11 d.

Body weight averaged 636 ± 18 kg on the first sampling day and increased ($P < 0.05$) throughout pregnancy (Table 1). Hematocrit averaged $27.7 \pm 0.4\%$ and was greater ($P < 0.05$) in trimesters 2 and 3 compared with trimester 1 (Table 1). Plasma protein averaged $6.8 \pm 0.1\%$ and did not differ based on trimester (Table 1).

Milk composition was normal for a well-managed Holstein herd. Log SCC and percentages of fat, protein, and lactose averaged 4.9 ± 0.1 cells/mL, $3.6 \pm 0.2\%$, $3.3 \pm 0.1\%$, and $4.7 \pm 0.1\%$, respectively. Milk yield for the p.m. milking averaged 17.7 ± 1.2 kg overall and was lower ($P < 0.001$) in trimester 3 compared with trimesters 1 and 2 (Table 1).

Recoveries of tritiated E_1 and E_2 standards through the extraction and isolation procedures averaged 98% for both plasma and milk E_1 and 75% for both plasma and milk E_2 . Overall, plasma E_1 concentration averaged 20.2 pg/mL (range = 0.6 to 77.6 pg/mL), and milk E_1 concentration averaged 11.0 pg/mL (range = 0.3 to 94.2 pg/mL). Plasma E_2 concentration averaged 1.1 pg/mL (range = nondetectable to 7.4 pg/mL), and milk E_2 concentration averaged 1.9 pg/mL (range = nondetectable to 22.3 pg/mL). Plasma and milk E_1 and E_2 concentra-

Figure 1. Mean concentrations of estrone (E_1) and 17 β -estradiol (E_2) in plasma and milk throughout pregnancy in 13 lactating Holstein dairy cows. Blood and milk samples were initially collected at an average of 11 d after confirmation of pregnancy and every 28 d thereafter throughout pregnancy. The average standard deviation for days pregnant on the x-axis was 4.5 d. Plasma E_1 standard error of the means = 2.8 pg/mL; milk E_1 standard error of the means = 2.4 pg/mL; plasma E_2 standard error of the means = 0.2 pg/mL; milk E_2 standard error of the means = 0.6 pg/mL.

tions each increased during pregnancy (Figure 1). Parity had no effect on the concentrations of E_1 and E_2 in plasma and milk.

Plasma E_1 concentrations increased ($P < 0.001$) from 0.8 pg/mL in trimester 1 to 41.8 pg/mL in trimester 3 (Figure 2). The E_1 concentrations in milk increased ($P < 0.05$) from <1 pg/mL in trimester 1 to 27.1 pg/mL by trimester 3 (Figure 2). The calculated E_1 milk-to-plasma ratio averaged 0.8, 0.5, and 0.6 in trimesters 1, 2, and 3, respectively, with only small differences among trimesters. The mass of E_1 accumulated in milk over a 12-h period (as calculated by multiplying milk E_1 concentrations by p.m. milk volume, mL) increased ($P < 0.01$; Table 1) throughout pregnancy.

Mean concentrations of E_2 in plasma increased from 0.5 pg/mL in trimester 1 to 2.0 pg/mL in trimester 3 (Figure 2). Plasma E_2 concentrations in trimester 3 were greater compared with trimesters 1 and 2 ($P < 0.001$). The E_2 concentrations in milk averaged 0.3 pg/mL in trimester 1 and 5.0 pg/mL in trimester 3. Milk E_2 concentrations in trimester 3 were greater compared with trimesters 1 and 2 ($P < 0.001$; Figure 2). The E_2 milk-to-plasma ratios averaged 0.4, 0.8, and 2.2 in trimesters 1, 2, and 3, respectively, and increased with advancing pregnancy ($P < 0.05$). The mass of E_2 accumulated in milk over a 12-h period (as calculated by multiplying milk E_2 concentrations by p.m. milk volume, mL) also increased throughout pregnancy ($P < 0.05$; Table 1).

Milk E_1 was most highly correlated with plasma E_1 ($r = 0.77$), days pregnant ($r = 0.67$), plasma E_2 ($r = 0.55$), percentage milk fat ($r = 0.50$), and milk yield ($r = -0.43$; each $P < 0.01$; Table 2). Milk E_2 was most highly corre-

Figure 2. Mean estrone (E_1 ; top panel) and 17β -estradiol (E_2 ; lower panel) concentrations in plasma and milk during pregnancy. Bars represent plasma and milk E_1 or E_2 concentrations (pg/mL) during trimesters 1 (50 to 93 d pregnant, $n = 22$ samples), 2 (94 to 186 d pregnant, $n = 47$ samples), and 3 (187 to 246 d pregnant, $n = 29$ samples). ^{a-c}Bars within sample type (plasma or milk) with different letters differ ($P < 0.001$).

lated with plasma E_2 ($r = 0.93$), plasma E_1 ($r = 0.71$), percentage of milk protein ($r = 0.63$), days pregnant ($r = 0.57$), and milk yield ($r = -0.57$; each $P < 0.01$; Table 2). The E_2 in milk was also positively correlated with log SCC in milk ($r = 0.40$; Table 2).

DISCUSSION

Cows in this study gained, on average, 53.6 kg between trimesters 1 and 2 and 53.0 kg between trimesters 2 and 3. The substantial weight gain during pregnancy is consistent with the developing uterine-conceptus mass as well as a significant increase in body condition. Hematocrit concentrations averaged 27.7%, which was similar to observations of Eley et al. (1981), Lee et al. (1978), and Smith (2002). Plasma proteins in the present study averaged 6.8 g/dL, which is similar

to the 6.9 g/dL observed in lactating cows (Eley et al., 1981).

Parity had no effect on the concentrations of E_1 and E_2 in milk, consistent with results from a previous experiment with a larger number of cows (Pape-Zambito et al., 2007). Plasma and milk E_1 and E_2 concentrations increased throughout gestation, with plasma E_1 increasing earlier than plasma E_2 (Figure 1). The greatest concentrations of E_1 and E_2 were found in the last plasma and milk samples (average = 238 d pregnant), when most dairy cows in the United States are not lactating. Plasma and milk E_1 increased throughout pregnancy, whereas E_2 significantly increased in trimester 3 of pregnancy in lactating pregnant Holstein dairy cows (Figure 2). Others have reported that plasma E_1 (Robinson et al., 1970) and E_2 (Monk et al., 1975; Robertson and King, 1979; Patel et al., 1999) increased during pregnancy in the cow. Monk et al. (1975) did not observe an increase in milk E_1 and E_2 during pregnancy, but they did not sample as late in pregnancy as in the current study. Pape-Zambito et al. (2007) reported that E_2 concentrations in raw whole milk were greater in cows that were 141 to 210 d pregnant than in cows < 141 d pregnant (3.0 vs. 0.9 pg/mL, respectively). Malekinejad et al. (2006) reported increased concentrations of E_1 and E_2 in raw milk throughout pregnancy; however, only 5 cows were sampled from each trimester. In addition, milk samples from each trimester were pooled before analyses, and statistical differences among trimesters were not presented. The repeated measures analysis in the present experiment has merit in that animal variation was not confounded with pregnancy stage. Heap and Hamond (1979) and Malekinejad et al. (2006) reported greater E_1 -sulfate concentrations in milk from pregnant cows, particularly during the latter half of pregnancy. The present experiment, to our knowledge, is the first to show a significant increase in E_1 and E_2 in milk during pregnancy using within-cow comparisons. Future studies will examine the concentrations of conjugated estrogens in milk from both non-pregnant and pregnant cows.

Plasma E_1 was correlated with plasma E_2 ($r = 0.74$, Table 2). Eley et al. (1981) also noted a positive correlation ($r = 0.54$) between plasma E_1 and E_2 in late pregnant dry cows. Plasma E_1 was correlated with milk E_1 in the present study ($r = 0.77$, Table 2), and plasma E_2 was significantly correlated with milk E_2 ($r = 0.93$, Table 2). The correlations between plasma and milk estrogens suggest that plasma E_1 and E_2 may passively diffuse from the plasma through mammary epithelial cells to the alveolar lumen where milk is accumulated. Batra et al. (1980) reported a high correlation between plasma and milk E_2 during pregnancy in buffaloes.

Table 2. Correlation coefficients among plasma and milk estrone (E₁) and 17 β -estradiol (E₂) concentrations and milk components¹

Item	MY ²	Fat	Protein	Lactose	Log SCC	Plasma E ₁	Milk E ₁	Plasma E ₂	Milk E ₂
Days pregnant	-0.65***	0.20	0.23*	-0.25*	0.33**	0.82***	0.67***	0.58***	0.57***
MY	1.00	-0.26*	-0.37***	0.49***	-0.67***	-0.45***	-0.43***	-0.47***	-0.57***
Fat		1.00	0.58***	0.33**	0.12	0.29**	0.50***	0.21*	0.25*
Protein			1.00	0.23*	0.27**	0.34***	0.29**	0.57***	0.63***
Lactose				1.00	-0.58***	-0.10	-0.03	-0.06	-0.16
Log SCC					1.00	0.18	0.16	0.29**	0.40***
Plasma E ₁						1.00	0.77***	0.74***	0.71***
Milk E ₁							1.00	0.55***	0.55***
Plasma E ₂								1.00	0.93***

¹Numbers in the table represent correlation coefficients for the comparisons of interest with n = 94 to 98 samples for each cell.

²MY = milk yield.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The milk:plasma ratio of E₁ remained below 1 throughout pregnancy (Figure 2). Perhaps the presence of binding proteins in plasma reduces diffusion of E₁ into the mammary glands. In contrast, the milk:plasma ratio of E₂ increased throughout pregnancy in the present experiment (Figure 2). This suggests that, as gestation progresses, either some specific blood-to-milk transport mechanism evolves or that cells within the mammary gland develop the ability to produce E₂. Keller et al. (1977) stated that the mammary glands might selectively accumulate E₂ or convert other estrogens to E₂, given that milk E₂ concentrations were greater than those in plasma. Maule-Walker and Peaker (1978), Maule-Walker et al. (1983), and Peaker and Taylor (1990) reported greater quantities of E₂ in mammary venous blood compared with arterial blood during late pregnancy and suggested the mammary glands produced E₂. Peaker and Taylor (1990) and Janowski et al. (2002) reported that mammary tissue from late pregnant animals converted ³H-androstenedione to ³H-E₂, a reaction that requires the aromatase (CYP19) enzyme. Belvedere et al. (1996) detected aromatase transcripts in mammary tissue from late lactation cows but not in nonlactating-nonpregnant cows. Other than aromatase, little has been reported on other enzymes that may be involved in mammary cell steroid biosynthesis.

Milk E₁ and E₂ in the present study were positively correlated with milk fat and protein percentages. The more lipophilic E₁ was more highly correlated with milk fat percentage than was milk E₂ with milk fat percentage ($r = 0.50$ vs. 0.25). Wolford and Argoudelis (1979) reported that 80% of milk E₁ but only 65% of milk E₂ was associated with the fat in milk after they incubated radiolabeled estrogens with milk and then separated and counted both fat and skim fractions. Our results are consistent with the conclusions of Wolford and Argoudelis (1979) but reflect partitioning of E₁ and E₂ during milk biosynthesis. Pape-Zambito et al. (2007)

reported a correlation of 0.20 between percentage of milk fat and E₂ concentration in milk from over 200 cows when adjusted for trimester of pregnancy. This is similar to the correlation in the present study (0.25).

Milk E₁ and E₂ concentrations in the present study were also correlated with percentage of milk protein ($r = 0.29$ and 0.63 , respectively). Wolford and Argoudelis (1979) reported that 84 to 85% of E₁ and E₂ found in skim milk were bound to proteins. Albumin is a minor whey protein in normal milk, but in plasma, albumin can nonspecifically transport both E₁ and E₂ throughout the body (Pardridge, 1986). Perhaps because E₁ is more hydrophobic than E₂, it associates to a greater degree with fat-soluble molecules, whereas the relatively more hydrophilic E₂ tends to associate with albumin or some other protein(s) during milk synthesis.

Milk E₂ concentrations were correlated with log SCC ($r = 0.40$, Table 2). It is possible that plasma proteins that transport E₂ may enter the alveolar lumen as tight junctions open during the passage of somatic cells or that somatic cells in milk have the ability to convert E₁ to E₂, leading to an increase in milk E₂ concentrations. The enzyme required for this conversion is 17 β -hydroxysteroid dehydrogenase. It would be interesting to determine if milk somatic cells possess 17 β -hydroxysteroid dehydrogenase activity. The correlations between milk E₁ and E₂ and milk components provide an insight into the complex manner by which E₁ and E₂ accumulate in the alveolar lumen. Results from the present study suggest that E₁ and E₂ accumulate in milk by somewhat different mechanisms.

The absolute concentrations of both plasma and milk E₁ and E₂ in our study are low relative to some reports in the literature (Monk et al., 1975; Erb et al., 1977; Patel et al., 1999) but are similar to others (Glencross et al., 1973; Glencross and Abeywardene, 1983; Abeywardene et al., 1984). The trend for increased plasma E₁ and E₂ concentrations during pregnancy is consistent across studies. Zdunczyk et al. (2001) did not detect E₂

in plasma or milk during pregnancy, and Hoffmann et al. (1997) were not able to detect E_2 in plasma until 10 d before parturition. These examples illustrate the sensitivity required to measure E_2 in bovine plasma and milk. The range of E_2 concentrations in milk in the current study (nondetectable to 22.3 pg/mL) agree with concentrations reported previously by our lab (Pape-Zambito et al., 2007). Pape-Zambito et al. (2007) and Monk et al. (1975) discussed factors including sample processing, the E_2 standard, and E_2 antibody that may explain differences in absolute concentrations reported in the literature. A specific issue may be related to the cross-reactivity of the E_2 antibody with other estrogens. The antibody used in the present study cross-reacted 0.68% with 17α -estradiol, whereas previous reports have noted a 17 to 32% cross-reactivity of the E_2 antibody with 17α -estradiol (Monk et al., 1975; Eley et al., 1981). Janowski et al. (2002) reported that 17α -estradiol concentrations in plasma were greater than E_1 or E_2 concentrations in late pregnancy. Thus, antibodies used to detect E_2 that significantly cross-react with 17α -estradiol would yield artificially high estimates of E_2 .

More recent technologies, such as GC-MS and liquid chromatography tandem mass spectrometry (**LC/MS-MS**), have been utilized to quantify estrogens in milk (Hartmann et al., 1998; Malekinejad et al., 2006). These methods are advantageous in that they do not rely on antibody-based detection. It is also possible to analyze multiple estrogens at a time ($E_1 + E_2 +$ estriol); however, it can be difficult to optimize a single method for all estrogens of interest (Giese, 2003). Recently, Farre et al. (2007) quantified E_2 from municipal waste, river, and ground waters using ELISA (an antibody-based detection system), HPLC mass spectrometry, and ultra-performance liquid chromatography quadrupole-time of flight mass spectrometry. Ultra-performance liquid chromatography quadrupole-time of flight mass spectrometry was the most sensitive method followed by ELISA then LC/MS-MS. The authors concluded that results from all techniques were in general agreement (Farre et al., 2007). Many investigators do not have access to the sensitive equipment required for GC-MS, LC/MS-MS, or ultra-performance liquid chromatography quadrupole-time of flight mass spectrometry analysis of estrogens in bovine samples where concentrations are generally below 100 pg/mL, or the cost for sample analysis using these techniques is too expensive. Therefore, antibody-based technologies such as RIA and ELISA are widely used due to the specificity of modern antibodies, sensitivity of the method, as well as the cost or sample.

One other notable difference between our study and others may be related to milk yield (**MY**). Cows in the Pennsylvania State dairy herd had an average 305-d

mature equivalent of 11,465 kg, which is likely greater than MY of dairy cows in European countries and US dairy cows in the 1970s and 1980s. Milk yield was negatively correlated with both milk E_1 and milk E_2 in the present study (Table 2), as well as in Pape-Zambito et al. (2007). Eley et al. (1981) compared E_1 and E_2 concentrations in plasma from cows that had been selected for increased MY to those in cows not selected for increased MY (control). Interestingly, cows that were selected for increased MY tended ($P < 0.10$) to have lower concentrations of plasma E_1 compared with control cows. Sangsritavong et al. (2002) reported that increased DM intake (observed in high-producing dairy cows) can increase liver blood flow and increase metabolic clearance rate of both progesterone and E_2 . Increased metabolic clearance rates of progesterone and E_2 can reduce circulating concentrations of these hormones (Lopez et al., 2004). Therefore, the low E_1 and E_2 concentrations in milk in the present study relative to others (Monk et al., 1975; Wolford and Argoudelis, 1979; Malekinejad et al., 2006), may be explained in part by greater MY of cows in the present study.

Ganmaa and Sato (2005) speculated that today a greater proportion of high-producing pregnant cows are being milked compared with 100 yr ago, and this contributes to greater quantities of estrogens in the consumable milk supply. Wiltbank et al. (2006) suggested that high-producing dairy cows have increased metabolic clearance rates of E_2 with resultant decreased circulating concentrations of E_2 , which, in turn, may affect fertility in modern high-producing cattle. The decrease in circulating concentrations of E_2 over time likely would result in decreased concentrations of E_2 in milk, because milk concentrations are significantly correlated with plasma concentrations (Table 2). Thus, in contrast to Ganmaa and Sato (2005), it is likely that milk produced by modern high-producing dairy cows actually would have less E_2 compared with milk from cows from 100 yr ago.

Although we did observe a significant increase in milk E_1 and E_2 concentrations during pregnancy, it is important to point out that the final trimester 3 samples were collected at a time when cows are usually nonlactating (>220 d pregnant). Milk from cows > 220 d pregnant would generally not be consumed by people or would represent an insignificant fraction of the milk supply. Much of the milk entering the bulk tank on a dairy farm is from nonpregnant cows with a small percentage (<20%) of cows in a herd producing milk during the last trimester of pregnancy. Based upon the data in Figure 1, data from Pape-Zambito et al. (2007), and the knowledge that early lactation cows have high milk yields, it is likely that the majority of cows producing milk for consumption would have <10 pg of E_1 /mL

of milk and <2 pg E_2 /mL milk. This estimate is based upon the assumption that the average Holstein cow is 201 DIM (Dairy Records Management Systems, Raleigh, NC), that average days open is 122 (Hare et al., 2006), that farmers generally dry off cows at 60 d before the next expected calving, and that pasteurization and homogenization do not affect concentrations of E_1 or E_2 in milk (Pape-Zambito et al., 2006). Using mean concentrations of E_1 and E_2 for pregnant cows in the present study, the combined amount of E_1 and E_2 consumed in 3 servings (711 mL or 24 fl. oz.) of whole milk would be 9.6 ng. This exposure value does not take into account the potential for lower E_1 and E_2 concentrations in bulk tank milk (because bulk tank milk would contain a considerable amount of milk from nonpregnant cows) and lower fat milk (Malekinejad et al., 2006; our own unpublished results), as well as either fecal excretion or first-pass metabolism by the gastrointestinal mucosa and liver of orally ingested estrogens (Ruoff and Dziuk, 1994; O'Connell, 1995). It has been reported that prepubertal girls produce 54,000 ng of $E_1 + E_2$ /d and that prepubertal boys produce 100,000 ng of $E_1 + E_2$ /d (as reported in Hartmann et al., 1998). Thus, a conservative estimate of the mass of $E_1 + E_2$ in 3 servings of milk represents 0.02% of what prepubertal girls produce daily. The low concentrations of E_1 and E_2 in milk, therefore, are unlikely to pose a health risk to humans. However, additional research on concentrations of estrogen conjugates in milk, as well as bioavailability and absorption rates of orally ingested estrogens in milk, is required to ascertain true biological risk to humans.

CONCLUSIONS

Plasma and milk E_1 and E_2 were greater in trimester 3 of pregnancy compared with trimesters 1 and 2 in lactating Holstein dairy cows. The E_1 concentrations were 0.91 and 0.69 pg/mL at 60 d of pregnancy in plasma and milk, respectively, and concentrations increased to 51.3 and 36.7 pg/mL by d 238 of pregnancy. The E_2 concentrations averaged 0.53 and 0.22 pg/mL at 60 d of pregnancy in plasma and milk, respectively, and increased to 3.1 and 7.4 pg/mL by d 238 of pregnancy. The E_2 concentrations in plasma were greater than those in milk during trimester 1 (milk:plasma ratio = 0.4), but in trimester 3 of pregnancy, milk concentrations exceeded those in plasma (milk:plasma ratio = 2.2). This change may indicate that cells within the mammary gland acquire the capacity to produce E_2 from a precursor steroid. Milk E_1 concentrations were positively correlated with plasma E_1 concentrations and percentage of milk fat and were negatively correlated with milk yield. The E_2 concentrations in milk were

positively correlated with E_2 concentrations in plasma and percentage of milk protein and negatively correlated with milk yield. Results suggest that E_1 and E_2 accumulate in milk by somewhat different mechanisms. The concentrations of E_1 and E_2 consumed from milk are extremely low relative to endogenous 24-h production rates of E_1 and E_2 in humans.

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