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Research Article

Steroid Hormone Exposure as a Potential Hazard in Milk Consumers: A Significant Health Challenge in Iran

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The presence of steroid hormones in milk is inevitable, and they can be considered as potential carcinogenic agents for consumers. The purpose of this study was to evaluate the level of 17β-estradiol, progesterone, and hydroxyprogesterone in sixty-nine bovine milk samples, collected from April to September 2020, in Hamadan, Iran. The milk samples were analyzed using enzyme-linked immunosorbent assay (ELISA). In this study, the mean contents of 17β-estradiol, progesterone, and hydroxyprogesterone in the milk samples were determined to be 330.5 ± 190.2 pg/ml, 3.57 ± 2.47 ng/ml, and 1.54 ± 0.41 ng/ml, respectively. However, the content of these steroid hormones in milk samples could be considered safe in children and adults, if the milk consumption is assumed to be in the reported range (175–240 ml/daily). Due to the effects of steroid hormones, especially 17β-estradiol, in the etiology of various cancers, regular monitoring of these hormones is recommended in milk and its dairy products in Iran.

1. Introduction

Milk is one of the most important sources of calcium and protein for the humans that plays the main role in the worldwide food pyramid [1, 2]. Based on the 2017 report of the United Nations food and agriculture organization (FAO), milk consumption per capita in the world is approximately 100 kg/years, which may be very variable in various regions [3]. Meanwhile, milk consumption per capita in Iran is lower than the consumption in the world and estimated to be 66.12 kg per year. According to the FAO's report, the average milk production in Iran has increased remarkably from 4 million tons in 1998 to 8.8 million tons in 2014 [4].

This food source is prone to a variety of environmental contaminants such as mycotoxins [5], pesticides [6], heavy metals [7], melamine [8], antibiotics, and different hormones [9, 10]. Among these, the presence of steroid hormones in milk and dairy products is inevitable and can be considered as a threatening factor in consumers [10, 11]. The part of milk hormones originates naturally from internal glands and, subsequently, the blood flow and are secreted in milk via diffusion [12]. In addition, steroid hormones are used to increase milk production and improve reproductive performance in animal husbandry [13]. Therefore, it is expected that milk is considered a rich source of endogenous and exogenous steroid hormones, whose content in the milk often exceeds that in the maternal blood plasma. The level of these lipophilic compounds depends on the fat level of the milk [14]. Food processing, such as churning and/or heating, does not appear to influence the amount of the steroid hormones [15].

The presence of steroid hormones in milk can be one of the most effective factors in the occurrence of hormonal disorders and their consequences [16]. For instance, Maruyama et al. [17] showed that men who consumed at least 600 mL/m^2 of the body surface area of cow's milk had higher contents of steroid hormones including estradiol, estrone, and progesterone in urine and serum. These men had declined serum levels of testosterone and pituitary gonadotropins about hours after drinking the milk [17]. In international comparisons, daily consumption of milk and dairy products is strongly associated with rates of various cancers, especially prostate and ovarian cancers [18, 19]. In addition, total milk and dairy consumption have been correlated with the occurrence of endometrial cancer, mostly among postmenopausal women who are not receiving hormone therapy, a finding probably associated to the steroid hormone content of dairy products [20].

Hamadan (the latitudes 33°33′ to 35′48′N and longitudes 47°45′ to 49′36′E), a region in western Iranthat, due to its geographical location and climatic status, provides a considerable part of milk for different regions of Iran. According to the estimates made from animal husbandries, approximately 80% of the milk produced comes from pregnant cows, which naturally has significant levels of steroid hormones [17]. There are also unofficial reports on the use of steroid medicines to increase milk production in Iranian animal husbandry. Given that 60–80% of steroid hormones enter the body through the consumption of milk and dairy products [14], it is very important to determine the appropriate amount of consumption of these products to prevent carcinogenic and destructive effects of these hormones. Therefore, in order to evaluate the risk of these hormones through milk consumption in consumers, the levels of three steroid hormones in Hamadan milk samples were measured by the enzyme-linked immunosorbent assay (ELISA) method, and its result was compared to FAO/WHO food standards.

2. Materials and Methods

2.1. Sampling. As shown in Figure 1, sixty-nine bovine milk samples (from four different zones) were randomly collected from Hamadan markets during the period of April to September 2020. All samples were kept at −20°C and analyzed as soon as possible.

2.2. Sample Preparation and Hormone Analysis. The sample preparation was down according to the method of Yang et al. [21] with some modifications. Briefly, 1 mL of the sample was transferred into a microtube. Then, 200 μL of acetate buffer (0.2 M, pH 5.2) was added and homogenized using a homogenizer for 1 min. For deconjugation of steroid hormones, 20 μL glucuronidase/arylsulfatase was added and incubated overnight at 37°C. Then, 0.5 ml of the sample was transferred into a microtube and 0.5 ml of methanol was added to precipitate milk proteins. Following 3 min of vortex shaking, the samples were centrifuged (at 25°C for 10 min/3500 g) and their supernatant was separated and cleaned using solid-phase extractions (SPE). The quantitative analysis of steroid hormones was carried out using competitive enzyme-linked immunosorbent assay (ELISA) kits (DiaMetra, Spello Perugia, Italy) according to the manufacturer's instructions.

2.3. Quality Assurance. Calibration curves for each steroid hormone were constructed using external standards, prepared by dilution of a stock solution in deionized water. Then, the limits of quantification were determined as (SD/S) × 10, where SD/S is the ratio of standard deviation/slope of the calibration curve [22]. Quality control samples were prepared by spiking milk samples with different concentrations of 17β-estradiol (25, 50 and 500 pg/ml), progesterone (5, 10 and 20 ng/ml), and hydroxyprogesterone (1.5, 3, and 6 ng/ml) standards. Following 1 min of vortex shaking, samples were homogenized in an ultrasonic water bath for 30 min. Finally, the ELISA technique was validated to ensure data quality using measurement of recoveries and the average variation coefficient for milk spiked with various concentrations of each hormone.

2.4. Health Risk Assessment. As shown in equation (1), the estimate daily intake (EDI) of steroid hormones was calculated using the average body weight of child and adult consumers (W_{bw}), the amount of milk consumed (F_{M}), and the level of steroid hormones in milk (C_{SH}).

$$\mathrm{EDI\ (ng/kgbw/day)} = \frac{\mathrm{FM\,(ml)} \times \mathrm{CSH\,(ng/ml)}}{W_{bw}\,\mathrm{(kg)}}. \quad (1)$$

According to the work of Abedi et al. [4], the average of milk consumption in children (4–10 years) and adults (18–50) in Iran was considered to be about 240 and 175 ml/day, respectively. In addition, the body weight for the child and adult consumers is equal 70 and 26 kg, respectively, based on the body weight studies by environmental protection agency [4, 23]. In addition, the hazard quotient (HQ) index was calculated based on the following equation:

$$\mathrm{HQ} = \frac{\mathrm{EDI}}{\mathrm{ADI}}. \quad (2)$$

3. Results and Discussion

The lactating race selection and improvement of the zootechnical situation in animal husbandry is ongoing, and therefore, the level of milk production per cow is much elevated [14]. This increase may be the outcome of alterations in the endocrinological system of the lactating cow which can affect the profile of steroid hormones in milk as a cancer risk factor [24]. Hence, the growing consumption of milk and dairy products has become a serious health challenge in recent years [25].

In the current study, the content of steroid hormone in milk samples from different regions of Hamadan was measured after validation of the ELISA procedure. As shown in Table 1, the r^2 indexes indicated an acceptable standard curve in the defined ranges. In addition, the results of recovery experiments and their relative standard deviation

Figure 1: Geographical locations of milk sampling sites in Hamadan city, Iran.

Table 1: Linearity range and quantification limits for steroid hormones analyses using the ELISA method.

Steroid hormones	Range	Linear equation†	r^2	Limit of quantification
17β-Estradiol (pg/ml)	20–2000 pg/ml	$Y = -4.0453X^2 + 0.6277X + 3.2752$	0.9948	8.68 pg/ml
Progesterone (ng/ml)	0.2–40 ng/ml	$Y = -1.1442X^2 + 0.0356X + 1.726$	0.9954	0.05 ng/ml
Hydroxyprogesterone (ng/ml)	0.2–20 ng/ml	$Y = -0.0322X^2 - 0.8332X + 1.4682$	0.9892	0.05 ng/ml

†The absorbance changes (at 450 nm) were plotted relative to the logarithmic concentrations of each hormone.

(RSD) values were within the acceptable ranges, showing good precision and accuracy of these analytical methods (Table 2).

The identification of steroid hormones in milk that have the potential to reproductive dysfunction has raised concern [10]. 17β-Estradiol is the main estrogen secreted by the premenopausal ovary. It is synthesized from testosterone primarily in the ovarian granulosa cells and placenta, but small levels can be produced in the adrenal gland [26]. In dairy cows, pregnancy is related to the faster reduction in milk production [27]. 17β-Estradiol can stimulate the growth of tumors and is associated with the occurrence of breast cancer [28]. In this study, 17β-estradiol was detected in all of the milk samples in the range 75.6–922.3 ng/L and its mean contamination level was determined as 330.5 ± 190.2 ng/L (Table 3). Several studies have reported 17β-estradiol levels in milk samples. For instance, Malkinejadet al. [14] reported 17β-estradiol levels in processed milk and raw milk at the range of 5.6 to 51 ng/L. Chen et al. [29] detected the level of the 17β-estradiol in commercial milk samples in the range of 0 to 70.12 ng/L. An important part of produced milk is obtained from pregnant cows; therefore, the content of the milk steroids depends on the different stages of the pregnancy. During pregnancy, the secretion of steroid hormones such as estrogens and progesterone increases [27]. Based on previous reports, the cumulative concentration of estrogens in the third trimester is about 27 times higher than that in the milk of cows in their first trimester of pregnancy [14]. In this regard, Japanese investigators have displayed that milk samples from a cow in the late stage of pregnancy contain up to 33 times as much estrone sulfate than milk from a nonpregnant cow [30]. Their findings could at least partially explain the high standard deviation in our data.

Progesterone is a steroid hormone generated by the cow during ovulation and could be assayed within milk samples [31]. This hormone rises and falls during various stages of the cow's reproductive cycle [32]. In the current study, progesterone was determined in all of the milk samples in the range of 0.69 to 12.1 ng/ml and its mean contamination level was detected 3.57 ± 2.47 ng/ml (Table 4). Previously, Ginther et al. [33] exhibited the range of milk progesterone contents between 15.1 and 26.2 μg/L during pregnancy [33]. In addition, Regal et al. [34] showed that the average contents of progesterone were 0.824 and 0.082 μg/L in raw milk from pregnant and nonpregnant cows, respectively [34]. In addition, in the study of Wu et al. [35], the average of progesterone level were reported about 0.5 ng/ml and 4.48 ng/ml in milk samples of nonpregnant and pregnant cows, respectively [35]. It seems that the stage of the pregnancy or estrous cycle can have a predominant role on milk progesterone level [36]. In this regard, there is an obvious relation between milk output and double ovulation, and it is now confirmed that this phenomenon could be related to the circulating progesterone levels near the time of follicle selection [37].

Hydroxyprogesterone is a biological progestin that is generated following steroid hormone synthesis and is raised during early pregnancy [38]. In this study, hydroxyprogesterone was measured in all of the milk samples in the range of 0.80to 2.61 ng/ml and its average contamination level was detected as 1.54 ± 0.41 ng/ml (Table 5). There is no evidence about the hazardous of hydroxyprogesterone in milk, but due to the role of this hormone in increasing the expression of progesterone receptors, a permissible limit should be considered for it.

In this study, the 17β-estradiol and progesterone daily intake in both age groups of children and adults was estimated in the Iranian population. The Joint FAO/WHO Expert Committee on Food Additives has reported an ADI

Table 2: Recoveries of steroid hormones in spiked milk samples.

Hormones	Hormone spiked	Hormone sample	Hormone found	Recovery (%)	RSD (%)†
17β-Estradiol (pg/ml)	25	198	197	88.3	5.4
	50	198	413	92.1	4.4
	500	198	659	94.4	4.8
Progesterone (ng/ml)	5	4.4	8.9	94.6	6.1
	10	4.4	14.1	97.9	5.5
	20	4.4	21.1	86.4	5.8
Hydroxyprogesterone (ng/ml)	1.5	0.13	1.5	93.7	4.6
	3	0.13	2.7	86.2	5.7
	6	0.13	5.6	91.3	3.9

†Relative standard deviation (RSD) was obtained from triplicate tests.

Table 3: Milk 17β-estradiol level and its daily intake in the different regions of Hamadan, Iran.

	17-β Estradiol (ng/L)			Daily intake (ng/kg body weight)			
				Child		Adult	
Zones	*n*	Mean ± SD	Min-max	Mean ± SD	Min-max	Mean ± SD	Min-max
Zone 1	19	318.0 ± 199.8	86.3–922.3	2.93 ± 1.84	0.79–8.51	0.79 ± 0.49	0.21–2.30
Zone 2	18	288.0 ± 182.5	75.6–681.7	2.65 ± 1.68	0.69–6.29	0.72 ± 0.45	0.18–1.70
Zone 3	16	443.4 ± 184.7	151.6–842.9	4.09 ± 1.70	1.39–7.78	1.10 ± 0.46	0.37–2.10
Zone 4	16	282.2 ± 133.0	93.8–503.0	2.60 ± 1.22	0.86–4.64	0.70 ± 0.33	0.23–1.25

Table 4: Milk progesterone level and its daily intake in the different regions of Hamadan, Iran.

	Progesterone (ng/ml)			Daily intake (ng/kg body weight)			
				Child		Adult	
Zones	n	Mean ± SD	Min-max	Mean ± SD	Min-max	Mean ± SD	Min-max
Zone 1	19	2.74 ± 1.85	1.26–7.81	25.30 ± 17.14	11.67–72.13	6.85 ± 4.64	3.16–19.53
Zone 2	18	3.69 ± 2.38	0.69–7.43	34.08 ± 22.02	6.45–68.62	9.23 ± 5.96	1.74–18.58
Zone 3	16	4.77 ± 3.12	1.38–12.0	44.04 ± 28.81	12.74–111.46	11.92 ± 7.80	3.45–30.18
Zone 4	16	3.10 ± 1.83	1.56–8.76	28.63 ± 16.90	14.40–80.93	7.75 ± 4.57	3.90–21.91

Table 5: Milk hydroxyprogesterone level and its daily intake in the different regions of Hamadan, Iran.

	Hydroxyprogesterone(ng/ml)			Daily intake (ng/kg body weight)			
				Child		Adult	
Zones	n	Mean ± SD	Min-max	Mean ± SD	Min-max	Mean ± SD	Min-max
Zone 1	19	1.48 ± 0.46	0.81–2.61	13.71 ± 4.30	7.54–24.12	3.71 ± 1.16	2.04–6.53
Zone 2	18	1.45 ± 0.45	0.80–1.22	13.45 ± 4.23	7.41–20.57	3.64 ± 1.14	2.00–5.57
Zone 3	16	1.72 ± 0.30	1.24–2.23	15.92 ± 2.78	11.50–20.66	4.31 ± 0.75	3.11–5.59
Zone 4	16	1.52 ± 0.33	0.90–1.94	14.11 ± 3.11	8.37–17.95	3.82 ± 0.84	2.26–4.86

Table 6: Calculation of HQ index based on the average of daily intake of steroid hormones by milk consumption in Hamadan, Iran.

Steroid hormones		Daily intake(ng/kg body weight)		Hazard quotient (HQ) index†	
		Mean ± SD	Min-max	Mean ± SD	Min-max
17β-Estradiol	Child	3.05 ± 1.75	0.69–8.51	0.06 ± 0.035	0.01–0.17
	Adult	0.82 ± 0.47	0.189–2.30	0.016 ± 0.009	0.003–0.046
Progesterone	Child	32.95 ± 22.8	6.36–111.69	<0.001	<0.001–0.003
	Adult	8.92 ± 6.17	1.72–30.25	<0.001	<0.001

†If the HQ value is less than 1, there is no risk through consumption of milk.

for 17β-estradiol and progesterone equal 50 ng/kgbw and 30 μg/kgbw, respectively [39], whereas the estimated exposure was calculated via dividing the daily intake of these hormones by their safe limits. As presented in Table 6, the results of this research show that the 17β-estradiol and progesterone contents in milk samples could be considered safe in children and adults, if the milk consumption is assumed to be in the defined range (175–240 ml/daily).

If the maximum contents of steroid hormones are considered in exposure assessment estimates, consuming more than 1.5 L/day of milk could put children at high risk for 17β-estradiol. Although consuming this amount of milk per day seems unlikely, due to the high consumption of dairy products in Iran, there is a possibility of exposure to significant amounts of 17β-estradiol.

4. Conclusions

Taken collectively, due to the fact that many dairy products are made from cow's milk and estradiol has a high stability in it, the risks of 17β-estradiol exposure through the consumption of milk and dairy products might be worrisome for consumers. In contrast, the risk of progesterone exposure through milk consumption seems very small and even unlikely. However, attention should be given to regular monitoring of steroid hormones in milk and its dairy products, due to the rising trend of consuming these products among the Iranian population.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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