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Full Research Article

ESTRONE, 17β-ESTRADIOL AND PROGESTERONE CONCENTRATIONS IN PROCESSED MILK WITH DIFFERENT FAT CONTENTS

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Abstract

Introduction. The aim of this study was to determine estrone (E1), 17β-estradiol (E2) and progesterone (P4) concentrations in processed milk with different fat contents and to compare the concentrations of these hormones in commercial ultrahigh temperature (UHT) processed milk and commercial pasteurized milk.

Materials and Methods. Commercial milks with different fat contents (UHT 0.5 %, UHT 1.5 %, UHT 3.5 % and pasteurized 3.5 % (10 samples of each type of milk)) were purchased in local stores. E1, E2 and P4 concentrations were determined by commercial ELISA kits.

Results and Conclusions. E1 concentrations were below the limit of detection (15 pg mL^{-1}) in all milks except in two UHT 3.5 % (out of 10) and two pasteurized 3.5 % (out of 10) milk samples. Mean E2 and P4 concentrations in UHT 3.5 % milk (25.37 ± 1.15 pg mL^{-1} and 10.76 ± 0.43 ng mL^{-1}, respectively) were significantly higher than in UHT 0.5 % milk (19.38 ± 0.79 pg mL^{-1} and 7.06 ± 0.26 ng mL^{-1}, respectively). Significant positive correlations were determined between hormone concentrations and milk fat contents. Relatively high E2 and P4 concentrations indicate that the bulk of milk in the commercial milks examined originated from pregnant cows.

Key Words: estrone, 17β-estradiol, progesterone, commercial milk

INTRODUCTION

Many endogenous and exogenous compounds are present in cows' milk as a result of excretion from the cow's body, and these can include bioactive steroid hormones. In cows, estrogens and progesterone (P4) are synthesized in the ovary and placenta and are excreted through bile, urine and saliva, and in lactating animals, also through milk. Estimation of reproductive status in cows by measuring fecal, urinary and milk

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estrogens and P4 concentrations is well-established (Pape-Zambito et al., 2008; Snoj, 1997; Hoffmann et al., 1977). Milk estrogens and P4 concentrations reflect their blood levels (Pape-Zambito et al., 2008), and their concentrations fluctuate during the estrus cycle and pregnancy. Both estrogens and P4 concentrations increase during pregnancy in cows and are present in the highest levels during the third trimester (Pape-Zambito et al., 2008; Pape-Zambito et al., 2007; Malekinejad et al., 2006). As cows in modern milk production are milked until the 7th or even 8th month of pregnancy, such milk contains relatively high amounts of estrogens and P4. The presence of estrogens in milk has opened a wide discussion regarding their possible role in endocrine disruption in consumers. Some studies suggest that consumption of milk and thus exposure to cows' estrogens might be involved in reproductive and developmental disorders and in increased incidence of hormone related cancers in humans (Maruyama et al., 2010; Ganmaa et al., 2005; Ganmaa et al., 2001; Sharp and Skakkbeak, 1993). Additionally, milk P4 might have an uterotrophic effect in experimental animals (Zhou et al., 2010). However, other studies failed to demonstrate the connection between milk consumption and endocrine disruption (Grgurevič et al., 2016; Larsson et al., 2015; Davoodi et al., 2013; Ganmaa et al., 2004). Estrogens and P4 levels in processed commercial milk have been reported in several studies; however, the results are not conclusive. Farlow et al. (2012) found 14.45 pg mL^{-1} of estrone (E1) in regular commercial milk, while the concentration of 17β-estradiol (E2) was 5.84 pg mL^{-1}. Malekeinejad et al. (2006) measured 8.2 – 20 pg mL^{-1} of E1 and 10.3 – 20.6 pg mL^{-1} of E2 in milk, and they also found a correlation between milk fat content and concentrations of estrogens. In another study, commercial milk E1 and E2 concentrations were reported to be 150 and 34 pg mL^{-1}, respectively (Zhou et al., 2010). In dairy products, P4 concentrations also correlated with milk fat content and were found to be, on average, 12 ng mL^{-1} in whole milk (Malekinejad and Rezabakhsh, 2015). The aim of our study was to determine E1, E2 and P4 levels in samples of processed milk with different fat contents and which had been subjected to different processing procedures.

MATERIALS AND METHODS

Milk samples

Forty packages of milk (1 L each) were purchased from different local stores. The details of the samples are presented in Table 1.

Table 1. Milks and their characteristics

Trade name	Alpsko mleko 0,5	Alpsko mleko 1,5	Alpsko mleko 3,5	Mu 3,5
Milk fat content	0.5 %	1.5 %	3.5 %	3.5 %
Processing	UHT homogenization	UHT homogenization	UHT homogenization	pasteurization homogenization
No. of samples	10	10	10	10
Time before expiry date (days)	28 - 61	36 - 66	19 - 58	2 - 5

UHT – milk was processed by ultrahigh temperature (135 °C for 3 seconds)

All milk samples studied were produced by the dairy plant 'Ljubljanske mlekarne' (Ljubljana, Slovenia) and were packed on different days. In the stores, UHT milks were kept at room temperature while pasteurized milk was kept at 4 to 8 °C.

In the laboratory, milk packages were shaken well, then opened and samples of 3 mL each were transferred into three plastic tubes. Samples were then stored at -20 °C before analysis

E1, E2 and P4 detection in milk

The milk E1 concentrations were determined by Estrone ELISA kit (catalogue number DE4174, Demeditec Diagnostics, Kiel-Wellsee, Germany) as described by Grgurevič et al. (2016). Partial validation of the test (recovery and precision) was performed and described previously (Grgurevič et al., 2016). Specificity was provided by the manufacturer. The quality of measurements was estimated by intra-assay coefficient of variation (CV) and was determined as 8.1 %. The range of detection was 15 – 1,000 pg mL^{-1}.

Similarly, the milk E2 concentrations were determined by Demeditec Estradiol-17β ELISA kit (catalogue number DE4399, Demeditec Diagnostics, Kiel-Wellsee, Germany) as described by Grgurevič et al. (2016). Partial validation of the test (recovery and precision) was performed and described in the same reference as validation of E1 measurements (Grgurevič et al., 2016). Specificity was provided by the manufacturer. The quality of measurements was estimated by intra-assay CV and was determined as 7.9 %. The range of detection was 1.5 – 200 pg mL^{-1}.

P4 concentrations were determined by Ovucheck Milk ELISA kit (product code TRM-547, Biovet, Sant-Hyacinthe, QC, Canada) following the instructions for users. As the assay is validated for the detection of P4 in bovine milk, no further validation was performed. The quality of measurements was estimated using an intra-assay CV, and was determined as 6.0 %. The range of detection was 1 – 30 ng mL^{-1}.

Statistical analysis

SPSS version 21 (IBM, Chicago, IL, USA) commercial software was used for statistical analyses. The Shapiro-Wilk test was used for the estimation of data distribution. E2 and P4 were normally distributed in milk of all fat contents; therefore, one-way ANOVA was used for the evaluation of statistical significance between milks with different fat contents. E1 concentrations did not have a normal distribution. As E1 concentrations were above the limit of detection (LOD) in only a few samples, statistical analysis of E1 concentrations was not performed. Additionally, the one-tailed Pearson's correlation test was used to determine possible correlations between E2 or P4 concentrations and milk fat content. Results of E2 and P4 concentrations are presented as mean ± SEM, and statistical significance was considered at $P < 0.05$.

RESULTS

E1 concentrations in all samples of UHT 0.5 % (UHT 0.5) and UHT 1.5 % (UHT 1.5) milk were below the LOD of the method. In UHT 3.5 % (UHT 3.5) milk, two samples (out of 10) had E1 concentrations above the LOD, and these were 44 pg mL^{-1} and 28 pg mL^{-1}. Similarly, only two samples from pasteurized 3.5 % (pasteurized 3.5) milk (out of 10 milks) had E1 concentrations above the LOD (30 pg mL^{-1} and 62 pg mL^{-1}).

Mean E2 concentrations in milks with different fat contents are presented in Table 2. UHT 3.5 milk contained a significantly higher ($P < 0.01$) mean concentration of E2 in comparison to UHT 0.5 milk. No significant differences in E2 levels were found between UHT 3.5 and pasteurized 3.5 milk.

Table 2. 17β-estradiol concentrations in milk with different fat contents (pg mL^{-1})

Milk type	UHT 0.5	UHT 1.5	UHT 3.5	Past. 3.5
E2 (mean ± SEM)	19.38 ± 0.79^{aC}	22.05 ± 1.43^{ab}	25.37 ± 1.15^{bD}	23.69 ± 1.38^{ab}
Range (min – max)	17.00 – 22.60	17.16 – 29.00	21.50 – 31.07	16.55 – 2.80

UHT – milk processed by ultrahigh temperature (135 °C, 3 seconds); Past. – pasteurized milk; E2 – 17β-estradiol; a, b – values indicated with the same letters do not differ significantly; C, D – values indicated with different letters differ significantly ($P < 0.01$)

Mean P4 concentrations in milks with different fat contents are shown in Table 3. Results indicate significantly higher ($P < 0.05$) P4 levels in UHT 3.5 milk in comparison to UHT 0.5 milk. No significant differences in mean P4 levels were found between UHT 3.5 and pasteurized 3.5 milk.

Table 3. Progesterone concentrations in milk with different fat contents (ng mL^{-1})

Milk type	UHT 0.5	UHT 1.5	UHT 3.5	Past. 3.5
P4 (mean ± SEM)	7.06 ± 0.26^{aC}	8.90 ± 0.38^{ab}	10.76 ± 0.43^{bD}	13.45 ± 1.41^{ab}
Range (min – max)	6.27 – 8.67	7.10 – 9.99	8.51 – 12.75	8.66 – 23.13

UHT – milk processed with ultrahigh temperature (135 °C, 3 seconds); Past. – pasteurized milk; P4 – progesterone; a, b – values indicated with the same letters do not differ significantly; C, D – values indicated with different letters differ significantly ($P < 0.05$)

E2 and P4 concentrations varied only minimally among the 10 samples of each of the four different types of milk examined (data not shown).

Both E2 and P4 concentrations showed significant correlation with milk fat contents. Correlation coefficients were 0.87 ($P < 0.01$) and 0.91 ($P < 0.01$) for E2 and P4, respectively.

DISCUSSION

There are no consistent data about concentrations of estrogens or P4 in commercial cows' milk at consumer level, and concentrations reported in different studies are very variable. Furthermore, it is not clear from some studies whether reported concentrations represent total estrogens (free plus conjugated form) or only free hormones, and in most studies, the number of milk samples tested is relatively low. Therefore, we tried to optimize the current study by examining a sufficient number of commercial milks with different fat contents. These results, thus, provided relevant and consistent E1, E2 and P4 concentrations in commercial, processed milk.

In the present study, concentrations of E1 in all UHT 0.5 and UHT 1.5 milks were below the LOD (15 pg mL^{-1}). Only in two out of 10 milks (UHT 3.5 and pasteurized 3.5 milk) were the E1 levels higher than the LOD. These results are somewhat in accordance with the study by Farlow et al. (2012), which reported E1 in commercial milk at a concentration of 14.45 pg mL^{-1}. Similarly, Malekinejad et al. (2006) and Ganmaa et al. (2001) reported low E1 milk concentrations (20 pg mL^{-1} and 21 – 26 pg mL^{-1}, respectively). However, Zhou et al. (2010) found 150 pg mL^{-1} of E1 in commercial milk. It is difficult to directly compare results of these studies, as milk was processed in different manners, milk fat content was not always documented, E1 was measured with different analytical methods, and in some studies there is no information about validation of the methods. Some reports suggest that concentrations of several steroid hormones, including E1, correlate with milk fat content (Pape-Zambito et al., 2008; Malekinejad et al., 2006). In our study, E1 concentrations above LOD (15 pg mL^{-1}) were found only in 3.5 % milk, which is somewhat in line with these studies.

Contrary to E1, E2 was detected in all milks, regardless of milk fat content. However, E2 concentration in UHT 3.5 milk was significantly higher ($P < 0.01$) than in UHT 0.5 milk (Table 2). This suggests that E2 concentration depends on milk fat content, which is not surprising, considering that steroid hormones are lipid molecules, soluble in fats. This is further supported by the correlation test, which showed significant positive correlation ($P < 0.01$) between E2 concentration and milk fat content. Similarly to this, Pape-Zambito et al. (2008) and Malekinejad et al. (2006) also reported a positive correlation between steroids and milk fat content. Several studies reported various concentration of E2 in milk such as 5 – 6 pg mL^{-1} (Goyon et al., 2017; Farlow et al., 2012) and 10 – 20 pg mL^{-1} (Malekinejad et al., 2006), levels similar to our results reported in this study, while Zhou et al. (2010) and Ganmaa et al. (2001) reported higher E2 concentrations of 25 – 33 pg mL^{-1} and 34 pg mL^{-1}, respectively. Since the E2 concentrations in our commercial milks was higher than 5.6 pg mL^{-1}, as was reported for milk of non-pregnant cows (Malekinejad et al., 2006), this most likely suggests that the majority of commercial milk we examined originated from pregnant cows. This was expected, since it was estimated that 75 % of milk in commercial dairy products originates from pregnant cows (Ganmaa et al., 2004). Interestingly, there we found no significant difference in E2 concentration between UHT 3.5 and pasteurized 3.5

milks, suggesting that the thermal processing procedure does not influence milk E2 concentration.

Statistical analysis of P4 levels produced similar results as we determined for E2 (see above). The mean P4 concentration in UHT 3.5 milk was significantly higher ($P < 0.05$) than in UHT 0.5 milk (Table 3). Other studies reported milk P4 levels comparable to those reported in the present study (Goyon et al., 2016; Snoj, 1997). In cows, milk P4 levels fluctuate during the estrus cycle and pregnancy. The lowest P4 concentrations were detected before and during ovulation (around 1 ng mL^{-1}), while the highest levels (more than 25 ng mL^{-1}) were found in metestrus and during pregnancy (Snoj, 1997). As reviewed by Malekinejad and Razabakhsh (2015), P4 levels in commercial milk and dairy products depend on fat content. The same authors reported that in commercial whole milk, the P4 level was 12 ng mL^{-1}, which is in accordance with the results of our study (10.76 and 13.45 ng mL^{-1} in UHT and pasteurized 3.5 milk, respectively). As described previously (Malekinejad and Razabakhsh, 2015), it is evident that, similar to other steroid hormones, concentrations of P4, a non-polar compound, also correlated with milk fat content, since significantly higher P4 levels were found in UHT 3.5 milk than in UHT 0.5 milk. Since the P4 concentration we determined in all types of milk was higher than was previously described for milk from non-pregnant cows (Snoj, 1997), this confirms the suggestion that the majority of the commercial milk we examined originated from pregnant cows.

Due to the technology and management of milk production, the proportion of non-pregnant and pregnant cows which are included in milking is more or less the same, since cows are initially inseminated approximately 60 days after calving and are milked until the 7th or 8th month of pregnancy. Therefore, in commercial milk which is produced from raw milk from different farms, negligible differences in milk estrogens and P4 levels are expected between different batches of commercial milk. As expected, the results of this study showed that E2 and P4 concentrations varied minimally between samples in each milk group. We believe that the main advantages of the present study were that milks were systematically obtained from one manufacturer and that the milks were sorted by fat content, which is considered as a dependent variable. Thus, standardization of sampling the milks was optimally performed, which is very important for the relevance of the results obtained.

CONCLUSION

In conclusion, in commercial milks grouped by their different fat contents, variations were determined in levels of estrogens and P4. Mean E2 and P4 levels were strongly correlated with fat content of the milk. Different thermal processing procedures did not influence milk estrogen or P4 concentrations. Considering information from the literature, it might be concluded that estrogen and P4 levels determined in this study were relatively high, which is characteristic for milk from pregnant cows. However,

these hormone levels were still below the levels that might cause estrogenic effects in mammals.

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Authors' contributions

TS: study design together with GM, SK, MŽ and NČK; samples collection together with SK, MŽ and NČK; laboratory analyses together with NČK; preparing the manuscript together with GM; statistical analysis; interpretation of the results together with GM, SK, MŽ and NČK.

GM: study design together with TS, SK, MŽ and NČK; preparing the manuscript together with TS; interpretation of the results together with TS, SK, MŽ and NČK.

SK: study design together with TS, GM, MŽ and NČK; samples collection together with TS and NČK; interpretation of the results together with TS, GM, MŽ and NČK.

MŽ: study design together with TS, GM, SK and NČK; samples collection together with TS, SK and NČK; interpretation of the results together with TS, GM, SK and NČK.

NČK: study design together with TS, GM, MŽ and SK; samples collection together with TS, SK and MCŽ; laboratory analyses together with TS; interpretation of the results together with TS, GM, SK and MŽ.

Declaration of conflicting interests

Hereby we confirm that we have no competing interest.

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KONCENTRACIJE ESTRONA, 17β-ESTRADIOLA I PROGESTERONA U TERMIČKI OBRAĐENOM MLEKU SA RAZLIČITIM SADRŽAJEM MASTI

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Kratak sadržaj

Uvod. Cilj ove studije je bio da se utvrde koncentracije estrona (E1), 17β-estradiola (E2) i progesterona (P4) u prerađenom mleku sa različitim sadržajem masti i da se uporede

koncentracije ovih hormona u mleku obrađenom ultravisokom temperaturom (UHT) i pasterizovanom mleku.

Materijal i metode. Konzumno mleko sa različitim sadržajem masti UHT 0.5 %, UHT 1.5 %, UHT 3.5 % kao i pasterizovano 3.5 % (po 10 uzoraka od svake vrste mleka) kupljeno je u lokalnim prodavnicama. Koncentracije E1, E2 i P4 utvrđene su komercijalnim ELISA setovima.

Rezultati i zaključak. Koncentracija E1 bila je ispod granice detekcije (15 pg mL-1) u svim uzorcima izuzev kod dva (UHT 3.5 % i pasterizovanog 3.5 %) uzorka mleka. Koncentracije E2 i P4 kod UHT 3.5 % mleka (25.37±1.15 pg mL-1 i 10.76±0.43 ng mL-1) bile su značajno veće nego kod UHT 0.5 % mleka (19.38 ± 0.79 pg mL-1 i 7.06 ± 0.26 ng mL-1). Značajna pozitivna korelacija utvrđena je između koncentracije hormona i sadržaja masti u mleku. Relativno visoke koncentracije E2 i P4 ukazuju da najveći deo konzumenog mleka potiče od steonih krava.

Ključne reči: estron, 17β-estradiol, progesteron, konzumno mleko