Concentrations of 17 β -Estradiol in Holstein Whole Milk

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ABSTRACT

Some individuals have expressed concern about estrogens in food because of their potential to promote growth of estrogen-sensitive human cancer cells. Researchers have reported concentrations of estrogen in milk but few whole milk samples have been analyzed. Because estrogen associates with the fat phase of milk, the analysis of whole milk is an important consideration. The objectives of this study, therefore, were to quantify 17 β -estradiol (E₂) in whole milk from dairy cows and to determine whether E₂ concentrations in milk from cows in the second half of pregnancy were greater than that in milk from cows in the first half of pregnancy or in nonpregnant cows. Milk samples and weights were collected during a single morning milking from 206 Holstein cows. Triplicate samples were collected and 2 samples were analyzed for fat, protein, lactose, and somatic cell counts (SCC); 1 sample was homogenized and analyzed for E₂. The homogenized whole milk (3 mL) was extracted twice with ethyl acetate and once with methanol. The extract was reconstituted in benzene:methanol (9:1, vol/vol) and run over a Sephadex LH-20 column to separate E₂ from cholesterol and estrone before quantification using radioimmunoassay. Cows were classified as not pregnant (NP, n = 138), early pregnant (EP, 1 to 140 d pregnant, n = 47), or midpregnant (MP, 141 to 210 d pregnant, n = 21) at the time of milk sampling based on herd health records. Mean E₂ concentration in whole milk was 1.4 $\pm$ 0.2 pg/mL and ranged from nondetectable to 22.9 pg/mL. Milk E₂ concentrations averaged 1.3, 0.9, and 3.0 pg/mL for NP, EP, and MP cows, respectively. Milk E₂ concentrations for MP cows were greater and differed from those of NP and EP cows. Milk composition was normal for a Holstein herd in that log SCC values and percentages of fat, protein, and lactose averaged 4.9, 3.5, 3.1, and 4.8, respectively. Estradiol concentration was significantly correlated (r = 0.20) with percentage fat in milk. Mean milk yield was 18.9 $\pm$ 0.6 kg for the morning

milking. The mean E₂ mass accumulated in the morning milk was 23.2 $\pm$ 3.4 ng/cow. Likewise, using the overall mean concentration for E₂ in milk, the mean E₂ mass in 237 mL (8 fluid ounces) of raw whole milk was 330 pg. The quantity of E₂ in whole milk, therefore, is low and is unlikely to pose a health risk for humans.

Key words: 17 β -estradiol, whole milk, pregnancy

INTRODUCTION

Milk is a valuable nutrient source for humans, but recent reports have questioned its safety because of the presence of steroid hormones in milk. Estrogens are present in milk (Monk et al., 1975; Erb et al., 1977a,b) and if present at high concentrations, could affect biological processes within humans who consume milk. Hartmann et al. (1998) reported that dairy products might account for up to 60% of true estrogens (compared with phytoestrogens) in a typical German diet. It has been hypothesized that long-term exposure to estrogens increases the risk of breast cancer (Yue et al., 2003) and others have specifically stated that the estrogens in milk may be responsible for increased cancer risk (Li et al., 2003; Qin et al., 2004). Consequently, individuals with estrogen-sensitive cancers are concerned with the potential biological consequences of estrogens in dairy products.

Several researchers have quantified estrogens in skim milk (Wolford and Argoudelis, 1979; Abeyawardene et al., 1984; Lopez et al., 2002), but few have quantified 17 β -estradiol (E₂) in whole milk. This distinction is important because E₂ is a lipophilic molecule and is at a greater concentration in the fat fraction of milk (Schwalm and Tucker, 1978; Wolford and Argoudelis, 1979). Monk et al. (1975) reported that E₂ concentrations in whole milk from cyclic (n = 6) and pregnant (n = 13) cows averaged 37 and 64 pg/mL, respectively. Erb et al. (1977a,b) reported a mean whole milk E₂ concentration of 13 pg/mL in 6 nonpregnant cows that were between 3 and 25 DIM. Wolford and Argoudelis (1979) quantified E₂ from 2 to 4 raw and commercial whole milk samples with E₂ averaging 12.3 and 6.7 pg/mL, respectively. These studies were valuable and gave estimates of E₂ in whole milk but the number of cows or samples used were small.

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Previous research has also shown that E_2 concentrations in plasma increase throughout pregnancy in the cow (Robertson and King, 1979; Patel et al., 1999) and are correlated with milk E_2 concentrations (Batra et al., 1980; Abeyawardene et al., 1984). It is less clear whether milk E_2 concentrations also increased throughout pregnancy. Given the reported concerns about estrogens in milk and because genetic variability and stage of pregnancy may lead to differences in E_2 concentration, it is important to analyze E_2 concentration in whole milk from a large number of cows.

The objectives of this study were to analyze E_2 concentrations in milk from a large number of cows (>200) and to determine whether the E_2 concentrations in milk from cows in the second half of pregnancy are greater than those in milk from cows in the first half of pregnancy or in nonpregnant cows.

MATERIALS AND METHODS

Sample Collection

Triplicate milk samples were collected during a single November morning milking (0500 h) of 206 Holstein cows in the Pennsylvania State University dairy herd in a milking parlor equipped with the AFIfarm system (S.A.E. Afikim, Kibbutz Afikim, Israel; US Distributor: Germania Dairy Automation, Waunakee, WI). Duplicate samples were sent to DHIA for fat, protein, lactose, and SCC analyses. The remaining milk sample (9 mL) was homogenized using a Polytron homogenizer with a PTA probe on setting 6 for 8 s before storage at -20°C .

Milk weights and reproductive status for each cow were obtained from farm records. Herd reproduction was conventionally managed with a 60-d voluntary waiting period before breeding was attempted. Cows were given a 60-d dry period. All cows sampled were >5 DIM. Based upon the date of sampling, cows were categorized as not pregnant (NP; $n = 138$) or in the first or second half of pregnancy. Cows in the first half of pregnancy were designated as early pregnant (EP; 1 to 140 d pregnant, $n = 47$), whereas cows in the second half of pregnancy were designated as midpregnant (MP; 141 to 210 d pregnant, $n = 21$). Sixty-nine cows calved in the 3-mo period before our sampling day, which contributed to a larger number of NP cows relative to EP or LP cows.

E_2 Extraction from Whole Milk

Solvent extraction procedures were adapted from Monk et al. (1975) and Wolford and Argoudelis (1979). Homogenized milk samples were thawed in a warm water bath and vortexed for 10 s before aliquoting 3 mL into a 50-mL screw cap glass extraction tube. Milk

samples were extracted with 9 mL of ethyl acetate (HPLC grade, J. T. Baker, Phillipsburg, NJ). The mixture was vortexed for 30 s and 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 resultant liquid organic layer was transferred to a glass test tube and dried under N_2 at 50°C . The ethyl acetate extraction was then repeated. After freezing, the organic layer from the second ethyl acetate extraction was transferred to a corresponding sample tube and dried again. Warm (50°C) methanol (2 mL, HPLC grade, Burdick and Jackson, Muskegon, MI) was added to the extract, and the mixture was incubated at 50°C for 1 h with thorough mixing at 0, 15, 30, 45, and 60 min. The mixture was subsequently incubated at -20°C for 1 h and then centrifuged at $1,370 \times g$ for 30 min at 4°C to precipitate triglycerides. The supernatant solution containing the steroid hormone fraction was quantitatively transferred to a clean glass test tube and dried under N_2 at 50°C .

The estrogen-containing extract was reconstituted in benzene:methanol (9:1, vol/vol; benzene from Sigma-Aldrich, St. Louis, MO). Column chromatography was used to separate E_2 from residual cholesterol and other steroids (Mikhail et al., 1970). A Sephadex LH-20 column (GE Healthcare, Piscataway, NJ) was packed to a height of 2.5 cm in glass columns with an internal diameter of 1 cm. Steroids were eluted with benzene:methanol (9:1, vol/vol) as described by Kensinger et al. (1986). The E_2 elution pattern was verified with 2, 4, 6, 7- ^3H - E_2 (GE Healthcare; cat no. TRK322), and the estrone (E_1) elution pattern was verified using crystalline estrone followed by RIA (Diagnostic Systems Laboratory, Webster, TX; cat no. DSL-8700). Estrone was eluted between 1.5 and 4.5 mL, and E_2 was eluted between 4.7 and 8.3 mL at 25°C . Other experiments in our lab have verified the E_1 elution pattern using 2, 4, 6, 7- ^3H - E_1 (GE Healthcare; cat no. TRK321). In addition, we have confirmed the absence of cholesterol in the E_2 fraction when this methodology is used. The E_2 fraction was dried under N_2 at 50°C .

E_2 Quantification by RIA

The dried E_2 fraction from each milk sample was reconstituted in 125 μL of pooled wether sera before quantification using an RIA specific for E_2 (cat. no. 07-138106; MP Biomedicals, Irvine, CA). The immunogen used to generate the antiserum in rabbits was 6-keto-estradiol-17 β -6-oxime-BSA. The cross-reactivity of the antiserum with other steroids is as follows: estrone, 20%, estriol, 1.5%, 17 α -estradiol, 0.68%, and <0.01% with cholesterol, progestins, and androgens. Samples

were run in duplicate according to the manufacturer's instructions.

Internal standards were run for each set of samples extracted to quantify percentage E_2 recovery from whole milk. Tritiated E_2 (0.01 μCi) was added to pooled homogenized whole milk samples. After the final reconstitution step in pooled wether sera, the internal standards (125 μL) were pipetted into 7-mL scintillation vials with 5 mL of Ecolite scintillation fluid (ICN, Costa Mesa, CA) and counted with 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 inter- and intraassay variation. Estradiol was analyzed in 4 assays with cows at all stages of pregnancy represented in each assay. The inter- and intraassay coefficients of variation were each 6.8%.

The E_2 concentrations were corrected for recovery of $^3\text{H}-E_2$. Fifty-six percent of the samples repeatedly predicted below the limit of quantification (LOQ) of the RIA (0.7 pg of E_2/mL of milk). Low concentrations of E_2 were detected in these samples, but the predicted value was less than the lowest concentration standard from the standard curve. Any milk sample that predicted below the LOQ was assigned the LOQ value of 0.7 pg of E_2/mL of milk. The data presented, therefore, are conservative estimates of E_2 concentrations in raw whole milk. Estradiol mass was calculated by multiplying E_2 concentration by the volume of milk produced at that milking. Milk volume (mL) was calculated using the following conversion factors beginning with pounds of milk: 1 gallon of milk/8.8 pounds of milk; 3.78 L of milk/1 gallon of milk; 1,000 mL of milk/1 L of milk.

Statistical Analyses

Version 8.2 of the SAS software was used for statistical analyses (SAS Institute, Cary, NC); PROC GLM was used to determine if E_2 concentrations were affected by reproductive status. Reproductive status was a fixed effect in the following model: E_2 concentration, E_2 mass, percentage fat, percentage protein, percentage lactose, log SCC, milk yield = reproductive status. The least squares means option was used to determine differences between reproduction categories. The MANOVA option within PROC GLM was used to determine partial correlations between E_2 concentrations, milk components, and milk yield after adjusting for reproductive status.

RESULTS

Reported E_2 concentrations were corrected for the mean recovery of E_2 from raw whole milk, which was

Figure 1. Concentrations of 17 β -estradiol in whole milk from individual cows ($n = 68$) vs. days of pregnancy (d 1 to 210). The best-fit equation to describe the relationship, a second-order polynomial ($y = 0.0001x^2 - 0.0163x + 1.1005$; $r = 0.61$, $P < 0.01$), is also shown.

$59.4 \pm 2.1\%$ (mean $\pm$ SD). The E_2 concentration in raw whole milk averaged 1.4 ± 0.2 pg/mL of milk (mean $\pm$ SEM) and ranged from nondetectable to 22.9 pg/mL of milk. Estradiol concentrations were 1.3 ± 0.2 , 0.9 ± 0.3 , and 3.0 ± 0.4 pg/mL milk for NP, EP, and MP cows, respectively. The concentration of E_2 did not differ between NP and EP cows; however, E_2 in whole milk of MP cows was greater compared with that in NP and EP cows ($P < 0.001$).

Figure 1 shows a scatter plot of E_2 concentrations in milk vs. days pregnant for the 68 pregnant cows. Milk E_2 increased as days pregnant increased. The best-fit equation to describe this relationship was a second-order polynomial ($r = 0.61$, $P < 0.01$).

Milk yield for the morning milking averaged 18.9 ± 0.6 kg overall and was lower ($P < 0.05$) for MP cows compared with NP and EP cows, which did not differ (Table 1). Milk fat averaged $3.5 \pm 0.1\%$ overall and 3.6, 3.3, and 3.6% in NP, EP, and MP cows, respectively. Total E_2 mass accumulated in the morning milking averaged 23.2 ± 3.4 ng/cow and was greater ($P < 0.05$) in MP cows than in NP or EP cows. Estradiol concentrations in whole milk were positively correlated with percentage milk fat and negatively correlated with milk yield (Table 2).

DISCUSSION

The concentrations of E_2 in raw whole milk were greater in cows >141 d pregnant compared with non-pregnant cows and cows <141 d pregnant, and E_2 concentrations tended to increase during pregnancy (Fig-

Table 1. Milk composition, milk yield, and mass of 17 β -estradiol (E₂) accumulated in milk for cows grouped by reproductive status

Analyte	Reproductive status ¹			SEM
	NP	EP	MP	
Fat, %	3.6 ^a	3.3 ^b	3.6 ^{ab}	0.08
Protein, %	3.1 ^b	3.2 ^a	3.3 ^a	0.03
Lactose, %	4.8 ^a	4.8 ^a	4.6 ^b	0.04
Log SCC	4.9 ^a	5.0 ^a	5.0 ^a	0.08
Milk yield, kg	19.5 ^a	18.7 ^a	15.8 ^b	0.61
E ₂ mass, ² ng	23.4 ^b	14.8 ^b	39.9 ^a	3.36

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

¹NP = nonpregnant (n = 138; mean DIM = 123); EP = early-pregnant (1 to 140 d pregnant, n = 47; mean DIM = 223); MP = midpregnant (141 to 210 d pregnant, n = 21; mean DIM = 289).

²E₂ mass was calculated by multiplying E₂ concentration by the volume of milk (mL) produced at the a.m. milking. Milk volume (mL) was calculated using the following conversion factors beginning with pounds of milk: 1 gallon of milk/8.8 pounds of milk; 3.78 L of milk/1 gallon of milk; 1,000 mL of milk/1 L of milk.

ure 1). In contrast, Monk et al. (1975) did not show increased E₂ concentrations in milk during pregnancy. Our results, however, were not surprising because milk E₂ is correlated with plasma E₂ (Batra et al., 1980; Abeyawardene et al., 1984) and plasma E₂ concentrations increase throughout pregnancy in cows (Robertson and King, 1979; Patel et al., 1999). If E₂ levels in plasma and milk are positively correlated and plasma concentrations increase throughout pregnancy, one would expect E₂ in milk to increase throughout pregnancy.

Mean E₂ concentrations in the present study were lower than those reported by others (Monk et al., 1975; Erb et al., 1977a; Wolford and Argoudelis, 1979). This could be attributed to differences in sample handling (i.e., homogenization), extraction and isolation methodologies, physiological states of the cows when the samples were collected, genetics, season during sample collection, purity of the E₂ standard used, or the fact that different antibodies were used for detection of E₂. Monk

et al. (1975) discussed these factors and suggested that within-study comparisons are more useful than comparisons of absolute concentrations across studies. Plasma E₂ concentrations steadily increase during pregnancy (Patel et al., 1999); however, plasma E₂ concentrations greatly increase 20 to 30 d prepartum because of increased placental conversion of progesterone to E₂ (Henricks et al., 1972; Patel et al., 1999). Estradiol concentrations in mammary secretions are greatest several days around parturition (Monk et al., 1975) coincident with the increase in plasma E₂. Erb et al. (1977b) reported a mean E₂ concentration of 13 pg/mL of milk in cows between 3 and 25 DIM. The values reported in the present study were representative of cows in various reproductive states (i.e., noncycling, follicular, luteal, and various stages of pregnancy) although all cows were >5 DIM. Milk samples collected during the first 25 DIM would likely have greater E₂ concentrations given the carryover effects of the recent calving. That the 138 NP cows in our study averaged 123 DIM on the milk sampling day may explain, in part, why our values were lower than those of Erb et al. (1977b). Wolford and Argoudelis (1979) did not report the reproductive status or DIM for the 2 to 4 cows sampled for their E₂ analyses. Their average milk E₂ concentration of 12.3 pg/mL, however, is consistent with the range presented in this study. Monk et al. (1975) reported a mean milk E₂ concentration of 63 pg/mL in 13 pregnant cows. Procedural differences including the antibody used may be one reason that our estimates of milk E₂ concentration were lower. We adjusted for recovery of tritiated E₂ standard from milk samples and showed that increasing the extracted milk volume led to greater E₂ recovery (parallelism). We also measured 158% recovery of E₂ added to whole milk in added-mass verification tests of our assay. The 158% is not perfect but it was deemed reasonable given the extensive sample manipulation throughout the assay procedure.

Table 2. Partial correlation coefficients among milk 17 β -estradiol (E₂) concentrations and milk components, E₂ mass, and milk yield (MY)¹

	Fat, %	Protein, %	Lactose, %	Log SCC	E ₂ mass, ² ng	MY, kg
E ₂ , pg/mL	0.20**	0.09	-0.13	0.12	0.95***	-0.15*
Fat, %		0.46***	-0.17*	0.23**	0.12	-0.28***
Protein, %			-0.08	0.27***	0.01	-0.32***
Lactose, %				-0.51***	-0.08	0.13
Log SCC					0.10	-0.14*
E ₂ mass, ng						0.07

¹Numbers in table represent partial correlation coefficients, adjusted for reproductive status, for the comparisons of interest with n = 203 to 206 for each cell.

²E₂ mass was calculated by multiplying E₂ concentration by the volume of milk (mL) produced at the a.m. milking. Milk volume (mL) was calculated using the following conversion factors beginning with pounds of milk: 1 gallon of milk/8.8 pounds of milk; 3.78 L of milk/1 gallon of milk; 1,000 mL of milk/1 L of milk.

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

The extraction and column chromatography procedures used in the present study led to the separation of E_2 from other whole milk components and potentially cross-reactive compounds such as cholesterol. The presence of structurally related compounds in the RIA could lead to inflated E_2 concentrations. The average cholesterol concentration in raw milk is $144 \pm 38 \mu\text{g/mL}$ (Bachman et al., 1976). Although the E_2 antibody in the RIA cross-reacted $<0.01\%$ with cholesterol, cholesterol could, at $\mu\text{g/mL}$ concentrations, interfere with antibody-based detection of E_2 in milk. If the antibody had significant cross-reactivity with cholesterol and measures were not taken to separate E_2 from cholesterol, erroneously high concentrations of E_2 would be reported.

The relatively low E_2 concentrations reported in the present study were verified by repeating this experiment at a later date with a smaller number of randomly selected cows ($n = 19$; NP = 8, EP = 11). Estradiol concentrations in milk averaged 0.9 pg/mL in the replicated study with values ranging from nondetectable to 3.0 pg/mL . In retrospect, we would expect the mean value to be somewhat lower than the mean for the data set with 206 cows (1.4 pg/mL of milk) because the smaller study did not contain any MP cows.

Estradiol concentrations in whole milk were correlated with milk fat percentage (Table 2). This is not surprising because E_2 is found in greater concentrations in the fat fraction of milk (Schwalm and Tucker, 1978; Wolford and Argoudelis, 1979; our own unpublished results). Previous research also showed that progesterone was significantly correlated ($r = 0.98$) with the fat percentage of milk (Ginther et al., 1976). Progesterone is less polar than E_2 , which could explain why its correlation with milk fat is greater than that observed between E_2 and milk fat in the present study. Skim milk was used by others to measure relative changes in E_2 concentrations associated with the estrous cycle (Abeyawardene et al., 1984; Scholey et al., 2005). Estradiol concentrations measured in skim milk, however, do not reflect E_2 in whole milk, because a large proportion of the E_2 associated with the fat would be removed before analysis. The r -value for the correlation between E_2 and percentage milk fat in the current study was significant but small (0.20), suggesting that the concentration of E_2 in milk is determined by multiple factors. Plasma E_2 concentrations in the mammary blood vessels influence the concentrations in milk (Monk et al., 1975). However, genetics, integrity of tight junctions, and the presence of a transport protein such as albumin may also influence E_2 concentration in milk. It is possible that mastitis could influence milk E_2 concentrations if the integrity of tight junctions within the mammary gland was compromised, allowing increased concentra-

tions of albumin (a nonspecific carrier of estradiol) into the alveolar lumen. Mastitis, therefore, might increase milk E_2 concentration in infected glands relative to non-infected glands. The accumulation of E_2 in milk is probably a complex process and is not well defined.

Fat percentage was greater in the milk of NP cows compared with EP cows and some may find this result surprising. We evaluated milk composition, however, in relation to reproductive status and reproductive status was confounded with DIM. Some of the NP cows were in an advanced stage of lactation and may have contributed to the greater milk fat percentages observed in NP cows compared with EP cows.

Although the E_2 concentration in the milk of cows $>141 \text{ d}$ pregnant was greater than that in nonpregnant cows or cows $<141 \text{ d}$ pregnant, the concentrations in raw whole milk were low. The mass of E_2 in 237 mL (8 fluid oz.) of raw whole milk would average 330 pg . This is a conservative estimate of E_2 mass in milk because 56% of the analyzed samples repeatedly predicted below the LOQ of the RIA but were assigned the value for the LOQ (0.7 pg/mL of milk). Guidelines provided by the USDA suggest consuming 3 servings of dairy foods per day (www.mypyramid.gov). Based on the raw whole milk data in the present study, if a person consumed 24 fluid oz. (or 711 mL) of whole milk per day, then he or she would ingest slightly less than 1 ng of E_2/d . This analysis assumes that pasteurization and homogenization do not affect concentrations of E_2 in milk (Pape-Zambito et al., 2006) and does not account for a lower percentage of fat in milk that is sold commercially. Reported 24-h production rates of E_2 in humans range from 400 ng/d in prepubertal girls ($<8 \text{ yr old}$; Joint FAO/WHO Expert Committee on Food Additives, 2000) to 37.8 mg/d in late-pregnant women (Hoffmann and Evers, 1986). Three servings of whole milk would contain 0.25% of the E_2 produced daily in the body of a prepubertal girl. Toxicologists often use the term "no observable effect level" to define the level at which a chemical has no observed biological effect. Whole milk consumption would have to be $>215,000 \text{ L/d}$ ($56,800 \text{ gallons/d}$) to observe the bioactivity of E_2 derived from whole milk based upon the "no observable effect level" of $300 \mu\text{g}$ of E_2/d as reported by the World Health Organization (Joint FAO/WHO Expert Committee on Food Additives, 2000). These calculations also do not take into account the potential for lower E_2 concentrations in reduced-fat milk, fecal excretion, and the extensive intestinal and hepatic metabolism of orally ingested estrogens (Ruoff and Dziuk, 1994). Based upon the results of our analyses, E_2 concentrations in milk are low and unlikely to pose a health risk for humans.

CONCLUSIONS

Concentrations of 17β -estradiol in cows >141 d pregnant were greater than those in nonpregnant cows or cows <141 d pregnant. 17β -Estradiol concentrations were correlated with milk fat. Concentrations of E_2 in raw whole milk are negligible relative to literature values for 24-h production rates of E_2 in humans. Therefore, the minute E_2 concentrations in milk are unlikely to pose a health risk for humans.

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