THE ABILITY OF HUMAN ELECTROCHEMILUMINESCENCE IMMUNOASSAY TO MEASURE TESTOSTERONE AND PROGESTERONE IN OVINE PLASMA

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ABSTRACT. The present study describes the use of electrochemiluminescence immunoassay (ECL) with the specific human kit for measuring plasma testosterone and progesterone in ovine. This study was carried out on 16 mixed age animals of the local breed. They were divided into two groups of both sexes, namely *Male-1 Group*, *Male-2 Group*, *Female-1 Group* and *Female-2 Group*. Blood samples were collected from the jugular vein into tubes containing EDTA and centrifuged at 1500 rpm for 20 min. Plasma was rapidly separated and stored at -20° C until assayed. The reproducibility inter- and intra-assay of the P4- and T-ECL is satisfactory (2.11-7.3 %). The accuracy (93-102 %) and the test of parallelism were largely acceptable. No cross-reaction was observed with the different hormones including PMSG, hCG, progesterone, testosterone, oxytocin and $PGF_{2\alpha}$ when concentrations of 10 UI/ml and 10^{-3} UI/ml were used. T concentrations were higher in *Male-1 Group* than in *Male-2 Group* (1.67 ± 1.15 and 0.38 ± 0.45 ng/ml, respectively). The *female-1 Group* showed very high P4 concentrations (15.17 ± 4.91 ng/ml) compared to the *Female-2 Group* (0.08 ± 0.04 ng/ml). The results obtained clearly show that human ECL system can be used to measure progesterone and testosterone in plasma ovine

Keywords: assay, electrochemiluminescence, testosterone, progesterone, ovine

INTRODUCTION

The physiology of reproduction is mediated through hormonal signals. The reproductive status can be assessed by endocrine measurements (Lasley and Kirkpartrick, 1991). The influence of sex hormones is a key factor underlying male reproductive behavior in mammals (Mooring *et al*., 2004). Indeed, many hormones are involved in the regulation of male reproductive functions. They control the sexual behavior; influence the sexual arousal, the onset of erection and ultimately the ejaculation process.

Testosterone (T) is one of the most important androgens secreted into the blood. It is produced primarily by the testes of the male and is responsible for the normal spermatogenesis (Mclachlau *et al*., 1996; Goeritz *et al*., 2003), the sexual behavior (Corteel, 1985) and the normal function of the reproductive tract (Luke and Coffey, 1994, Walkden-Brown *et al*., 1999).

Progesterone (P4) is a reproductive hormone produced by the *corpus luteum* (CL) of the ovary and the placenta. It is measured for several reasons including the determination of the pregnancy (Karen *et al*., 2003), the monitoring of the ovarian activity and the study of early mortality (Engeland *et al*., 1999). Measurement of the progesterone concentration in the blood is a reliable indicator of the functional CL. A positive relationship exists between the number of fetuses and the mean plasma progesterone concentrations after the second half of pregnancy in sheep (Kalkan *et al*., 1996).

The familiar techniques used to measure the reproductive hormones are radio immunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) in milk and in plasma of the animal. Traditionally, RIA has been employed to quantify the testosterone and progesterone concentrations in serum using specific antibodies that prevented the cross-reaction arising from corticoid binding globulin. Because of their inherent radiation hazards and the inaccuracy in sample preparation steps, RIAs are less popular for routine analysis. They also generate radioactive waste that causes environmental contamination.

Many alternatives have been recommended and developed to replace the radioactive label in immunoassays because of concerns for radiation safety, short shelf-lives of radioactive reagents and radioactive waste disposal. Rongen and collaborators (1994) listed several advantages of analysis techniques based on chemiluminescence (CL). They demonstrated excellent sensitivity and fast assay technique. In addition, the reagents and the hormone-enzyme conjugates are stable and not toxic.

The goal of the present study is the use of electrochemiluminescence immunoassay (ECL) method with the specific human kit (*Elecsys 2010, Roche diagnostics*) for measuring plasma testosterone and progesterone in ovine.

MATERIALS AND METHODS

The study protocol was approved by the Scientific Committee of the University A. Mira, Bejaia (Algeria). This study was carried out on different animal groups of the local breed (n=16 with mixed sex and ages). The animals were divided into two groups of both sexes as *Male-1 Group* (n=3, aged 2 to 3 months), *Male-2 Group* (n=5, aged 9 to 36 months), *Female-1 Group* (n=4, non pubescent and aged 1 to 3 months) and *Female-2 Group* (n=4, diagnosed pregnant by ultrasonographic). The experiment was conducted during the period of April 2012 in Bass Kabylie, Algeria (36°43'N, 5°04'W). Blood samples from the jugular vein were collected between 09:30 and 11:00 a.m. from all the males, in order to determine the testosterone blood concentration. Blood samples were collected in EDTA-containing tubes and further centrifuged at 1500 rpm for 20 min. Plasma was rapidly separated and stored at -20°C until assayed (Delahaut *et al*., 1979).

The assay of T and P4 was carried out by a method of immunological competition revealed by electrochemiluminescence (substrate: ruthenium) on the automat Elecsys® 2010 (Roche diagnosis). The antibodies of capture and revelation

are monoclonal specific to testosterone or progesterone of human origin. The estimated dose of testosterone and progesterone measurement extends from 0.025 to 15 ng/ml and from 0.03 to 60 ng/ml, respectively.

Validation of ECL immunoassay

Reproducibility

To test the reproducibility of the ECL, one sample with testosterone or progesterone concentration was used. Samples were obtained from pregnant and adult sheep for progesterone and testosterone measurement, respectively. The reproducibility was determined by calculating the intra- and inter-assay coefficients of variation (CV) as follow: [% CV = (SD/mean)*100]. For intra-assay CV, the same serum was assayed 3 times in duplicate within the same assay. The inter-assay reproducibility was assessed by analyzing 6 times consecutive assays in duplicate (Rodbard *et al.*, 1974).

Accuracy

The accuracy for ECL was determined by adding increasing concentrations of testosterone or progesterone to ovine sera. The mean (±SD) concentration of each mixture was determined in duplicates. The percentage of recovery was calculated as follows: [observed value (ng/ml)/expected value (ng/ml)] × 100.

Parallelism

Parallelism was assessed by serially diluting pregnant and adult sheep serum containing relatively high testosterone and progesterone concentrations with distilled water. Parallelism for ECL system was determined by evaluating a sample at its initial strength (1/1), and at dilutions of 1/2, 1/4 and 1/8.

Specificity

The specificity was tested against the following hormones: PMSG (Folligon 1000 IU; Intervet International B.V., Boxmeer, Holland), hCG (Chorulon 1500 IU; Intervet International B.V. Boxmeer, Holland), progesterone (Progesterone® Retard pharlon, Bayer Schering pharma; France) ocytocine (Oxytekel Synth, Kala N.V. St. Hoogstraten, Belgium), prostaglandin $F_{2\alpha}$ (Enzaprost, Ceva Animale, Libourne, France). Each hormone was dissolved and then diluted in distilled water to concentrations of 10 UI/ml and 10^{-3} UI/ml, respectively. Each dilution of tested compounds was assayed twice and considered as an unknown sample.

Data analysis

Statistical analyses were carried out in STATVIEW (Version 4.55). The T and P4 concentrations measured in the males and females were used to calculate the mean ± standard error.

RESULTS

The reproducibility of both T and P4 ECL immunoassay are summarised in Table 1. The values of inter-assay CV in both T and P4 ECL immunoassay are similar, whereas intra-assay CV for P4-ECL are lower than those calculated for T-ECL (2.11 and 7.30, respectively).

The accuracy of both ECL systems is shown in Table 2. Higher recovery rates were obtained by the use of T-ECL (> 95.5%), while lower values were recorded in the PP4-ECL (< 94%). Parallelism of plasma samples diluted with distilled water for T- and P4-ECL systems is presented in Table 3.

As far as the specificity is considered, no cross reaction of both T- and P4-ECL was recorded, except in the case of ocytocin (1/1 and 1/1000), which slightly interfered in P4-ECL (e.g. 0.541 and 0.521 ng/ml, respectively).

The T and P4 concentration (mean ± SE) determined in plasma samples from ovine males and females are represented in Table 4. The T concentrations are largely higher in Male-1 Group than in Male-2 Group (1.67±1.15 and 0.38±0.45, respectively). In contrast, the *female-1 Group* shows very high P4 concentrations (15.17±4.91). Whereas, the Female 2 group samples revealed very low content (0.08 ± 0.04).

DISCUSSION

The determination of testosterone is of great importance in the evaluation of the endocrine activity of the ram, particularly in its reproductive state. The progesterone

Table 1. Intra- and inter-assay coefficients of variation of testosterone and progesterone ECL immunoassay.

	Intra-assay		Inter-assay	
	Concentration (±SE ng/ml) (n=3)	CV (%)	Concentration (±SE ng/ml) (n=3)	CV (%)
T-ECL	0.41 ± 0.03	7.30	0.40 ± 0.02	5.00
P4-ECL	13.37 ± 0.28	2.11	13.85 ± 0.80	5.78

Table 2. Recovery of different concentrations of testosterone and progesterone added to a plasma sample containing low concentrations of T and P4 respectively as measured by ECL immunoassay.

	Theoretical hormone concentration (ng/ml)	Observed hormone concentration (ng/ml)	Recovery (%)
T-ECL	3.10	2.96	95.5
	1.04	1.06	102
P4-ECL	15.94	14.81	93
	12.76	12.00	94

Table 3. Serial dilutions (mean ± SE) of a plasma sample containing relatively high T and P concentrations as measured by ECL immunoassay.

Dilution of sample	T-ECL (ng/ml)	P4-ECL (ng/ml)
1/1	0.352	21.76
1/2	0.176	9.99
1/4	0.142	4.61
1/8	0.111	1.93

Table 4. Mean (± SE) T and P4 concentrations obtained by ECL immunoassay in male and female ovine.

	Hormone concentration (± SE ng/ml)	
T-ECL	*Male-1 Group* (n=5)	*Male-2 Group* (n=3)
	1.67 ± 1.15	0.38 ± 0.45
P4-ECL	*Female-1 Group* (n=4)	*Female-2 Group* (n=4)
	15.17 ± 4.91	0.08 ± 0.04

is the principal hormone of reproduction secreted by the corpus luteum and by the placenta during gestation (Linzell and Heap, 1968). The essential role of progesterone in maintaining gestation is known since the beginning of last century. It was at the base of the development of the first methods of hormonal diagnosis by assay in blood and milk.

For several years, various methods have been developed to determine the concentrations of the steroids of the man. These competition type techniques employ iodises, enzymatic or chimiluminescent tracer. Electrochemiluminescence technique is a form of chemiluminescence where the emission of light from a chemical reaction, is preceded by an electrochemical reaction (Richeter, 2004). In the present study, we describe the ability of electrochemiluminescence assay kit specific human (Elecsys 2010, Roche diagnostics) for measuring the plasma testosterone and progesterone in ovine.

The minimal limit of detection of the system corresponds to the smallest concentration of the measurable progesterone. The MLD obtained from the ECL system is lower than that calculated using radioimmunoassay method (0.03 ng/ml *vs* 0.2 ng/ml, respectively) (Benyounes *et al*., 2006). However, the ELISA techniques are less sensitive (0.05 ng/ml) than the T-ECL (Casao *et al*., 2010). On the other hand the T measurement by chromatography technique followed of a spectrophotometer and enzyme immunoassay revealed a LMD of 0.2 ng/ml (Robert *et al*., 1996, Moghaddam *et*

al., 2012). We can say that the ECL technique of P4 and T measurement are very sensitive. This difference in MLD value obtained in the present study and in the previous can be attributed to technical aspects, such as the labeled method, the incubation time step or antibody typical.

The reproducibility of the T and P4 by ECL evaluated with ovine plasma is very satisfactory. CV inter- and intra-assay of T as measured by Vidas were reported by Gasser and co-authors (2002) to be 6.7 and 13%, respectively. In addition, Zamiri *et al*. (2010) used a commercial RIA kit for measuring testosterone with the CV intra- and inter-assay of 5.5 and 7.7%, respectively. Recently, Maghaddam and co-authors (2012) reported different values of 6.5 and 9.3% by using EIA kit. In previous studies, the CV intra and inter-assay of P4 registered high values by using RIA (14.1 and 18.7%, respectively) and method EIA (6.1 and 12.3%, respectively) that our results (Karen *et al*., 2006; Del Vecchio *et al*., 1995).

Parallelism was assessed by a serial of dilution (1/1, 1/2, 1/4 and 1/8) with serum of adult male and pregnant female containing respectively a high T and P4 concentration. In the present study, the values show that the T and P4 concentrations obtained are practically parallel. Concerning the precision of T- and P4-ECL, the results obtained are acceptable with the rate of recovery ranging from 93 to 103%. The percentages of covering 100 % higher values could be explained by the presence of interferences. Our results showed that the T- and P4-ECL systems are specific for the detection of P4 and T in ovine with regard to GnRH, PMSG, Oxytocin, Progesterone, Testosterone and Prostaglandin $F_{2\alpha}$.

As shown in Table 3, the T concentrations in *Male 1 Group* are very low which indicates an absence of leydig cells secretion because these animals are under the age of puberty. In the developmental stage, the neuro-secreting cells of complex hypothalamo-hypophyse are immature. They secrete low amounts of hormones (GnRH, FSH and LH) which are insufficient to induce maturation and activation of testicular cells (i.e. cell of Leydig) (Thibault *et al*., 2001). Except T concentrations obtained in the male aged of 2 years (4.48 ng/ml) in *Male 2 Group*, the T values are quite lower ranging from 0.35 to 1.73 ng/ml. The season of reproduction and the endocrine mechanisms involved can largely be influenced by behavioral stimuli released through the social relationships that an animal (both the ram and the ewe) establishes with others of the same species (Rosa and Bryant, 2002). Photoperiodic changes related to season have a direct effect on the reproductive activities due to a change in the levels of the hormones secreted by hypothalamus, pituitary and gonads (Pérez and Mateos, 1995). The high T concentration of 4.48 ng/ml could be due to the individual instability of the sexual activity or to a testicular anomaly. Issa and collaborators (2006) reported a change in T concentration in two ram breeds (Tuaregs and Peuls) during October and April. The results showed high T concentrations of 4.17±1.82 and 4.55±2.71 ng/ml in Peul and Tuareg rams, respectively. Palacios *et al*. (2009) have also showed an effect of the age

and the race on the T concentration in three breeds of rams (Assaf, Churra and Castilian).

As for *Female-1 Group*, the P4 measurements were very low ranging from 0.03 to 0.14 ng/ml. The P4 concentrations in the control group are lower than the physiological concentrations of the follicular phase (0.3 ng/ml). These results indicate an absence of a luteal secretion and also confirm that the females were not yet pubescent. Concerning the females in post-partum, a value of 0.057 ng/ml agrees with the results reported previously (Benyounes *et al*., 2006; Ranilla *et al*., 1997). The *Female-2 Group* is ewes pregnant which have normally high progesterone concentrations secreted by the placenta (Linzell and Heap, 1968). In the day of estrus, the minimal progesterone concentration observed is 0.2-0.3 ng/ml. Then, it rises gradually from days 3-4 of the cycle to reach a maximum of 2 ng/ml between days 7 and 10 (Cunningham *et al*., 1975). The P4 concentration remains stable until days 14 and 15 of estrus cycle. Afterwards, it decreases gradually with the CL luteolyse. After fecundation, the CL is stabilised and the plasma P4 concentration exceeds that observed in luteal stage. The P4 measurement provides information throughout gestation because the P4 concentrations increase regularly during the pregnancy. The present results obtained from an assay of the plasma as measured by the method of electrochemiluminescence revealed high plasma progesterone values within the range of 10.12-21.76 ng/ml in pregnant females. The differences in P4 concentration in females could be explained by the differences in gestation periods and the number of carried fetuses. Indeed, Benyounes and colleagues (2006) reported that the progesterone concentrations increase from 4.5±0.4 to 8.5±0.9 in 13 and 17 weeks, respectively. Then, it stabilises at a value of 10.9±1.1 ng/ml until the end of gestation. Others reported that P4 concentrations are much higher in the ewes carrying 2 or 3 fetuses than in female carrying only one foetus (19.2, 29.9 and 9.2 ng/ml, respectively) (Chauhan and Waziri, 1991; Kalkan *et al*., 1996; Barbato *et al*., 2009). The molecular structure of the steroids, especially testosterone and progesterone, are not species specific. In 1997, the IAEA adapted a human progesterone kit to be used in determining progesterone in domestic animals. Some studies validated ELISA, EIA and ECL for measuring P4 by using heterologous systems in plasma bovine, equine, ovine and canine (Eckersall and Harvey, 1987; Bayemi *et al*., 2007, Ayad *et al*., 2014)

In conclusion, the present study shows clearly that human electrochemiluminescence kit can be used to measure testosterone and progesterone in plasma ovine for the detection of heats and the diagnosis of complementary gestation in the ewe. They can also be used for the characterisation of reproduction seasons, the evaluation of reproduction performance and possibly for the diagnosis of some pathology in testicle.

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