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A sensitive EIA for 17β-estradiol and progesterone in culture medium for oocyte *in vitro* maturation procedures

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A sensitive heterologous enzyme immunoassay (EIA) was validated to determine 17β-estradiol (E2) and progesterone levels, without previous extraction, in culture medium from rabbit oocytes matured *in vitro* with and without the addition of IGF-I. Polyclonal E2 (C902), and progesterone (C914) antibodies were raised in rabbits using 6-keto-17β-estradiol 6-carboxymethyloxime:BSA, and 11α-hydroxyprogesterone 11α-hemisuccinate:BSA. Horseradish peroxidase was used as label, conjugated to 17β-estradiol 3-hemisuccinate, and to progesterone 3-carboxymethyloxime. Standard dose response curves covered a range between 0 and 1 ng/well (100 µl). The low detection limits of the technique were 1.99 pg/well for E2, and 13.21 pg/well for progesterone. Intra- and interassay coefficient of variation percentages (% CV) were < 6.3 and < 7.8 for E2 and progesterone, respectively (n = 10). The recovery rate of known E2 or progesterone concentrations added to a pool of culture maturation medium averaged 96.39 %, and 98.65 %, respectively. Compared with RIA, EIA values were in close agreement for E2 (n = 15, R = 0.96, P<0.001), and progesterone (n = 15, R = 0.99, P < 0.001). Medium samples were obtained after oocyte maturation *in vitro* for 16 h. Use of IGF-I significantly elevated steroids production in the oocyte surrounded cumulus cells. The EIA described here is highly sensitive and specific assay, and provides a rapid, simple, inexpensive, and non-radiometric alternative to RIA for determining E2 and progesterone levels in oocyte culture medium.

Key words: 17β-estradiol, Progesterone, EIA, Culture medium, Oocyte.

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Many isotopic immunoassays have been developed for the measurement of steroid hormone levels in the last years (5, 16, 31). Isotopic immunoassays have some disadvantages making necessary the development and validation of new non-isotopic techniques. Enzymeimmunoassay (EIA) techniques are an alternative 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 (7, 12, 22, 26, 28). However, to date, 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 mammalian oocyte maturation involves certain hormonal signals that regulate the synthesis of proteins specifically associated with this event (23). Although the precise mechanism of steroid effects on the oocyte maturation both *in vivo* and *in vitro* is poorly understood, they play a decisive role sustaining ooplasmic maturation *in vitro* and, therefore, they have traditionally been added to oocyte maturation media (32).

In these terms, validation of EIA methods for determining steroid hormone levels in these culture medium samples must be made with precision, especially the raising and characterization of 17β-estradiol and progesterone antibodies, since the estrogen and progesterone composition may be different in culture medium from *in vitro* oocyte maturation than in other biological fluids, so that improvement of the antibody specificity and an additional amplification step are necessary (6, 10, 15). For all the above mentioned reasons, our objective was to validate sensitive and precise heterologous enzymeimmunoassay suitable for quantification of 17β-estradiol and progesterone levels, without previous extraction, in culture medium samples from oocyte maturation procedures.

Materials and Methods

Reagents.– Horseradish peroxidase (EC 1.11.1.7., RZ = 3, type VI) was purchased from Boehringer Mannheim, bovine serum albumin (BSA, Cohn Fraction V) from Sigma and TMB (3,3',5,5'-tetramethylbenzidine dihydrochloride) from Pierce Europe B.V. (The Netherlands). All steroids were purchased from Steraloids Inc. (Wilton, NH, USA). The rest of the solvents and reagents were analytical grade and were purchased from Merck and Panreac (Spain), as well as the culture medium reagents. Ninety-well flat bottomed polystyrene microtiter plates were obtained from Dynatech (M-29A; Germany), the microplate reader from Bio Tek Instruments Inc. (Winooski, VT, USA). RIAs for 17β-estradiol and progesterone were purchased from DPC (Diagnostic Products Corporation, Los Angeles, CA), Petri dishes from Bibby (England) and human-recombinant IGF-I from Boehringer Mannheim (Germany).

Animals.– Sexually mature New Zealand White (NZW) rabbits (3.5-4.5 kg) were used. The animals were housed individually in metal cages on perforated sheets of dimensions 32 cm x 52 cm in air-conditioned rooms (20 °C, 45 % relative humidity) under a 12 h light-12 h dark cycle. Artificial light was on from 8:00 h to 20:00 h. Pelleted commercial diet (Lab Rabbit Chow, Purina Mills Inc: 16.2 % protein, 2.5 % fat, and 13.5 % fiber crude) was restricted to about 125 g/animal, once daily. Water was supplied *ad libitum*. Animals were treated according to the EC Council Directive (86/609, 1986) for the Care of the Experimental Animals.

Polyclonal antibodies and enzyme conjugates.– Polyclonal antibodies were produced in eight adult NZW rabbits. As immunogens for 17β-estradiol and for progesterone 6-keto-17β-estradiol 6-carboxymethyloxime: BSA, and 11α-hydroxyprogesterone 11α-hemisuccinate: BSA (both at 20 moles steroid: 1 mol BSA), were used. Animals were immunized by intradermal multiple site technique following the schedule described by ILLERA *et al.* (11). Titer of the antiserum was assessed during immunization by EIA, and once the required amount was achieved, polyclonal antibodies were purified (14). Characterization of these antibodies was done by the method of RODBARD (24). 17β-estradiol and progesterone were labelled with horseradish peroxidase (HRP) by the mixed anhydride method (4) using 17β-estradiol 3-hemisuccinate, and progesterone 3-carboxymethyloxime. Conjugates were stored freeze-dried until assay. Assessment of the conjugates was also done using the reported methods (21).

Enzyme immunoassay procedure.– Assays were conducted essentially as reported by JONES and MADEJ (15) and SILVÁN *et al.* (7, 17). Ninety-five of the 96-well Dynatech flat bottomed polystyrene microtiter plates were coated with 100 µl/well of appropriately antibody solutions (progesterone: 1/8,000 and 17β-estradiol: 1/4,000, in coating buffer: sodium carbonate, 50 mmol/L, pH 9.6), and incubated overnight at 4 °C. The first well acted as a plate/assay blank. Before the assay 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 prepared by solubilizing them in ethanol, evaporating the solvent and redissolving in assay buffer (sodium phosphate, 100 mmol/L, pH 7.0, with sodium chloride, 8.7 g/L, BSA, 1 g/L). Standard curves covered a range between 0 to 1 ng/well (100 µl), and were constructed by using eleven standard solutions: 0.5, 1, 2.5, 5.0, 10.0, 25.0, 50.0, 100, 250, 500 and 1,000 pg/well. Standards and culture medium samples were analyzed in duplicate, and 100 µl were transferred to each well from the 2-11 rows. In the wells of the 1 and 12 rows, assay buffer (100 µl) was added. These served as maximum binding wells or "zero standard" wells (Bo), and were used to determine the enzyme activity bound to solid phase antibody in absence of free hormone (1). Plates were covered with a self-adhesive plastic sheet and incubated 3 h at 37 °C. Then plates were emptied by inversion and washed 5 times with wash solution. Conjugate working solutions were prepared by diluting a 1:100 concentrate stock solution of both progesterone-HRP and 17β-estradiol-HRP, 1:80,000 and 1:30,000 respectively, in cold assay buffer (4 °C), and 100 µl of these solutions were added to each well across the entire plate. Plates were covered and incubated again for 2 h at room temperature in darkness. To separate free from bound hormone plates, these were emptied by inversion, washed 5 times with wash solution, and blotted dry.

Fresh substrate solution (100 µl, pH 5.0) was added to all wells, to determine the amount of conjugate bound to solid phase antibodies. The enzyme conversion of the substrate, manifested as a colour change, was stopped after 15 min by the addition of 100 µl of 1M phosphoric acid. Absorbance was read at 450 nm in an automatic microplate reader. Progesterone and 17β-estradiol concentrations were calculated by means of software developed for this technique (ELISA AID, Eurogenetics, Belgium). Standard-dose response curves were constructed by plotting the binding percentage (B/Bo x

100) against 17β-estradiol and progesterone standard concentrations being added. 17β-estradiol and progesterone concentrations were expressed in ng/ml.

RIA procedure.– The RIAs for progesterone and 17β-estradiol were performed as instructed on DPC Coat-A-Count total progesterone and 17β-estradiol kits counting for gamma emissions. For the adaptation of this method to culture maturation medium samples an extraction step was necessary. Fifteen culture medium samples obtained from IVM procedure were extracted with diethyl ether (100 µl of sample in 2.0 ml of ether), the solvent was evaporated under a nitrogen stream at 45 °C, the extract being dissolved in assay buffer.

In vitro *maturation of oocytes.*– In this experiment does were killed with an overdose of pentobarbitone sodium (iv), their ovaries were immediately removed, and placed in 5 ml Brackett's defined medium (2) in a 60 mm Petri dish. Four does acted as ovary donors on the same day. The medium was filtered through 0.22 µm cellulose filters for sterilization. Selected follicles (> 1 mm in diameter) were sliced under a dissecting microscope according to JELINKOVA *et al.* (14). Collected oocytes were transferred onto 35-mm plastic Petri dishes containing 1.0 ml of Brackett's medium (equilibrated at 37 °C, in an atmosphere of 5 % CO_2 in air, 100 % humidity and pH 7.3-7.4), followed by 5 washes. According to pre-defined criteria (17), healthy cumulus-oocytes were divided for culturing either as cumulus-oocyte complexes (with intact and unexpanded cumulus: COC) or denuded oocytes (without layers of cumulus cells: DO), and placed under 1.0 ml of Brackett's medium and cultured in 35-mm Petri dishes, at 37 °C in 5 % CO_2 in air and 100 % humidity, for 14-16 h. Neither serum nor hormones were added to the maturation medium. The oocyte maturation experiment was repeated seven times with different batches of ovaries. The collection of oocytes and preparation of cultures were routinely completed within 1.5 to 2 h. At the end of the culture period, the oocytes were fixed in acetic acid:ethanol (1:3) and stained with aceto-orcein to ascertain their meiotic stage under phase-contrast microscope, as previously reported (20). Oocyte maturation rate was calculated from the percentage of oocytes that had achieved a metaphase II stage after 16 h of culture. Culture maturation medium samples for immunoassay were collected after completion of oocyte cultures, centrifuged at 2,500 *g* for 10 min, and supernatants stored at –30 °C for quantification of 17β-estradiol and progesterone by competitive EIA.

Addition of IGF-I to oocyte culture.– To validate the use of our EIAs in a biological study, oocytes (COC and DO) were cultured in presence of IGF-I at 0, 50 and 100 ng/ml. At the end of culture, steroid levels were analyzed as mentioned above.

Statistics.– The statistical analysis of hormone results was performed by using the BMDP (Biomedical Data Program) (8) an analysis of variance and a regression analysis between EIA and RIA being carried out. All values were expressed as mean ± SE. Intra- and interassay coefficients of variation (%) were calculated by RODBARD'S method (24). The significance of cumulus cells and IGF-I effects on steroid production during oocyte maturation were determined by Catmod procedure of Statistical Analysis System (SAS/STAT) (25). Percentages were compared using the Chi-square test. Only P values less than 0.05 were considered significant.

Results

Antibody production and characterization.– Polyclonal 17β-estradiol and progesterone antiserum reached the required titer for purification after an immunization period of four months (1/1,000 by EIA). After purification, 17β-estradiol antibodies (C902) showed a titer of 1/4,000, and progesterone antibodies (C914) showed a titer of 1/8,000, estimated by EIA. Specificity of the antibodies expressed as percentage of cross-reactivity to related steroids, is summarized in table I. The percentage of cross reactivity is defined as: 50 % inhibition point of respective dose-response curves expressed as ng 17β-estradiol or progesterone/ng related steroid x 100. The main cross-reactant steroids for progesterone were: 11α-hydroxyprogesterone (14.03 %) and pregnenolone (21.73 %); and for 17β-estradiol were: 6-keto-17β-estradiol (20.00 %) and 16-keto-17β-estradiol (16.70 %). Other cross-reactants were insignificant (less than 10 %).

Conjugate assessment.–The assessment of the conjugates showed the following results: 17β-estradiol:HRP ratio was 1.2:1 moles of 17β-estradiol per mol of HRP, while the progesterone:HRP ratio was 0.9:1. The recovery of the enzyme activity after conjugation was more than 85% in both cases, and the determination of the immunoreactivity of the conjugate resulted in a working solution of 1/30,000 for 17β-estradiol, and 1/80,000 for progesterone.

Table I. *Percentage of cross-reactivity of various steroids compared to 17β-estradiol for its antibody, and to progesterone for its antibody.*

Steroid compound	17β-estradiol	Progesterone
17β-estradiol	100.00	–
6-keto-17β-estradiol-carboxymethyloxime	130.00	–
16-keto-17β-estradiol	16.70	–
6-keto-17β-estradiol	20.00	–
2-hydroxyestradiol	8.74	–
6-hydroxyestradiol	9.30	–
17β-estradiol 3-benzoate	3.28	–
17α-estradiol	0.05	<0.01
Estrone	0.50	<0.01
Estriol	0.05	<0.01
Testosterone	0.10	<0.46
Cortisol	<0.01	<0.01
Progesterone		100.00
11α-hydroxyprogesterone-hemisuccinate	–	110.00
11α-hydroxyprogesterone	–	14.03
17α-hydroxyprogesterone	–	<0.01
20α-hydroxyprogesterone	–	<0.01
20β-hydroxyprogesterone	–	<0.01
Pregnenolone	–	21.73

Enzymeimmunoassay 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 EIAs was tested by determining the recovery rates of known amounts of 17β-estradiol and progesterone 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 % for 17β-estradiol and 98.65 ±1.07 % for progesterone. Precision of 17β-estradiol and progesterone EIAs was determined by calculating the intra- and inter-assay CV (%). The intra-assay CV (%) was calculated by replicate measurements of three standard concentrations of 17β-estradiol and progesterone: low: 30 pg/ml, medium: 300 pg/ml and high: 3 ng/ml, 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 interassay CV (%) are summarized in table II, being less than 8% in all cases. The sensitivity of the EIAs was tested by means of low detection limit, and calculated from Bo values minus 2 SD in 10 consecutive assays. Low detection limits were: 1.99 pg/well and 13.21 pg/well (or pg/100 µl) for 17β-estradiol and progesterone, respectively. In order to determine the effects of culture maturation medium in the 17β-estradiol and progesterone standard curves, several dose response curves were run with 17β-estradiol and progesterone standards diluted both in assay buffer 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 curves for both hormones. All dose response curves are parallel, indicating that there is little or no culture maturation medium effect in the progesterone and 17β-estradiol EIAs, since the curves of culture maturation samples diluted in EIA buffer are parallel to the standard curves. Regression analysis between RIA and EIA using the same culture maturation samples showed correlation coefficients of R = 0.96 and R=0.99 (n = 15, $P < 0.001$ in both cases), for 17β-estradiol and progesterone, respectively.

17β-estradiol and progesterone concentrations in culture maturation medium samples.– Assay of culture maturation medium samples yielded the results expressed in table III. In the IGF-I treatments, the obtained concentrations of progesterone and 17β-estradiol were significantly higher compared to control in the COCs. Denuded oocytes significantly originated lower both 17β-estradiol and

Table II. *Intra- and interassay coefficients of variation (%) for 17β-estradiol and progesterone EIA added to control maturation medium and for six culture medium samples from oocyte IVM.*

	17β-estradiol		Progesterone	
pg/ml	Intra-assay	Inter-assay	Intra-assay	Inter-assay
30	3.9	5.1	5.1	6.8
300	4.2	4.8	4.3	5.2
3000	3.1	5.2	2.4	5.8
Medium samples	5.3	7.8	6.3	7.4

Table III. *17β-estradiol and progesterone concentrations (ng/ml) in culture maturation medium samples from rabbit oocytes matured* in vitro.
Different superscripts between rows denote significative differences (a vs b, $P < 0.05$; a,b vs c, $P < 0.01$; a, b vs d, $P < 0.05$). COC; cumulus-oocyte complexes, DO: denuded oocytes, n: number of samples.

Treatment	Type of oocytes	N° oocytes cultured	n	17β-estradiol (mean ± SE)	Progesterone (mean ± SE)
(no oocytes cultured)		–	5	0.00	0.00
Control	COC	104	7	2.55 ± 0.06^{a}	1.52 ± 0.05^{a}
	DO	97	6	0.19 ± 0.02^{b}	0.25 ± 0.02^{b}
IGF-I					
50 ng/ml	COC	114	7	6.27 ± 0.27^{c}	3.70 ± 0.21^{d}
	DO	119	7	0.29 ± 0.01^{b}	0.21 ± 0.01^{b}
100 ng/ml	COC	148	7	6.08 ± 0.31^{c}	2.95 ± 0.09^{d}
	DO	101	7	0.27 ± 0.01^{b}	0.26 ± 0.02^{b}

progesterone concentrations at the end of culture period, compared to those oocytes with cumulus cells, in control both and in the IGF-I treatments.

Discussion

The quantification of 17β-estradiol and progesterone concentrations is decisive for the knowledge of the ovarian physiology, such as the follicular growth and development, oocyte maturation, ovulation, and atresia among others (3, 9, 13, 30). From a functional viewpoint, interactions between some follicular fluid factors (i.e. growth factors) and cumulus cell-enclosed oocytes *in vivo* may result in changes in steroid production in follicular cells, and thus indirectly affect oocyte maturation. Therefore, 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 hormone 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, progesterone and their metabolites in culture maturation media. EIA methods have demonstrated that they unite these features (15, 16, 22), and this led us to validate a direct, simple and precise heterologous amplified EIA.

Culture maturation medium composition differs from the biological fluids in which the EIA has been applied for determining 17β-estradiol and progesterone concentrations, so that the specificity of the assay should be enhanced, this being made possible by improving antibody specificity (10-15). The titers of our polyclonal antibodies were 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 (18, 26). These results allowed excellent discrimination between 17β-estradiol and progesterone and their possible metabolites after culture maturation period. The sensitivity of the assay is also improved by the use of a heterologous bridge system (20) and requires a good labeling of the hormone with the enzyme, allowing for development of the competitive reaction between the labeled and unlabeled 17β-estradiol and progesterone under the best assay conditions (21). The assessment of our conjugates showed optimum ratios, 17β-estradiol-progesterone: HRP of 1.2:1 and 0.9:1, respectively (7, 21). The recovery of enzyme activity after conjugation (85 %) confirmed optimal use over an

extended period of time if the storage of the conjugate is adequate.

Parallelism tests confirm the excellent discrimination between 17β-estradiol and progesterone 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 coefficients between both techniques (more than 95 %), confirming once more the specificity, sensitivity and precision of our EIA system. In this sense, both the EIA intra- and interassay CV (%) are within acceptable limits, and are similar to those obtained with a more traditional RIA (31); moreover, the characteristics of standard curves, the specificity of the EIA, and the addition of known amounts of steroid hormones (17β-estradiol or progesterone) to culture maturation medium samples showed results also comparable with those obtained with the RIA.

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 (18, 19, 21, 27, 28). The introduction of an amplification step increases sensitivity and precision and unlike previous EIAs for measurement of steroids in biological fluids (15, 19), we found that extraction of culture maturation medium samples with organic solvents to determine progesterone and 17β-estradiol levels was not required prior to sample measurement, confirmed once more by parallelism tests, thus considerably simplifying the assay procedure and reducing the time of performance. However, since all the culture maturation medium samples used in the present study were obtained from serum-free medium, extraction requirements should be evaluated prior to use EIA with serum-containing medium.

In this study, the culture of denuded oocytes significantly originated lower concentrations of both, 17β-estradiol and progesterone at the end of the culture, compared to oocytes with intact cumulus cells (COCs). These data are consistent with the results obtained in nuclear maturation (metaphase II stage; data not shown), and demonstrated that the cumulus cells mediate in the signal that initiates oocyte maturation (29). These observations confirm the possibility that the steroid status of the cumulus cells surrounded oocyte regulates the modulation of c-AMP dependent meiosis arrest, and the nuclear maturation of the oocyte (32). Use of IGF-I supplementation greatly elevated 17β-estradiol production by cumulus cells. These results could be due to changes in the steroidogenesis pathways, in this species, produced by the cumulus cells in response to IGF-I stimulation.

In conclusion, we report here the validation of a simple, reliable, efficient, specific and precise EIA for determining 17β-estradiol and progesterone 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. Further effect of growth factors on cumulus cells steroidogenesis during the oocyte maturation are under way.

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ILLERA. *EIA para estradiol-17β y progesterona en medio de cultivo para procedimientos de maduración* in vitro *de oocitos.* J. Physiol. Biochem. (Rev. esp. Fisiol.) 53 (3), 271-280, 1997.

Se valida un enzimoimmunoensayo (EIA) para determinar estradiol-17β (E2) y progesterona en medio de cultivo de oocitos de coneja madurados *in vitro*, en presencia de IGF-I, sin extracción previa. Los anticuerpos policlonales anti-E2 y anti-progesterona se obtienen de conejos utilizando 6-keto-17β-estradiol 6-carbometiloxima:BSA y 11α-hidroxiprogesterona 11α-hemisuccinato:BSA; la peroxidasa de rábano picante se utiliza como enzima marcadora. La curva estándar cubre un intervalo desde 0 hasta 1 ng/pocillo. El límite de detección menor de la técnica es de 1,99 y 13,21 pg/pocillo para el E2 y la progesterona, respectivamente, mientras que los coeficientes de variación intra- e interensayo son menores a 6,3 % y 7,8 % para el E2 y la progesterona, respectivamente (n = 10). El porcentaje de recuperación es del 96,39 % y 98,65 %, respectivamente para el E2 y la progesterona. Los valores obtenidos por EIA se correlacionan con los obtenidos en el RIA para el E2 (R = 0,96; P < 0,001) y la progesterona (R = 0,99; P < 0,001). Las muestras del medio de maduración se obtienen tras 16 horas de maduración *in vitro* de los oocitos. La suplementación con IGF-I aumenta los niveles de esteroides en los oocitos rodeados de cúmulo celular. El EIA descrito es un ensayo sensible y específico, y además es una alternativa simple, barata y no radioactiva al RIA para la determinación de E2 y progesterona en el medio de cultivo de oocitos.

Palabras clave: Estradiol-17β, Progesterona, EIA, Medio de cultivo, Oocito.

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