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Detection and elimination of human exposure to environmental hormone disrupting substances.

Sertoli cell primary cultures assay protocol

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Experimental procedure Murine Sertoli cell primary cultures.

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A Reagents

All reagents, unless specified, were purchased from Sigma (St. Louis, USA). Recombinant human FSH (rhFSH; Puregon 100 I.E.) was granted by Organon (Oss, the Netherlands). Dulbecco's modified Eagle's medium: Ham's F-12 medium (DMEM:Ham's F-12) and fetal calf serum (FCS) were purchased from Life Sciences International (Zellik, Belgium). Mayer's Hematoxylin was purchased from Merck (Darmstadt, Germany).

B Isolation and culture of Sertoli cells

Sertoli cells were isolated from the testes of 18-day-old Sprague-Dawley rats using a method adapted from Monsees et al., 1996. Decapsulated testes were minced into small fragments and incubated for 30 min at 37°C in DMEM: Ham's F-12 (1:1) containing collagenase (1mg/ml) and Dnase (20µg/ml). For the second and third digestion a solution of collagenase (2mg/ml), and Dnase (20µg/ml) in DMEM: Ham's F-12 (1:1) was used for 30 min. at 37°C, and 15 min. at 37°C respectively. Enzymatic reactions were stopped by a brief treatment with soybean trypsin inhibitor at 400µg/ml DMEM: Ham's F-12. Remaining Sertoli cell clusters were broken up by gentle homogenization using a homogenizer. The resulting cell fraction was cultured overnight in DMEM: Ham's F-12 (1:1) supplemented with 10% FCS, 2 10⁻³ M L glutamine, 100 U/ml penicillin, 10ng/ml epidermal growth factor, 5µg/ml human transferrin, 2µg/ml insulin, 10⁻⁸ M hydrocortisone, 200ng/ml vitamin A, 200ng/ml vitamin E, 2.88ng/ml (10⁻⁸ M) testosterone, and 3ng/ml cytosin arabinoside (medium B). Sertoli cell preparations were plated at 1 million cells/ml or at 2 million cells/ml per well (2 cm²) in a 24-well plate in 1mL. The next day (day 2) the medium was replaced by medium without FCS. On day 3 of culture the medium was removed and the Sertoli cell monolayer was treated with a hypotonic solution (20mM Tris-HCl, pH 7.5, 5 min., 20°C) to remove contaminating germ cells (Galdieri et al., 1981); thereafter medium B without FCS and cytosin arabinoside was added (medium C).

Control or basal cultures were maintained in medium supplemented with 10ng/ml epidermal growth factor, 5µg/ml human transferrin, 2µg/ml insulin (medium D).

C Analysis of myoid and germ cell contamination of the Sertoli cell cultures

To check the purity of the culture, four millilitres of 1 10⁶ cells/ml were distributed in 8-well Biocoat® cultureslides (BD Biosciences, Erembodegem, Belgium) and incubated at 34°C in a humidified, 5% CO₂ atmosphere. Sertoli cells and germ cells were identified by staining with Mayer's hematoxylin and examination of morphology.

D Effect of incubation time on inhibin B secretion

On day 6 of culture, the medium was removed and Sertoli cells were incubated with medium D. In order to compare the inhibin B accumulation in the culture media after 48 or 72 hours with the total inhibin B accumulation in two or three 24 hour periods, during the next three days of culture, the medium was renewed every 24 hours, or after 48 or 72 hours. Each time the removed medium was aliquoted and stored at -80°C until measurement of inhibin B.

E Influence of rh FSH on inhibin B secretion

On day 6 of culture, the medium was removed and Sertoli cells were incubated with medium C supplemented with different concentrations of rh FSH (1 U/l, 2.5 U/l, 5U/l and 10 U/l) for 24, 48 and 72 hours. On day 10 the medium was removed and replaced with medium lacking rh FSH, in order to allow the cells to “rest” for one day. The next day (11th) the medium was removed and the same wells were incubated again with medium containing different concentrations of rh FSH (1 U/l, 2.5 U/l, 5U/l and 10 U/l) for another 24, 48 and 72 hours.

The removed medium was aliquoted and stored at -80°C until measurement of inhibin B.

F Influence of testosterone (T) on inhibin B secretion

To study the effect of T on inhibin B production by immature rat Sertoli cells, the Sertoli cells obtained on day 6 were cultured for another six days in medium D supplemented with 2.884ng/ml, 5ng/ml or 50ng/ml of T, in the presence or absence of rh FSH (2.5 U/l and 5U/l). The medium was removed every 24 hours, aliquoted and stored at -80°C until measurement of inhibin B.

G Influence of 17 α -estradiol (E2) on inhibin B secretion

The purified Sertoli cells were cultured for 24, 48 or 72 hours in culture medium D supplemented with 0.01ng/ml or 0.10ng/ml of E2, in the presence or absence of rh FSH (2.5 U/l and 5U/l) or T (5ng/ml or 50ng/ml). The medium was removed every 24, 48 or 72 hours, aliquoted and stored at -80°C until measurement of inhibin B.

H Measurement of inhibin B

Inhibin B was measured in duplicate using the inhibin-B dimer assay kit (Serotex Limited, Oxford, UK) according to the manufacturer’s protocol. It is a double antibody enzyme-immunometric assay using a monoclonal antibody raised against the inhibin β B-subunit in combination with a labeled antibody raised against the inhibin α -subunit. The assay is based on a previously published method for measuring inhibin B (Groome et al., 1996).

The detection limit was 15.3pg/ml; the intra-plate and inter-plate coefficients of variation were < 5.2% and < 10.5% respectively for each assay performed.

I Statistical analysis

The results are reported as the mean value calculated from 2 different plates for each parameter measured in duplicate.

The effect of each of the different concentrations of rh FSH, T and E2 on inhibin B secretion in the medium was studied by analysis of variance (ANOVA), followed by Student-Newman-Keuls test for pairwise comparisons, and $P < 0.05$ was considered significant. Statistical analysis was performed using the MedCalc® program (MedCalc Software, Mariakerke, Belgium)(Schoonjans et al., 1995).

J References

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