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Determination of the levels of 17-β Estradiol and Progesterone in Cow milk and Baby Follow-on milk by ELISA

Determinación de los niveles de 17-β estradiol y progesterona en leche de vaca y leche de continuación para bebés mediante ELISA

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ABSTRACT

The current study was undertaken to determine the 17-β Estradiol (E2) and Progesterone (P4) levels in different milk products and some baby follow-on milk samples collected from retail markets in Türkiye. For this purpose, a total of 50 samples from 8 different product groups with different fat levels, production technologies, and production series were analyzed for natural P4 and E2 levels using a commercial ELISA kit. The highest E2 level was determined in UHT whole milk (3%) (39.20 ± 6.73 pmol·L^{-1}) while the lowest level was found in buttermilk (26.50 ± 1.85 pmol·L^{-1}) samples. For P4 levels, the highest concentration were found in baby follow-on milk (<1 year old) (45.83 ± 2.95 nmol·L^{-1}) and the lowest values were found in pasteurized milk samples (23.00 ± 6.66 nmol·L^{-1}). Also, in this study, differences among the product groups for E2 and P4 were not found statistically (*P*>0.05). In conclusion, the natural P4 and E2 were detected in all milk and baby follow-on milks at various levels and generally their levels associated with the product fat level.

Key words: Progesterone; estradiol 17-β; milk, baby follow-on milk; ELISA

RESUMEN

El estudio actual se llevó a cabo para determinar los niveles de 17-β estradiol (E2) y progesterona (P4) en diferentes productos lácteos y en algunas muestras de leche de seguimiento para bebés recolectadas en mercados minoristas de Turquía. Para ello, se analizaron un total de 50 muestras de 8 grupos de productos diferentes con diferentes niveles de grasa, tecnologías de producción y series de producción para determinar los niveles naturales de P4 y E2 utilizando un kit ELISA comercial. El nivel más alto de E2 se determinó en leche entera UHT (3%) (39,20 ± 6,73 pmol·L^{-1}), mientras que el nivel más bajo se encontró en muestras de suero de leche (26,50 ± 1,85 pmol·L^{-1}). Para los niveles de P4, la concentración más alta se encontró en leche de continuación para bebés (<1 año) (45,83 ± 2,95 nmol·L^{-1}) y los valores más bajos se encontraron en muestras de leche pasteurizada (23,00 ± 6,66 nmol·L^{-1}). Además, en este estudio, no se encontraron estadísticamente diferencias entre los grupos de productos para E2 y P4 (*P*>0.05). En conclusión, los niveles naturales de P4 y E2 se detectaron en todas las leches y leches de continuación para bebés en varios niveles y, en general, sus niveles se asociaron con el nivel de grasa del producto.

Palabras clave: Progesterona; estradiol 17-β; leche; leche de continuación para bebés; ELISA

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INTRODUCTION

Cow milk is a food that looks simple but quite a complex food in composition. Some of the chemicals that it contains are hormones. The presence of 18 different hormones has been identified in cow milk thus far. Hormones such as Progesterone (P4), α and β Estradiol, Estrone, α and β Testosterone, Dehydroepiandrosterone and Androstenedione can be given as examples for steroid hormones [12]. The steroid hormones, P4, and Estradiol (E2) are quite stable and preserve their structure during the processing stages of milk. When consumed, they are absorbed and enter to the blood stream of the consumer and may affect their cells. However, hormones in protein structure can lose their function through denaturation by pasteurization, sterilization, or other processing methods [38]. Hormones like Parathyroid hormone are reported to be found in similar rates in fresh and pasteurized milk. The effects of pasteurization and Ultra High Temperature (UHT) techniques were variable on hormones depending on their structure [25].

The most prevalent source of endogenous steroid hormones are pregnant cows. In an economically sustainable dairy farming, it is recommended that the cows become pregnant at the postpartum 2nd and 3rd months and continue producing milk for human consumption until 2 months before delivery. This means that the milk is obtained for approximately 7 months during the cows' pregnancy. This is the conventional type of modern dairy cow farming in the world. Progesterone, known as the pregnancy – hormone, found in the plasma of cows, and passes into milk at levels of 2–33 nanograms·microliters^{-1} (ng·µL^{-1}) (3.3–42.27 nanomol·liters^{-1} (nmol·L^{-1})) [16]. Its level decreases 1–2 weeks (wk) before delivery. During this time, colostrogenesis, production of colostrum, occurs. The level of estrogen hormone begins to increase at the second trimester of pregnancy or at 4–5 months of pregnancy and prepares the genital organs for delivery. Consequently, during 10–months of lactation, the cow milk includes high levels of P4 during 7 months and high levels of both P4 and estrogen hormones during 4 months. Pape–Zambito *et al*. [35] reported that 17β–estradiol (E2) levels in the milk samples taken from 1st, 2nd, and 3rd third from the same cows were 5.4 ng·mL^{-1} (6.92 nmol·L^{-1}), 16.2 ng·mL^{-1} (20.75 nmol·L^{-1}), and 39.4 ng·mL^{-1} (50.47 nmol·L^{-1}), respectively.

P4 is secreted from the corpus luteum (CL), placenta and the adrenal glands. The blood concentration varies depending on species, pregnancy, sexual cycle, and various disorders [26, 49]. Regal *et al*. [39] determined the steroid hormone levels in raw milk obtained from pregnant and non-pregnant animals. While the level of P4 was 2.86 nmol·L^{-1} in pregnant ones, level of P4 was reported to be 0.31 nmol·L^{-1} in the milk of non-pregnant cows. It has been reported that the amount of steroids such as P4, which has lipophilic properties, varies according to the fat level of milk and dairy products [21, 29].

A limited number of studies is available investigating the effects of steroid hormones of milk origin. The available studies are mostly about E2. The reason for this is that E2 plays a role in hormone-dependent cancers, mainly breast cancer. E2 is in the list of Group 1 carcinogens prepared by the International Agency for Research on Cancer (WHO-IARC).

Concerns about the effects of steroid hormones in milk are divided into 2 groups as cancer and reproductive system-related. It has been reported that increased consumption of animal-derived food may have adverse effects on the development of hormone-dependent cancer in humans. It has been claimed that drinking the milk of pregnant cows, known to contain significantly high P4 levels, or eating products derived from this milk may increase the hormone-related health risks [18]. Farlow *et al*. [13] reported that the risk of ovarian cancer in women consuming more than 4 glasses of milk or equivalent dairy products per day was 100% higher than those who consumed 2 glasses. Gao *et al*. [19], on the other hand, reported that consumption of 3 glasses per day (600 mL milk = yogurt, = 130 grams (g) cheese, 15 g = butter) increased the risk of prostate cancer in men by 39%.

There are data available reporting that early puberty, acne and especially breast cancer can be seen in humans who consume foods obtained from synthetic or natural P4-treated animals, and that the rate of such diseases may increase in men [2, 5, 13, 27, 28, 32]. However, there are also publications reporting that P4-containing milk does not cause such effects [41]. The possible side effects on human health have recently been mentioned, especially since milk and dairy products obtained from pregnant cows contain high natural P4.

In the study of Maruyama *et al*. [31] men, women and children were given 600 mL of milk from cows known to be at an advanced stage of pregnancy, and urine and blood samples were obtained at 15, 30, 45, 60, 90 and 120 minutes (min) and it was observed that the estrone and P4 levels increased and the luteinizing hormone (LH), follicle stimulating hormone (FSH) and Testosterone concentrations decreased in males. In the same study, it was revealed that the Estrone, E2, Estriol and Pregnandiol concentrations increased in the urine samples taken from men, women, and children.

Aksglaede *et al*. [4] stated that due to the fact that the amount of endogenous steroid hormone in children is very low, children may be very sensitive even to steroids taken with food. As a result, it has been reported that girls have an increased risk of early puberty and developing breast cancer at an advanced age, and that boys have a risk of a decrease in Sertoli cells and semen quality in advanced age as a result of FSH suppression, especially by E2, in addition to a risk of diabetes and obesity in all children.

In a study conducted to determine the daily milk and dairy products consumption habits of Iranian people and the levels of steroid hormones they take into their bodies using the ELISA method, the mean E2 and P4 levels in milk samples were reported to be 330.5±190.2 picograms·mL^{-1} (pg·mL^{-1}) (1.953±1.12 nmol·L^{-1}) and 3.57±2.47 ng·mL^{-1} (0.12±0.09 nmol·L^{-1}), respectively. However, it was stated that milk consumption was within the reported range (175–240 mL·day^{-1}), and the content of steroid hormones detected in milk samples could be considered safe in children and adults. Due to the effects of steroid hormones, especially E2, regular monitoring of these hormones in milk and dairy products has been recommended in Iran due to its effect in the etiology of various cancers [33].

Some researchers reported their concerns about that E2 exposure could lead to health risks due to the facts that most of the dairy products are made from cow milk and E2 is a quite stable molecule. In addition, compared to the past, milk, baby follow-on milks and dairy food consumption is higher today. Therefore, it is important to carry out surveys for the levels of steroid hormones in dairy products [33]. In the light of the information provided above, the objective of the present study was to determine the natural E2 and P4 hormone levels in milk and infant follow-up milk samples in Türkiye.

MATERIALS AND METHODS

Sample collection

In this study, a total of 50 samples of cow's milk (semi-skimmed UHT milk), 3% fat UHT milk, organic UHT milk, pasteurized milk, lactose-free

UHT milk, buttermilk) and baby follow-on milk (baby follow-on milk (under 1 year old), infant follow-on milk (1 year old)), with different fat levels and properties belonging to 8 product groups, from 2 different production series produced by various companies in Türkiye and collected from retail points were used. Information on the samples collected within the scope of the research has been presented in TABLE I.

The research was approved by Fırat University Animal Experiments Local Ethics Committee (case number: 2017/02-20).

TABLE I
Sample information of the milk and baby follow-on milk analyzed in the current study

No	Dairy Foods	Numbers of commercial brands	Numbers of samples collected
1	UHT Half-Fat Milk (1.5%)	3	6
2	UHT Whole Milk (3%)	3	6
3	UHT Organic Milk	2	4
4	Pasteurized Milk	3	6
5	UHT Lactose-free milk	3	6
6	Buttermilk	3	6
7	Infant Follow-On Milk (>1 year old)	5	10
8	Baby Follow-On Milk (<1 year old)	3	6
Total		25	50

Sample preparation

The collected samples were stored in deep freeze (Bosch, GSD2111/01 FD6811, Robert Bosch Hausgeröte, Germany) at -18°C until the time of analysis. When using liquid products such as milk and buttermilk after they are being thawed and diluted with a standard diluent at a ratio of 1/10. Then, the resulting suspension was centrifugated (Hettich Rotofix 32A, Germany) at 1008 Relative Centrifugal Force (RCF) or G-force for 10 min abd the supernatant was removed.

The preparation of samples of baby follow-on milks for ELISA was carried out according to the manual containing sample preparation information for ELISA in solid samples (samples preparation procedure for ELISA of SunRed).

ELISA

Natural P4 and E2 levels in the collected samples were determined in the ELISA reader (Bio Tek Instruments, USA) at 450 nanometers (nm) wavelength using the commercial ELISA kit. In the analysis, a plate (plate of 96 samples for each hormone) produced and verified by the relevant company in accordance with the standards for P4 and estrogen analysis was used (Sundred E2 kit catalogue no: 201–12–1009, progesterone kit catalogue no: 20112–1008 Shanghai, China) [34, 44]. Hormone levels in milk and milk products were analyzed according to the procedures by the kit manufacturer (SunRed).

Statistical analyses

In the study, first, the descriptive statistics of natural E2 and P4 hormones were calculated in the product groups. The Kruskall Wallis analysis of variance was then used for comparisons between product groups in terms of natural estradiol E2 and P4 hormone levels. The Mann Whitney U test was then used in the follow-up for the parameters that were found to be important as a result of these analyses [3, 10, 46]. These analyses were carried out using the SPSS program [45].

RESULTS AND DISCUSSION

The descriptive statistics of natural E2 and P4 hormone levels in cow's milk and infant follow-on milk have been presented in TABLE II.

The highest concentration for natural E2 hormone levels in the product groups was determined as 39.20 ± 6.73 pmol·L^{-1} in UHT Whole Milk (3%) and the lowest value was found as 26.50 ± 1.85 pmol·L^{-1} in buttermilk (TABLE II).

The highest concentration for P4 levels in the product groups were found in Baby Follow-On Milk (<1 year old) (45.83 ± 2.95 pmol·L^{-1}) and the lowest value were found in Pasteurized Milk (23.00 ± 6.66 nmol·L^{-1}) (TABLE II). Also, in this study, differences among the product groups for E2 and P4 were not found statistically (*P*>0.05) (TABLE II).

TABLE II
Natural E2 and P4 levels in milk and infant follow-on milk

Dairy Foods	n	E2 (pmol·L^{-1})		P4 (nmol·L^{-1})	
		X̄ ± SEM	Median	X̄ ± SEM	Median
UHT Half-Fat Milk(1.5%)	6	36.33 ± 6.12	38.00	25.00 ± 10.28	20.00
UHT Whole Milk (3%)	6	39.20 ± 6.73	37.00	40.80 ± 7.45	50.00
UHT Organic Milk	4	31.67 ± 2.73	30.00	27.00 ± 6.11	31.00
Pasteurized Milk	6	29.50 ± 2.87	27.00	23.00 ± 6.66	19.00
UHT Lactose-free milk	6	37.40 ± 4.85	38.00	37.17 ± 4.89	38.50
Buttermilk	6	26.50 ± 1.85	25.00	24.20 ± 4.51	20.00
Infant Follow-On Milk (>1 year old)	10	32.19 ± 2.29	33.50	39.57 ± 4.52	42.00
Baby Follow-On Milk (<1 year old)	6	32.00 ± 4.62	32.00	45.83 ± 2.95	49.00
		ns		ns	

SEM: Standard error of the mean. ns: Not significant (*P*>0.05)

Milk and dairy products have an important role in human nutrition. Worldwide, cow-derived milk or dairy products are consumed more frequently than milk or dairy products derived from other species. Although there are many studies on the components of cow's milk, the steroid hormones in dairy foods have not been much emphasized. However, there are previous publications stating that the steroid hormones carried by milk and dairy products from cows may have negative effects on human health [14]. There are data showing that steroid hormones found naturally in milk and dairy products have effects ranging from accelerating body growth [6] to carcinogenic properties [47] in humans and animals. There are studies demonstrating that there is a very strong relationship between high consumption of milk and dairy products and a high incidence of testicular and prostate cancer [17].

Concentration of P4 and E2 in milk and milk products were determined in several studies using different analysis methods. These; in a study, the P4 levels in skimmed milk, buttermilk, skimmed milk powder, and regular milk were reported to be 1.4, 6, 17, and 12 $ng \cdot mL^{-1}$ or $ng \cdot g^{-1}$ (1.79, 7.69, 21.77, and 15.37 $nmol \cdot L^{-1}$), respectively [23]. In another study, the P4 level in milk was determined as 9.81 $ng \cdot mL^{-1}$ or $ng \cdot g^{-1}$ (12.57 $nmol \cdot L^{-1}$) [21]. Also, Snoj *et al.* [42] found that mean P4 concentration in UHT 3.5% milk (10.76 ± 0.43 $ng \cdot mL^{-1}$) were significantly higher than in UHT 0.5% milk (7.06 ± 0.26 $ng \cdot mL^{-1}$). In addition, previous studies have shown that P4 is also significantly associated with the percentage of fat in milk ($r = 0.98$) [20]. However, the relationship between milk fat percentage and P4 is less than that of E2.

Previous studies report that approximately 60-80% of estrogens in human diets come from milk and dairy products [40]. In a study, the E2 level in milk was determined as 0.02 $ng \cdot mL^{-1}$ [21]. The distribution of estrogens, especially E2, in the fat or fat-free portions of milk is still controversial. Studies have shown that there is no difference in E2 concentrations in whole milk or composite skimmed milk [1, 30]. E2 concentrations in milk correlate with the percentage of milk fat and are found in higher concentrations in the fat fraction of milk [43, 50]. The level of E2, which is related to the fat ratio of milk, comes out as a result of the removal of most of the fat during production, causing the E2 concentrations measured in skimmed milk to differ from estradiol in whole milk. Pape-Zambito *et al.* [36] reported that E2 concentration in raw whole milk averaged 1.4 ± 0.2 $pg \cdot mL^{-1}$ of milk (mean ± SEM) and ranged from non detectable to 22.9 $pg \cdot mL^{-1}$ of milk. Snoj *et al.* [42] found that mean E2 concentration in UHT 3.5% milk (25.37 ± 1.15 $pg \cdot mL^{-1}$) were significantly higher than in UHT 0.5% milk (19.38 ± 0.79 $pg \cdot mL^{-1}$). In the same study, it was suggested that there were significant positive correlations between hormone concentrations and milk fat content, and that high E2 and P4 concentrations indicated that most of the milk in the examined commercial milks came from pregnant cows.

Generally, the P4 and E2 levels found in this study are higher than those reported by Vicini *et al.* [48] and Pape-Zambito *et al.* [37]. Potential explanations for these differences may cover the differences in analysis methods and limited sample size used in the current study as well as biological factors such as pregnancy period, sexual cycle period, genetics, mastitis, the animal's diet, and the presence of transport proteins. For this reason, it has been suggested that the concentration of E2 in milk and dairy products varies and is not well defined [36].

Judging the level of steroid hormones in food whether it is safe to consume is complicated. To the present knowledge, there is no toxic or harmful level, such as Maximum Residue Limits (MRL) for the steroid hormones determined for any type of food. However, there are the acceptable daily intake (ADI) level, Non-observed effect level (NOEL), and lowest-observed effect level established by the Joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) Expert Committee on Food Additives (JECFA) [24]. Among those, NOEL is particularly worthy because it is accepted as the maximum allowed daily intake with no harmful effect for humans. NOEL values for adults are 3.3 mg· kilograms $(kg)^{-1}$ bw per day for P4, and 5 micrograms (μg)·kg^{-1} bw per day for βE2 [24]. However, Food and Drug Administration (FDA) exhibited a different approach on evaluating the risk from steroid hormones [15]. Simply, daily endogenous production of P4, Testosterone, and βE2 were calculated and a permitted increase exposure has been established, which is 1% of the daily production. For instance, daily production and permitted increase exposure were reported as 150 μg and 15 μg for P4 and 6 μg and 0,06 μg for βE2. According to FDA, it can be said that total daily intake of these steroid hormones should not exceed the permitted increase exposure, which are too low compared to NOEL values of JECFA [24]. This inconsistency in the information may make interpreting the results of steroid hormone levels determined in foods difficult.

Nili-Ahmadabadi *et al.* [33] carried out a health risk assessment using the data they obtained on the estimated daily steroid hormone intake, average body weight of child and adult consumers, the amount of milk consumed and the level of steroid hormones in milk with the help of a formula. As a result, it was stated that the levels of steroid hormones taken with milk and dairy products were close to the levels found in the human body. However, in the study, it was reported that due to the hormone levels of some products, attention should be paid to their intense consumption.

In the present study, the highest level of E2 was found in UHT Whole Milk (3%) (39.20 ± 6.73 $pmol \cdot L^{-1}$) and the P4 level was found to be highest in Baby Follow-On Milk (<1 year old) (45.83 ± 2.95 $nmol \cdot L^{-1}$) (TABLE II).

It was concluded that this situation was due to the fact that the levels of estradiol and P4 in milk and baby Follow-On Milks changed depending on different factors other than the fat ratio. In generally, according to the results of this study, the hypothesis that P4 and estrogen can be expected in higher frequency and intensity in high-fat dairy products due to their lipophilic properties has been proven. However, the differences between the product groups for E2 concentrations were not significant ($P>0.05$).

Although the ELISA method is a successful method in the analysis of steroid hormones in blood plasma, its use in steroid hormone analysis in foods is very rare. No study has been found on the determination of steroid hormones by ELISA method in dairy products, except milk samples. In this study, ELISA method was used for the first time in buttermilk, and Baby Follow-On Milks not milk only, for this purpose. It is also reported that the ELISA method is useful as it is inexpensive compared to the advanced methods, does not require a well-equipped laboratory, yields fast results, and can analyze more samples at the same time [22]. However, confirmation of results with more advanced analysis methods could have been better in terms of the reliability of the results.

Another issue that should be discussed in the current study is the sample size. It would have been definitely more appropriate to have a higher sample numbers than it was in the current study. Although an increased sample size increases the power of the population proportionally, the increased sample size also increases the time spent for the research and costs [10, 46]. The most proper sample size varies according to the objectives of the study and limiting factors (time, cost, number of staff, and others). The sample size used in this study is considered as a short coming of the study.

Infant formulas have special functions in infant diets as they are the main and unique source of nutrients during the first months of life. These products are within the scope of Commission Directive 2006/141/EC, in particular, as amended by the Commission Directive 2013/46/EU [11]. Dairy preparations for infants and children are recognized as an important source of natural hormones, despite available information on their concentration in these products being very limited [9, 51]. Hence, it is proposed that more of these hormonal compounds should be investigated in different products in order to make an estimation of their consumption and make a comparison with their level in breast milk [7]. Barreiro *et al*. [8] reported that there was no reference level or legal limit for hormones in infant formulas, that there is no obligation to declare hormone values on product labels, and accordingly, no legal conclusions could be drawn in terms of food safety.

In the presented study, the concentrations of E2 and P4 in the follow-on milk of infants over 1 year of age were 32.19 ± 2.29 (pmol·L^{-1}) and 39.57 ± 4.52 (nmol·L^{-1}), respectively, and 32.00 ± 4.62 (pmol·L^{-1}) and 45.83 ± 2.95 (nmol·L^{-1}) in the follow-on milk of infants under 1-year-old and these rates were found to be within acceptable limits and not different from other milk and dairy products. Although the importance of these data for infant health and development is not fully known today, this study has once again confirmed that formulas are a source of steroid hormones.

The differences between the E2 and P4 levels in milk, buttermilk, and Baby Follow-On Milks between the current study and previous studies can be explained by multiple factors ranging from the differences in analysis methods and sample numbers to farm factors including nutrition and care conditions. Also, the breeds of the cows and lactation periods may cause by these different. The question of whether these levels of P4 and E2 may pose a health risk to the consumers does not have a simple answer and requires a risk assessment based on the exposure level.

CONCLUSIONS

In this study, it was confirmed by the ELISA method that P4 and E2 are naturally present at various levels in whole milk, buttermilk and baby follow-on milk, and It was also concluded that, in general, there appears to be a correlation between the concentrations of these hormones and the fat content of each product but may also be affected by other factors. Also, more has been determined that the levels of these hormones in the products in question are not at the levels that may be harmful to human health as stated in previous publications.

Conflict of Interest

The authors declare that they have no conflicts of interest in the research.

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