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

Pituitary LβT2: assay protocol

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Experimental procedure Pituitary LβT2 cell line.

A detailed description of this procedure is in preparation, and will be presented for publication in a peer reviewed international journal.

A Chemicals and Media

Dulbecco's Modified Eagle Medium (DMEM), phenol red free DMEM, Hanks Balanced Salt Solution (HBSS), D-glucose, L-glutamin, Phosphate Buffered Saline (PBS), trypsin/EDTA, penicillin/streptomycin, fetal calf serum (FCS), agarose, TBE-buffer (10x) and DNA ladders were obtained from Invitrogen N.V. (Merelbeke, Belgium). The Fluo3-probe for the calcium mobilization assay was obtained from Molecular Probes (Leiden, The Netherlands). Charcoal was purchased from Merck (Darmstadt, Germany). Dextran T70 was obtained from Amersham Biosciences Europe GmbH (Roosendaal, The Netherlands). Primaria six well plates and Matrigel were obtained from BD Biosciences (Erembodegem, Belgium). RNA extraction was performed using a commercial RNA extraction kit (High Pure RNA isolation kit) from Roche Diagnostics (Brussels, Belgium). PCR buffer, MgCl2, Amplitaq Gold, MuLV reverse transcriptase, RNase inhibitor and dNTP's were purchased from Applied Biosystems (Applera, Lennik Belgium). Ethidium bromide was from Sigma-Aldrich N.V. (Bornem, Belgium). Oligonucleotides were custom made by BioSource Europe, N.V. (Nivelles, Belgium).

B Cell culture:

LβT2 cells (kindly provided by Dr. Pamela Mellon, University of California, San Diego) were maintained in phenol red DMEM, supplemented with 10% Fetal Calf Serum (FCS), 100 U/mL penicillin and 100µg/mL streptomycin (growth medium1) in a humidified atmosphere (37°C) of 5% CO2. At weekly intervals, cells were passaged (1/3-1/5) using trypsin/EDTA.

C Mycoplasm test:

To check the LβT2 cell line for a possible Mycoplasm infection, cells were cultured on cover slides. After 2-3 days, they were fixed with ice-cold ethanol (-20°C for 15 minutes) and stained with 4',6-diamidino-2-phenylindole (DAPI) (1/100 dilution of 40 µg/ml in PBS) for 15 minutes at room temperature under dark conditions. The cover glasses were fixed on a carrier and analyzed using fluorescence microscopy. No mycoplasm infection was observed.

D Calcium mobilization assay:

Cells were trypsinized and seeded at a concentration of 25xE3 cells per labtek chamber and incubated at 37°C. After 24h, cells were incubated for 45 minutes with 240 µl of a Fluo-3 solution (20 µl Fluo3-stock (=1 mg/ml DMSO + 12% pluronic acid), dissolved in 1980 µl HBSS) under dark conditions. After addition of GnRH (diluted to the desired concentration in HBSS), calcium mobilization was analyzed for 3 minutes using a confocal microscope.

E Determination of cell concentration

E.1 Bürker counting chamber:

After trypsinization, cells were incubated with Trypan blue (0.4%; 1/1) and counted using a Bürker counting chamber. Living cells excrete the dye and are transparant under the microscope, in contrast to dead cells that stain blue.

E.2 Coulter counter:

After trypsinization, 20 µl of the cell suspension is diluted with 10 ml of an isotonic solution and counted with a Coulter counter (Beckmann Coulter Inc, distributed by Analis, Ghent, Belgium).

F Sulforodamine B (SRB) assay:

In preparation.

G Charcoal-dextran treatment of Fetal Calf Serum:

In preparation.

H Stimulation experiments:

In preparation.

I LH radioimmunoassay

LH concentrations were determined using the RPA552 RadioImmunoAssay (RIA) kit for rat Luteinizing hormone from Amersham Biosciences Europe GmbH (Roosendaal, The Netherlands). The kit was basically executed according to the protocol of the manufacturer, except that we used the half of the necessary volumes. This didn't alter the results and allowed us to analyze the double amount of samples. To determine the influence of the medium matrix (DMEM with or without FCS), 100 µl of medium containing 0.5 ng of rat LH standard, was incorporated in the test. The obtained results were 0.6±0.03 ng/100 µl for DMEM without FCS and 0.58±0.01 ng/100 µl for DMEM supplemented with FCS.