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EVALUATION OF STEROID HORMONES ANABOLIC USE IN CATTLE IN CROATIA

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Abstract: Natural sex hormones are part of the endocrine system and are found in animal biological material. On analysis of residual substances with anabolic effect and detection of their abuse, it is necessary to know the physiological levels of these hormones to be able to differentiate physiological concentrations from the illegal use of anabolics. The hormone concentrations exceeding the physiological ones, found on monitoring for illegal substance use, would point to the abuse of these substances for anabolic purpose. In the present study, concentrations of the natural hormones 17β-estradiol, progesterone and testosterone were determined in bovine plasma according to animal age and sex. Natural hormone concentrations were determined using quantitative validated ELISA methods in plasma samples from cattle of different breed composition collected at several farms in Croatia during the 2006-2009 period. Methods validation showed good mean recovery and repeatability (approx. 75-87%), demonstrating the methods efficiency in determination of 17β-estradiol, progesterone and testosterone level in cattle plasma, respectively. The level of sex hormones was statistically significantly higher in yearling plasma as compared with calf plasma ($P<0.05$). The highest levels of 17β-estradiol (0.03±0.01 ng/mL) and progesterone (4.87±1.63 ng/mL) were recorded in female yearlings, and of testosterone (9.44±5.47 ng/mL) in male yearlings. Results showed the steroid hormone levels to vary with animal age and sex, indicated that illegal use of anabolic substances could not be suspected in none of the study animals.

Key words: steroid sex hormones, anabolics, physiological levels, plasma, bovine

Introduction

The sex hormones 17β-estradiol, progesterone and testosterone are steroid molecules involved in endocrine regulation of growth in humans and animals. They

are synthesized in sex glands and act through specific gene activation. Besides influencing the development of sex characteristics, testosterone also influences protein synthesis, 17β-estradiol has a major role in protein deposition, and progesterone exerts antagonistic action to estrogen hormones *(Griffin and Wilson, 1998; Meyer, 2001)*. These very properties provoke their illegal use in fattening animals for anabolic purpose.

The physiological levels of sex hormones in animal plasma vary according to animal species, categories, sex and age *(Heitzman, 1994)*. Their body concentration is influenced by sexual maturity of the animal, presence of hormone in the diet, and overall rearing conditions *(Scippo et al., 1993; Schilt et al., 1996)*. The occurrence of estrus and thus the level of sex hormones can be influenced by dozens of plants found in animal feed, i.e. by the estrogenic effect of their constituents such as isoflavones, resorcyclic acid lactones, coumestans, etc. *(Barnes, 2010)*. Considering all these factors, it appears quite difficult to definitely determine the standard physiological levels of particular sex hormones in each animal category.

Steroid sex hormones are part of the endocrine system and are found in physiological ranges in animal biologic material. Therefore, their mere presence in animal blood need not always be taken as a proof of illegal anabolic use. The physiological presence and variation of these hormones according to age, sex and many other factors make identification of abuse of these substance for anabolic purpose still problematic *(Le Bizec et al., 2009)*. On determination of these hormone concentrations in biologic material, all data of animals and history data should also be taken in consideration when assessing the hormone levels as physiological or associated with substance abuse.

In the past, estradiol, testosterone, progesterone and synthetic steroids were used as growth promoters in the form of various implants, tablets with estradiol or a combination of estradiol and testosterone *(Annon., 1998; Annon., 2005)*. However, ban has been placed on their use for anabolic purpose because of their adverse effects on human and animal health *(Council Directive 1996/22/EC; Council Directive 2003/74/EC; Stephany, 2010)*. Therapeutic use of these substances has also been restricted (disorders of reproduction and pregnancy), while the possible accumulation of their residues in animal products and adverse effects on human health are prevented by strictly professional drug administration *(Lone, 1997; FAO/WHO, 2000)*. Therapeutic administration of hormones and their effects on productivity have been investigated for years in numerous studies *(Kesler et al., 1981; El-Zarkouny and Stevenson, 2004; Colazo et al., 2007; Alnimer and Husein, 2007)*. In Croatia, the use of sex hormones is currently allowed in veterinary practice, exclusively for therapeutic purpose, in accordance with the Directive on Placing a Ban on the Use of Certain Beta-Agonists and Substances with Hormonal and Thyrostatic Effects on Farm Animals *(Official*

Gazette of the Republic of Croatia, 2008) and application should be properly documented.

The administration of sex hormones as anabolics to farm animals results in meat with a higher proportion of muscle tissue and lower proportion of adipose tissue, i.e. meat of better organoleptic properties *(Lone,1997; Deshpande, 2002)*. The anabolic effect is obtained through direct and indirect mechanisms of action resulting in enhanced nitrogen retention and increased protein synthesis, i.e. animal growth *(Van Der Wal and Berende, 1983; Meyer, 2001)*. The efficiency of animal growth promotion depends on the animal breed, age, reproductive status, and route of hormone administration *(Michel and Baulieu, 1980)*; a growth gain by up to 20% can thus be achieved *(Meyer, 2001)*. Of natural hormones, estrogens in the form of 17β-estradiol or estradiol-benzoate have been most widely used. Progesterone, testosterone and some synthetic hormones have generally been used in combination with estrogens *(Andersson and Skakkebaek, 1999)*. According to literature data, some other substances (insulin-like growth factors) exert a synergistic effect on gonadotropic hormones *(Lucy, 1999)*.

On the analysis of anabolic substance residues, the ability of demonstrating the presence of a particular substance in animal biologic material by the analytical method employed is used as a criterion for the respective substance abuse in meat production. Samples of the liver, kidney, fat and muscle at the slaughterhouse, and urine, feces and hair *(Cacciatore et al., 2009; Duffy et al., 2009; Divari et al., 2010)*, and serum *(Scalas et al., 2007)* in live animals are most frequently used to determine anabolic substance residues.

The aim of the present study was to determine the levels of sex hormones (17β-estradiol, progesterone and testosterone) in cattle plasma of various sex and age using validated ELISA methods, to get an insight into the hormonal levels that might indicate to the illegal use of steroid hormones on farm animals in this region.

Materials and Methods

Plasma samples. Natural hormone concentrations were determined in plasma samples from 40 male animals (20 calves and 20 yearlings) and 40 female animals (20 calves and 20 yearlings) collected at several farms in Croatia during the 2006-2009 period. Animal blood was sampled into EDTA tubes and centrifuged for 10 min at 2000 rpm. Upon complete plasma separation, it was transferred to tubes by a micropipette and stored at -20 ºC until analysis. Plasma samples were divided according to sex (male and female) and age (calves and yearlings) and the concentrations of 17β-estradiol, progesterone and testosterone were determined in all samples.

Sample purification. Plasma purification was done by the liquid-liquid extraction. Five mL of ether mixture (tertiary butylmethyl ether/petrol ether 30/70

v/v) were added to 1 mL of the sample and left in a shaker for 20 min at room temperature. Then the content was frozen at -25 ºC for 60 min, the ether supernatants were decanted and evaporized on a vacuum vaporizer at 60 ºC (Laborota 4001-Efficient, Heildolph). Hormone residues were then dissolved in 0.5 mL (progesterone and 17β-estradiol) or 1 mL (testosterone) of the buffer for sample dilution. The content was vigorously shaken for 1 min and warmed in water bath (Haake SWB25, Thermo) at 37 ºC for 5 min. The last step was repeated two more times. The supernatants were used on ELISA analysis.

Hormone analysis. Hormone concentrations were determined by use of the commercial ELISA kits (Immunolab GmbH, Kassel, Germany), according to the manufacturer's instructions. Standard solutions of 17β-estradiol, progesterone and testosterone (six concentration levels) and prepared solutions of plasma samples were instilled in the microwells. Then solutions of the dissolved enzyme conjugate (peroxidase), substrate (tetramethylbenzidine -TMB) and antibodies were added, with microplate incubation at room temperature in the dark. The microwells were washed in phases with the use of ELISA washer (ELx50, Bio-Tek Instruments, USA). The reaction was stopped by the addition of stop solution (0.5 M sulphuric acid) and absorbance was measured on an ELISA reader at a wavelength of 450 nm (ELx800TM, Bio-Tek Instruments, USA). Upon plotting the calibration curve, the plasma hormone concentration was determined by use of the R-Biopharm Ridasoft Win software. Results were expressed in ng/mL (ppb) taking the plasma dilution factors into account. In order to assess differences of hormone concentrations in plasma of different sex and age categories of cattle ANOVA was performed. When ANOVA assumptions were not meet, after testing for data normality and homoscedasticity by Shapiro-Wilk W test, non-parametric Spearman correlation was used. Statistical analyses were performed using Stata 10.0 (StataCopr. 2005. Stata Statistical Software: Release 10.0, College Station, TX). Statistical significance was tested at the level of $P<0.05$.

Results

Validation results of quantitative ELISA methods include determination of the recovery, repeatability and detection capability (CCβ) of the test methods. Values of the validation parameters on determination of 17β-estradiol, progesterone and testosterone by ELISA method in plasma of different animal categories are presented in Table 1. The mean hormone concentrations (mean ± SD) according to animal sex are shown in Table 2. Hormone values according to animal age and sex are graphically presented in Figures 1-3.

Table 1. Mean values of the validation parameters of ELISA methods

Analite	Validation parameter		
	Recovery (n=18)	Repeatability (n=54)	CCβ (n=20)
17β-estradiol	84.5 %	86.3 %	0.02 ng/mL
Progesterone	80.3 %	78.9 %	0.09 ng/mL
Testosterone	75.6 %	82.3 %	0.11 ng/mL

Table 2. Natural sex hormone concentrations in bovine plasma according to animal sex

Animal sex	Number of samples	Hormone concentrations (ng/mL)					
		17β-Estradiol		Testosterone		Progesterone	
		min	max	min	max	min	max
Male	40	0.025	0.038	1.283	16.502	0.076	0.145
Female	40	0.026	0.043	0.145	0.518	0.225	7.402

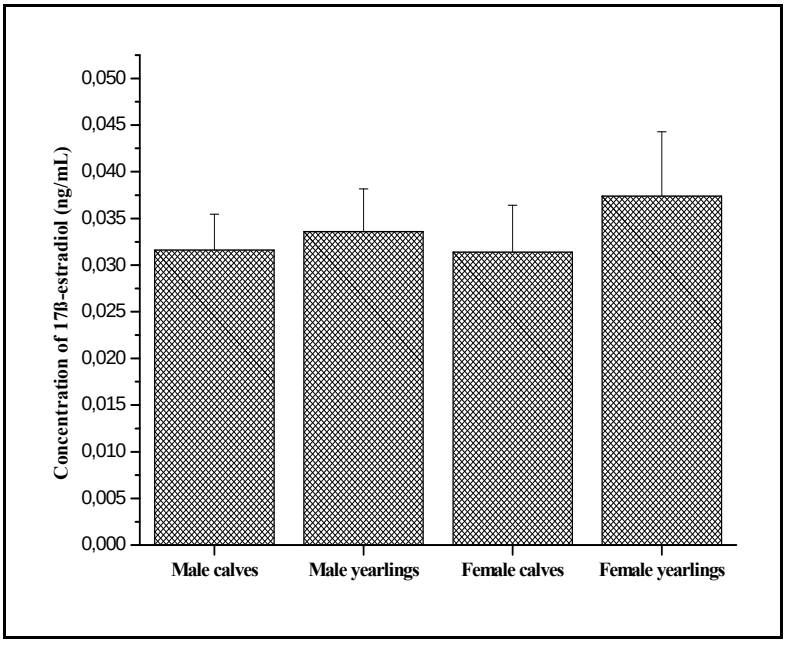

Figure 1. Concentration of 17β-estradiol (mean ± SD) in bovine plasma.

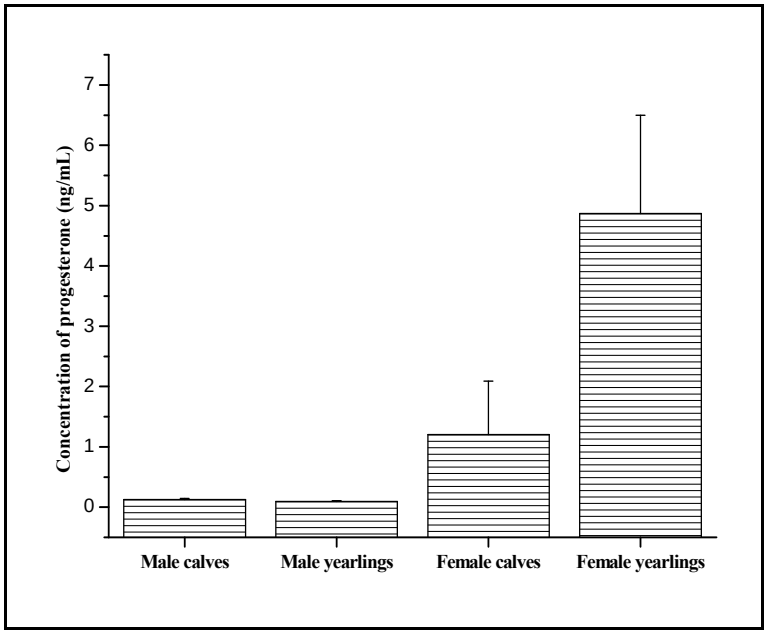

Figure 2. Concentration of progesterone (mean ± SD) in bovine plasma.

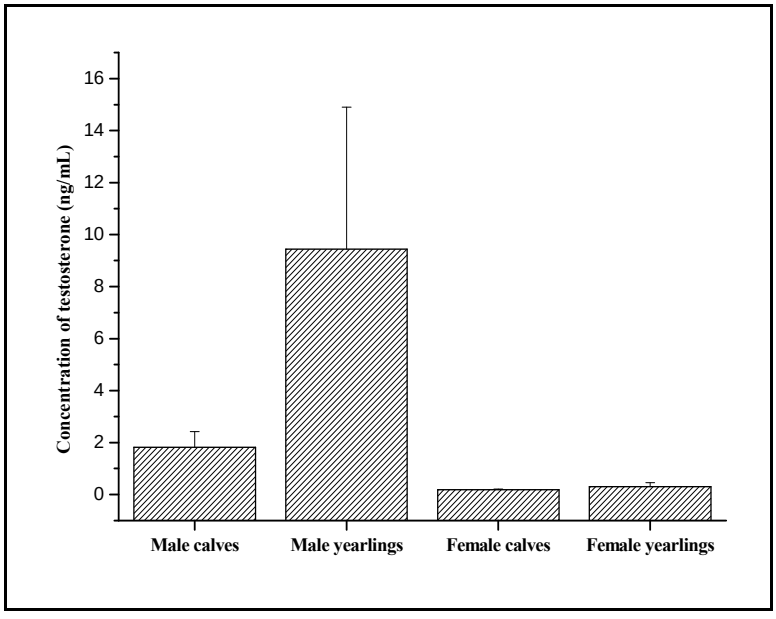

Figure 3. Concentration of testosterone (mean ± SD) in bovine plasma.

Results shown significant difference in testosterone and progesterone levels in plasma between male calves and male yearlings (P<0.05). In females, significant differences between calves and yearlings were found for progesterone levels (P<0.05). When comparing to animals sex of the same age categories (male calves with female calves and male yearlings with female yearlings) significantly differences were found in plasma testosterone and progesterone levels (P<0.05).

Discussion

Endogenous sex hormones are synthesized in the gonads, adrenal gland and placenta, and bind to protein plasma to be transported to target organs. By binding to specific receptors in particular tissues, these hormones cause numerous physiological effects in humans *(Lone, 1997)* and animals *(Berisha et al., 2002)*. Residual sex hormones taken with food of animal origin can cause the same physiological activity in humans as endogenous hormones. The influence on the physiological processes in the body depends on the amount taken relative to their natural level *(Andersson and Skakkebaek, 1999)*. Toxicological studies have shown that chronic animal exposure to anabolic doses of sex hormones induces mutagenic and carcinogenic lesions of sex organs *(Zimmerman, 1998; Delatour and Parisch, 1986)*.

Besides natural origin, residual sex hormones may also derive from various feed additives used for therapeutic and prophylactic purpose and can significantly increase the level of productivity. The effect of endogenous hormones in animals is potentiated by the administration of estrogens, gestagens and androgens in therapeutic or anabolic doses. Unprofessional use of veterinary drugs for the treatment of reproductive disorders can also cause accumulation of residues in animal tissues. Therefore, the dosage, route of administration and withdrawal period between the last dose and slaughter or using milk as food in humans are strictly regulated to eliminate the risk of residues in food of animal origin and adverse effects on human health. Natural gonadotropin and synthetic gonadotropin-releasing factor products used in the management of anestrus, and progesterone products and their synthetic analogs (gestagens) for estrus synchronization are currently most common veterinary medicine products on the Croatian market.

In the present study, concentrations of the natural hormones 17β-estradiol, progesterone and testosterone were determined in bovine plasma by use of previously validated ELISA methods. Methods validation resulted in mean recoveries ranging from 75.6% to 84.5%, repeatability from 78.9% to 86.3%, and detection capability (CCβ) ranging from 0.02 ng/mL to 0.11 ng/mL (Table 1). Acceptability of the validation parameter results and thus the appropriateness of the analytical methods for determination of hormone concentrations in animal plasma were demonstrated by comparison with the validation criteria given in *Commission*

Decision 2002/657/EC. The described ELISA methods should be used in monitoring of steroid hormones abuse as an anabolics in meat production, with a confirmation method required in case of non-compliant sample.

Determined hormone levels in bovine plasma vary greatly according to animal sex (Table 2). As expected, determination of sex hormone levels according to animal age showed statistically significantly higher levels in yearlings as compared with calves ($P<0.05$). The highest levels of 17β-estradiol (0.03±0.01 ng/mL) and progesterone (4.87±1.63 ng/mL) were recorded in female yearlings, and of testosterone (9.44±5.47 ng/mL) in male yearlings (Fig. 1-3), which is consistent with physiological values for that categories.

In most EU countries, studies of anabolic effects and abuse control mostly refer to 17β-estradiol because of its pronounced anabolic activity and growth increase, in cattle and sheep in particular (*Meyer, 2001*). Previous studies found plasma to be the most reliable matrix to discriminate between physiological concentrations and elevated hormone levels due to the administration of natural anabolics, with very low limits of detection for 17β-estradiol and testosterone *(Arts et al., 1991; Scippo et al., 1994)*. Literature data show the concentration of progesterone in bovine plasma to range from 0.2 to 8 ng/mL, and from 8 to 12 ng/mL in pregnancy *(EMEA, 1999)*. In the present study, progesterone concentration was 0.10-7.40 ng/mL, which is consistent with data reported from other studies for non-pregnant cattle. In a study by *Shafie et al. (1982)*, progesterone levels according to phases of sex cycle ranged from less than 0.1 ng/mL at the beginning of the cycle, reached a peak of 5.5±1.4 ng/mL during the luteal phase, then decreased abruptly to less than 1 ng/mL. The concentration of 17β-estradiol reached peak level of 0.02 ng/mL during the follicular phase of the cycle. The same study found the levels of 17β-estradiol and progesterone to show seasonal variation as well. However, little data have been published on the levels of various sex hormone metabolites, and this information would be of great importance knowing that some of them are also biologically active in the body *(Andersson and Skakkebaek, 1999)*.

The levels of 17β-estradiol and testosterone in calf plasma, which require due measures to be taken for suspect abuse are defined according to animal age and sex by the *Council Directive 1996/22/EC*. The borderline plasma level of 17β-estradiol demanding due measures has been set at 0.04 ng/mL in both male and female calves to obviate the possibility of a great number of false-positive results. The concentration of 17β-estradiol ranging from 0.1 to 1 ng/mL is only measured in plasma obtained from pregnant cows or illegally treated animals. In male and female calves, the allowed level of progesterone is 0.1 ng/mL and 0.4 ng/mL, respectively. The testosterone level characteristic of female yearlings is <0.5 ng/mL, whereas in male yearlings it may range from 10 to 30 ng/mL, depending on

animal age. The hormone concentrations exceeding those mentioned above point to abuse of these substances as anabolics.
In comparison with physiological values reported in the literature, the results obtained in the present study and data on study animals indicated that illegal use of anabolic substances could not be suspected in none of the studied animals.

As data on plasma hormones concentrations have not yet been precisely determined, are quite inadequate for different animal species and categories, and depend on numerous factors, additional studies are definitely necessary. Future studies should be focused on the hormone pattern of the population because these results would contribute to better identification of the possible abuse. Evaluation of the analytical results on hormone concentrations should always consider all the known factors that may potentially influence the result interpretation and apply some of the corroborative analytical methods if necessary. Furthermore, scientific concepts on the effects of some substances are modified and their potential undesired effects are discovered on a daily basis. Therefore, besides determination of the physiological levels of 17β-estradiol, progesterone and testosterone in particular cattle categories, the fate of other as yet uninvestigated substances that may exert a similar anabolic effect should also be monitored.

Ocena anaboličkog korišćenja steroidnih hormona kod goveda u državi Hrvatskoj

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Rezime

Prirodni polni hormoni su deo endokrinog sistema i nalaze se u biološkom materijalu životinja. U analizama rezidua supstanci sa anaboličkim efektom, radi otkrivanje njihove zloupotrebe, potrebno je znati fiziološke nivoe ovih hormona kako bi mogle da se razlikuju fiziološke koncentracije od nezakonite upotrebe anabolika. Koncentracije hormona koje prevazilaze fiziološki nivo, otkrivene prilikom nezakonitog korišćenja anabolika, ukazuju na zloupotrebu ovih supstanci za anaboličke svrhe. U ovom radu, koncentracije prirodnih hormona, 17β-estradiola, progesterona i testosterona merene su u plazmi goveda različite starosti i pola.

Koncentracije prirodnog hormona utvrđene su pomoću kvantitativne ELISA metode u uzorcima plazme iz goveda raznih rasa sa nekoliko farmi u Hrvatskoj tokom 2006-2009. godine.

Korišćene metode su pokazale efikasnost (oko 75-87%), za određivanje koncentracija 17β-estradiola, progesterona i testosterona u plazmi goveda.

Koncentacije polnih hormona bile su statistički značajno više u plazmi jednogodišnjih grla u odnosu na plazmu sa teladi ($p < 0,05$). Najviši nivo 17β-estradiola (0,03 ± 0,01 ng / ml) i progesterona (4,87 ± 1,63 ng / mL) zabeležen je u plazmi ženskih jednogodišnjih grla, a testosterona (9.44 ± 5,47 ng / mL) u plazmi jednogodišnjih muških grla goveda. Rezultati su pokazali da koncentracije steroidnih hormona variraju u zavisnosti od starosti i pola životinja, ukazujući da sumnja o ilegalnoj upotrebi anaboličkih supstanci nije mogla biti postavljena ni kod jedne od ispitivanih životinja.

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