VALIDATION OF 17β-ESTRADIOL EIA IN CULTURE MEDIUM FROM RABBIT OOCYTES MATURED *IN VITRO*

LORENZO P.L.[1], ILLERA J.C.[1], SILVAN G.[1], MUNRO J.C.[3], REBOLLAR P.G.[2], ALVARINO J.M.R.[2], ILLERA M.J.[1], ILLERA M.[1]

[1] Dpto. de Fisiología Animal, Facultad de Veterinaria, UCM, 28040 Madrid, Spain.
[2] Dpto. de Producción Animal, E.T.S.I. Agrónomos, Universidad Politécnica de Madrid, Spain
[3] Dpt. of Population Health and Reproduction, School of Veterinary Medicine, UC Davis, USA.

Abstract - A direct heterologous enzyme immunoassay (EIA) was validated to determine 17β-estradiol levels in culture medium, without previous extraction, from rabbit oocytes matured *in vitro* (IVM). Polydonal 17β-estradiol (C902) antibody was raised in rabbits using 6-keto-17βestradiol 6-carboxymethyloxime: BSA. Horseradish peroxidase was used as label, conjugated to 17β-estradiol 3-hemisuccinate. Standard dose response curve covered a range between 0 and 1 ng/well. The low detection limits of the technique was 1.99 pg/well. Intra- and inter-assay coefficient of variation percentages (CV) were <5.3 and <7.8, respectively (n= 10). The recovery rate of known 17β-estradiol concentrations added to a pool of culture maturation medium averaged 96.39 ± 1.40%. Compared with RIA, EIA values were in close agreement (n=15, R=0.96, P<0.001). Culture medium samples were obtained after IVM oocyte (n=401) culture for 14-16 h. Mean ± SE culture maturation medium concentrations were 2.55 ± 0.06 ng/ml and 0.19 ± 0.02 in both, oocytes with and without cumulus cells, respectively. We concluded that our EIA is a highly sensitive and specific assay that provides a rapid, simple, inexpensive, and non-radiometric alternative to RIA for determining 17β-estradiol concentrations in oocyte culture maturation medium.

INTRODUCTION

Isotopic immunoassays have some disadvantages making necessary the development and validation of new non-isotopic techniques. Enzyme immunoassay (EIA) techniques are an altemative to the above mentioned methods, and in recent years a great number of studies have been reported for the determination of steroid levels in many biological fluids (MUNRO and LASLEY, 1988; SILVAN *et al.*, 1993; ILLERA *et al.*, 1993). However, use of this technique for measurement of steroids in cumulus oocyte-complexes culture samples has not yet been reported. The interest of these hormonal determinations is supported by the fact that somatic control of oocyte maturation involves certain hormonal signals that regulate the synthesis of proteins specifically associated with this event (OSBORN and MOOR, 1983). The 17β-estradiol effect on oocyte maturation is poorly understood, but it plays a decisive role sustaining ooplasmic maturation *in vitro.*

In these terms, validation of EIA methods for determining steroid hormone levels in culture medium samples must be made with precision, especially the raising and characterization of 17β-estradiol antibodies, since the estrogen composition may be different in culture medium from *in vitro* oocyte maturation than in other biological fluids. For all the above mentioned reasons, our objective was to validate herein direct, reliable and precise heterologous enzyme immunoassays for quantification of 17β-estradiol levels in culture medium samples from oocyte maturation.

MATERIAL AND METHODS

Animals

Sexually mature New Zealand White (NZW) rabbits (weighing 3.5-4.5 kg) were used. The animals were housed individually in metal cages on performated sheets of dimensions 32 cm x 52 cm in air-conditioned rooms under a 12 h light-12 h dark cycle, starting at 8:00 h. Pelleted commercial diet (Lab Rabbit Chow, Purina Mills Inc.) was restricted to about 125 g/animal, once daily. Water was supplied *ad libitum.* Animals used in this study were treated according to the CEE Council Directive (86/609, 1986) for the Care of the Experimental Animals.

Polyclonal antibodies and enzymes conjugates

Polyclonal antibodies were produced in eight adult NZW rabbits. 6-keto-17β-estradiol 6-carboxymethyloxime: BSA (20 moles steroid: 1 mol BSA), was used as immunogen. Animals were immunized by intradermal multiple site technique with the necessary modifications (SILVAN, 1991). Titer of the antiserum was assessed during immunization by EIA, and once it was the required, polyclonal antibodies were purified (ILLERA *et al.*, 1992). 17β-estradiol was labelled with horseradish peroxidase (HRP) by the mixed anhydride method (DAWSON *et al.*, 1978) using 17β-estradiol 3-hemisuccinate. Conjugate was stored freeze-dried until assay. Assessment of the conjugate was also done using the reported methods: calculation of 17β-estradiol: HAP ratio, recovery of enzyme activity after conjugation, and titration of conjugate in EIA to determine the working solution (MUNRO and STABENFELDT, 1984).

Enzyme Immunoassay Procedure

Assays were conducted essentially as reported by SILVAN *et al.* (1993). Ninety-six well Dynatech flat bottomed polystyrene microtiter plates were coated with 50 µl/well of appropriately antibody solutions (17β-estradiol: 1/4,000, in coating buffer: sodium carbonate, 50 mmol/L, pH 9.6) except for the first well which acted as plate/assay blank, and incubated overnight at 4°C. Afterwards, non-bound antibodies were removed from the wells by washing plates five times with wash solution (NaCl, 150 mol/L, Tween 20, 0.5 ml/L), inverted and dried. Standards were solubilized in ethanol, evaporating the solvent and resolubilizing them in working conjugate solution (17β-estradiol: 1:30,000) in assay buffer: sodium phosphate, 100 mmol/L, pH 7.0, with sodium chloride, 8.7 g/L, BSA, 1 g/L). Standard curve covered a range between 0 to 1 ng/well and was constructed by using ten standard solutions: 1; 2.5; 5; 10; 25; 50; 100; 250; 500 and 1,000 pg/well. 50 µl of culture maturation medium samples were mixed with 250 µl of conjugate working solution. Conjugate working solution was added into the wells of the first and last rows. These rows were called Bo. For Bo and standard samples 50 µl of assay buffer were pipetted hlto the wells followed by the addition of 50 µl of conjugate working solution (row 1 and 12) or standard solutions in duplicate (rows 2 to 4). For culture maturation medium samples 40 µl of assay buffer were pipetted into the wells of 5 to 11 rows followed by the addition of 60 µl of each sample (prepared as above, where 60 µl is equivalent to 10 µl of culture maturation medium and 50 µl conjugate solution) in duplicate. Plates were covered and incubated at room temperature in darkness, and competitive reaction was completed approximately in two hours. Bound/free separation was achieved by emptying plates by inversion and washing them, five times with wash solution. To evaluate the amount of labeled 17β-estradiol bound to the antibody, 100 µl of substrate solution (3,3',5,5'-tetramethylbenzidine dihydrochloride, pH 5.0) were added to all wells and incubated for 15 min. at room temperature, this reaction was stopped by the addition of 100 µl of 1 M phosphoric acid. Absorbance was read at 450 nm in an automatic microplate reader. 17β-estradiol concentrations were calculated by means of software developed for this technique (ELISA AID, Eurogenetics, Belgium). Standard-dose response curve (Figure 1) wase constructed by plotting the binding percentage (B/Bo x 100) against 17β-estradiol standard concentrations added. 17β-estradiol concentrations were expressed in ng/ml.

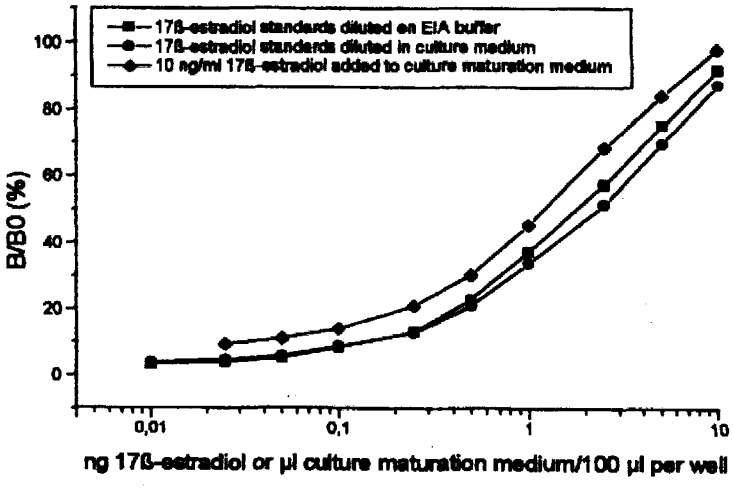
RIA procedure

The RIA for 17β-estradiol was performed as instructed on DPC Coat-A-Count total 17β-estradiol kits counting for gamma emissions. For the adaptation of this method to culture maturation medium samples an extraction step was necessary.

***In vitro* maturation of oocytes**

Does were killed with an overdose of pentobarbitone sodium (i/v), and their ovaries were immediately removed. Selected follicles (> 1 mm in diameter) were sliced under a dissecting microscope. Collected oocytes were cultured in 1.0 ml of Brackett's

Figure 1 : Parallelism of 17β-estradiol standard curves *vs* oocytes culture maturation medium dilution curve



medium (at 37 °C, 5% CO_2 in air, and 100% humidity, pH 7.4), for 14-16 h. According to pre-defined criteria (LORENZO *et al.*, 1994), healthy cumulus-oocytes were divided for culturing either as cumulus-oocyte complexes (with intact cumulus: COC) or denuded oocytes (without layers of cumulus: DO). Neither serum nor hormones were added to maturation medium. Oocyte maturation experiment was repeated seven times with different batches of ovaries. At the end of the culture period, the oocytes were fixed and stained with acetic-orcein to ascertain their meiotic stage. Culture maturation medium samples for EIA were collected after completion of oocyte cultures, centrifuged at 2,500g for 10 mn, and supernatants stored at -30 °C for quantification of 17β-estradiol by competitive EIA.

Statistics

Statistical analysis of the hormone results was performed using the BMDP (Biomedical Data Program) carrying out an analysis of variance and a simple regression analysis between EIA and RIA. All values were expressed as mean ± SE. Intra- and inter-assay coefficients of variation (%) were calculated by Rodbard's method (1974). The significance of cumulus cells effect on 17β-estradiol concentration was determined by Catmod procedure of Statistical Analysis System (SAS/STAT). Only P values less than 0,05 were considered significant.

RESULTS

Antibody Production and Characterization

Polyclonal 17β-estradiol antiserum reached the required titer for purification after an immunization period of four months (1/1,000 by EIA). After purification, 17β-estradiol antibody (C902) showed a titer of 1/4,000, estimated by EIA. Specificity of the antibody expressed as percentage of cross-reactivity to related steroids, is summarized in Table 1.

Table 1 : Percentage of cross-reactivity of various steroids compared to 17β-estradiol for 17β-estradiol antibody

Steroid compound	% Cross-reactivity
17β-estradiol	100.00
6-keto-17β-estradiol 6-carboxynethyloxime	130.00
16-keto-17β-estradiol	16.70
6-keto-17β-estradiol	20.00
2-hydroxyestradiol	8.74
6α-hydroxyestradiol	9.30
17β-estradiol 3-benzoate	3.28
17α-estradiol	0.05
Estrone	0.50
Estriol	0.05
Progesterone	< 0.01
Cortisol	< 0.01

Conjugate Assessment

The assessment of the conjugate showed the following result: 17β-estradiol: HRP ratio was 1.2:1 moles. The recovery of the enzyme activity after conjugation was more than 85%.

Enzyme immunoassay validation

The validation of the EIAs in the culture maturation medium samples was based on the results of accuracy, precision, sensitivity, parallelism, and their correlation with RIA. The accuracy of the EIA was tested by determining the recovery rates of known amounts of 17β-estradiol added to culture maturation medium without oocytes (control) and to a pool of culture maturation medium samples (with oocytes; n=6). The recovery rate averaged 96.39 ± 1.40%, and it is shown in Table 2.

Table 2 : 17β-estradiol concentrations (ng/ml) and recovery rate (%) after addition of 17β-estradiol to different pools of culture maturation medium

17β-estradiol added (ng)	17β-estradiol concentrations (ng/ml) and recovery rate (%)	
	Control culture medium (no oocytes cultured)	Pool of culture maturation medium (oocytes cultured)
0	0.00	1.18
1	0.93 (93.00)	2.16 (99.31)
5	5.10 (102.14)	5.96 (96.48)
10	8.93 (89.30)	11.06 (98.93)
15	14.49 (96.64)	15.42 (95.32)
Average	95.27 ± 2.73	97.51 ± 0.96

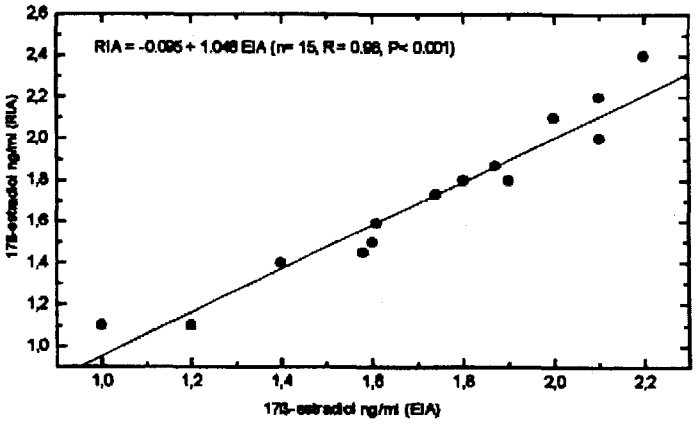
Precision of 17β-estradiol EIA was determined by calculating the intra- and inter-assay Coefficiens of Variation (CV). The intra-assay CV (%) was calculated by replicate measurements of three standard concentrations of 17β-estradiol, added to control medium, and six culture maturation medium samples with oocytes. Each sample was tested in duplicate, ten times within an assay. Inter-assay CV (%) was calculated by the replicate measurements of the above mentioned samples in ten consecutive assays. Both intra- and inter-assay CV (%) are summarized in Table 3.

Table 3 : Intra- and inter-assay CV (%) for 17β-estradiol EIA added to control maturation medium and for six culture maturation medium from oocyte IVM

	INTRA-ASSAY (%)	INTER- ASSAY (%)
30 pg/ml	3.9	5.1
300 pg/ml	4.2	4.8
3000 pg/ml	3,1	5.2
Culture maturation medium samples	5.3	7.8

The sensitivity of the EIA was tested by means of low detection limit, as defined by Abraham and calculated from Bo values minus 2 SD in 10 consecutive assays. Low detection limit was: 1.99 pg/well (or pg/100 µl). To determine the effects of culture maturation medium in the 17β-estradiol standard curve, several dose response curves were run with 17β-estradiol standards diluted both in assay burlier and control medium. The addition of high standard doses (10 ng/ml) to a pool of culture maturation medium samples, and serially diluted were run in parallel with the standard dose response curve. All dose response curves are parallel (Figure 1), indicating that there is little or no culture maturation medium effect in the 17β-estradiol EIA.

Figure 2 : Simple regression analysis of 17β-estradiol EIA *versus* in culture medium maturation samples



Correlation coefficients between RIA and EIA using the same culture maturation medium samples was depicted in Figure 2. Regression analysis between RIA and EIA showed correlation coefficient of R=0.96 (n=15, P < 0.001).

17β-estradiol concentrations in culture maturation medium samples

Assay of culture maturation medium samples yielded the results expressed in Table 4.

Table 4 : 17β-estradiol concentrations (ng/ml) in culture maturation medium samples from rabbit oocytes matured *in vitro*

	Hormone concentrations (ng/ml)		
Type of oocyte	N° oocytes cultured	N° of samples	17β-estradiol (mean ± SEM)
Control (no oocytes cultured)	-	5	0.00
Cumulus-oocyte complexes	204	7	2.55 ± 0.06^a
Denuded oocytes	197	7	0.19 ± 0.02^b

Different superscripts between rows denote significative dillierences (a *vs* b, $P < 0.01$)

DISCUSSION

The quantification of 17β-estradiol concentrations is decisive for the knowledge of the ovarian physiology, such as the follicular growth and development, oocyte maturation, ovulation, and atresia among others (FORTUNE, 1994; CAMPBELL *et al.*, 1995). The steroid effect on oocyte nuclear maturation *in vitro* in mammals is the result of the steroid modulation produced by follicular cells during culture. In order to investigate the steroid hormones levels at the end of IVM period, it is necessary to validate simple, reliable, and precise methods, highly specific, and able to discriminate between 17β-estradiol and their metabolites in culture maturation media. EIA methods have demonstrated that they unit these features (MUNRO and LASLEY, 1988), and this led us to validate a direct, simple and precise heterologous competitive EIA. The titers of our polyclonal antibody was very high, and the percentages of cross-reactivity against related steroids were comparable and even lower than others previously reported for these hormones using EIA methods. Our results allowed excellent discrimination between 17β-estradiol and their possible metabolites after culture maturation period. The assessment of our conjugate showed optimum ratio, 17β-estradiol: HRP of 1.2:1 (MUNRO and STABENFELDT, 1984). The conditions under which EIA has been carried out are based on the perfect statement of the working solutions both antibody and conjugate, and are in agreement with those previously reported (MUNRO and STABENFELDT, 1984; SILVAN *et al.*, 1993). Precision of our direct (without previous extraction of the sample) system is also elevated, and simplifies largely the method and reduces the time of performance. Parallelism test confirm the excellent discrimination between 17β-estradiol and their metabolites in culture maturation medium samples, showing no interferences of the culture medium components in the assay. Comparison with the results obtained by RIA showed elevated correlation coefficient between both techniques (more than 95%). In this sense, both the EIA intra- and inter-assay CV (%) are within acceptable limits, and are similar to those obtained with a more traditional RIA (XU *et al.*, 1988). In this study, the culture of denuded oocytes significantly originated lower concentrations of 17β-estradiol at the end of the culture, compared to oocytes with intact cumulus cells, suggest that the cumulus cells mediate in the signal that initiates oocyte maturation. Our data confirm the possibility that the steroid status of the cumulus cells surrounded oocyte regulates the modulation of cAMP dependent meiosis arrest, and the nuclear maturation of the oocyte.

In conclusion, we report here the validation of a simple, reliable, efficient, specific and precise EIA for determining 17β-estradiol concentrations in the culture medium from oocyte *in vitro* maturation. This EIA does not require extraction of samples prior to assay, and it is rapid and inexpensive with the potential for determining a large number of samples in a short period of time.

REFERENCES

ABRAHAM G.E., 1975. Characterization of anti-steroid antisera. In: Steroid Immunoassay (Cwneron, E.H.D., Hillier, S.G., Griffiths, K., eds.), pp. 67-78. Alpha-Omega Publishers Ltd. Cardiff.

CAMPBELL B.K., SCARAMUZZI R.J., WEBB R., 1995. Control of antral follicle development and selection in sheep and cattle. *J. Reprod. Fert.*, **Supp. 49**, 335-350.

FORTUNE J.E., 1994. Ovarian Follicular growth and development in mammals. *Biol. Reprod.*, **50**, 225-232.

ILLERA J.C., SILVAN G., ILLERA M., 1992. Antibody production against steroids for immunological studies. *An. Real Acad. Farm.*, **58**, 475-482.

ILLERA J.C., SILVAN G., ILLERA M.J., ILLERA M., 1993. Steroid hormone profiles in several domestic species and the methodology for determination thereof. *In: Trends in Comparative Biochemistry and Physiology* (Council of Scientic Research Integration, Research Trends ed.), pp 869-879, Trivandrum, India.

LORENZO P.L., ILLERA M.J., ILLERA J.C., ILLERA M., 1994. Enhacement of cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factor and insulin-like growth factor I. *J. Reprod. Fort.*, **101**, 697-701.

MUNRO C., STABENFELDT G., 1984. Development of a microtitre plate enzyme immunoassay for the determination of progesterone. *J. Endocrinol.*, **101**, 41-49.

MUNRO C.J., LASLEY B.L., 1988. Non-Radiometric methods for immunoassay of steroid hormones. In: Non Radiometric assays: Technology and application in polypeptide and steroid hormone detection (Albertson B.D. and Haseltine F.P., ed.), pp. 289-329, Alan R. Liss, Inc., New York.

OSBORN J.C., MOOR R.M., 1983. The role of steroid signals in the maturation of mammalian oocytes. *J. Steroid Biochem.*, **19**, 133-137.

RODBARD D., 1974. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Of. Chem.*, **20**, 1255-1270.

SILVAN G., 1991. Correlations between follicular size and steroid hormone levels in cattle. PhD Thesis, pp. 111- 114, U.C.M. Spain.

SILVAN G., ILLERA J.C., ILLERA M., 1993. Determination of follicular fluid estradiol levels by enzyme-linked immunosorbent assay. *Steroids*, **58**, 324-329.

XU K.P., HOIER R., GREVE T., 1988. Dynamic changes of estradiol and progesterone concentrations during *in vitro* oocyte maturation in cattle. *Theriogenology*, **30**, 245-255.

Validacion del estradiol-17β mediante eia en el medio de cultivo de oocitos de coneja madurados *in vitro*

- En este estudio se ha validado un enzimo inmunoensayo heterólogo (EIA) para cuantificar, sin extracción previa, niveles de 17β-estradiol en un medio de cultivo procedente de la maduración *in vitro* de oocitos de coneja. El anticuerpo policlonal (C902) fue producido en conejos utilizando 6-keto-17β-estradiol 6-CMO: BSA. La peroxidasa se empleo como enzima marcadora y se conjugó al 17β-estradiol 3-HS. La curva estándar se construyó cubriendo un rango entre 0 y 1 ng/pocillo. El límite de detección fue de 1.99 pg/pocillo. Los coeficientes de variación (%), intra-e inter-ensayo fueron <5.3 y <7.8, respectivamente (n= 10). El porcentaje media de recuperación de concentraciones conocidas de 17β-estradiol, añadidas a un pool de medio de maduración fue 96.39 ± 1.40%. Las muestras de medio de cultivo fueron obtenidas después de un periodo de maduración de 14-16 h. Las concentraciones (Media +± ES) de 17β-estradiol en el medio de cultivo al final de la IVM fueron 2.55 ± 0.06 y 0.19 ± 0.02 ng/ml en COC y DO. Comparados con el RIA, los resultados del EIA fueron similares (n=15, R=0.96, P<0.001). Podemos concluir diciendo que este enzimo inmunoensayo es un método muy sensible y especifico, proporcionando una alternativa rápida, simple, barata y no radiactiva al RIA para determinar 17β-estradiol en un medio de maduración de oocitos.