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A simple short term method to study thyroid disruption using a fetal rat thyroid culture

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ABSTRACT

Introduction: Thyroid modulation activity has not been investigated for many chemical substances. Due to ethical, practical and financial reasons, *in vivo* evaluation of a large number of compounds is not feasible. It has been proposed that an *in vitro* mechanism-based strategy could be more adequate for the identification of thyroid hormone disrupting chemicals. Here we describe a simple and mostly inexpensive, short term culture assay to study thyroid disruption.

Methods: Fetal thyroids collected from gestation day 20.5 were cultured up to 24 h in Hank's saline solution, at 37 °C with oxygenation at 0 and 12 h. Viability of the cultured explants was evaluated by the MTT assay. Positive (thyroid stimulating hormone, TSH) and negative (6-propyl-2-thiouracil, PTU) modulation of cultured thyroids was assessed with morphometrical analysis of H & E stained gland sections. Thyroxine expression was evaluated by immunohistochemistry.

Results: Viability was shown to increase with time of culture with higher metabolic activity being achieved at 24 h as compared to shorter periods of incubation. Follicular epithelial cells exhibited a statistically significant dependence on thyrotropin concentration, although more evident in the inner than in the outer portion of the glands. As expected, TSH induced expression of thyroxin while PTU inhibited it.

Discussion: GD20.5 fetal thyroids may be cultured up to 24 h under relatively simple laboratory conditions during which viability and function of the gland are preserved showing that it is possible to reproduce *in vivo* response under *in vitro* conditions. This culture could be a suitable short term assay to study mechanism of thyroid disruption.

1. Introduction

Thyroid hormones (TH) are essential for normal physiological processes and play a significant role in brain development during pregnancy (Patel, Landers, Li, Mortimer, & Richard, 2011). Synthesis of thyroid hormones begins with iodide uptake into the follicular cells, a transport that is mediated by the sodium/iodide symporter (NIS). The thyroid epithelium then secretes Tg into the follicular lumen where thyroperoxidase (TPO) catalyzes coupling of iodine into Tg forming triiodothyronine (T3) and thyroxin (T4). Both hormones are reabsorbed into the epithelial cells before being released into circulation (Mihai, 2014).

A series of chemicals, including dietary components and biogenic and anthropogenic environmental pollutants, have shown thyroid disruption activity (Duntas & Stathatos, 2015; Maqbool, Mostafalou, Bahadar, & Abdollahi, 2016). However, thyroid modulation activity has not been investigated for many other chemical substances (Murk et al., 2013). Due to ethical, practical and financial reasons, *in vivo* evaluation of a large number of compounds is not feasible and it has been proposed that an *in vitro* mechanism-based strategy could be more adequate for the identification of thyroid hormone disrupting chemicals (Murk et al., 2013). All steps along thyroid axis including TH synthesis, catabolism and clearance, binding to transport proteins and entry into cells, deiodinase activity, transcriptional activity of TH receptors and TH modulation or gene expression, may be targets to thyroid disruptors (Jugan, Levi, & Blondeau, 2010).

Action of chemicals on TPO activity isolated from human or rodent follicles, microsomes or partially purified protein fractions has been evaluated (Freyberger & Ahr, 2006; OECD, 2014). Likewise, NIS inhibition may be studied by ^{125}I capture in human-NIS-transfected-HEK293 cells (Lecat-Guillet et al., 2007) and by a non-radioactive spectrophotometric method in FRTL5 cells (Waltz, Pillette, & Ambroise, 2010). However, biological meaning of TPO and NIS *in vitro* activity may be distorted by the lack of functional relationships with up- and downstream events in the thyroid gland. For example, TPO is co-localized in the apical membrane with dual oxidase and both enzymes are required

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for TH synthesis. On the other hand, monolayer cell cultures are not able to form polarized follicles, something that is a structural requirement for iodine organification in the *in vivo* gland (Massart et al., 2011).

Short term assays using zebrafish, sea urchin, and xenopus embryo or organ explants cultures may be used to overcome cell culture limitations when studying thyroid function (OECD, 2014). Other assays include thyroid follicle culture (Spinel-Gomez, Colin, Van den Hove, & Denef, 1990), organotypic thyroid culture on a tridimensional collagen matrix (Toda et al., 2002) and whole organ culture, the latter being the only model capable to show not only interaction among cellular matrix, cytokines, growth factors and other cell types including endothelial and nervous (Delmarcelle, Villacorte, Hick, & Pierreux, 2014; Toda et al., 2011) but also to maintain the tridimensional architecture required to model the *in vivo* conditions (Vickers et al., 2012). *Xenopus laevis* explant cultures are described in the literature (Hornung et al., 2010; OECD, 2014).

Fetal rat thyroids may also be cultured under relatively simple conditions (Young & Bakert, 1982) and respond well to physiological stimuli such as iodine, thyroxine and insulin (Nataf, 1968). Rodents are more sensitive than humans to thyroid axis disruption due to their higher expression of NIS and lower thyroid hormone reserve capacity (Lewandowski, Seeley, & Beck, 2004; Vickers et al., 2012). This higher sensitivity may be advantageous for an *in vitro* model for the qualitative study of thyroid disruptors (Lewandowski et al., 2004). On the other hand, rat fetal thyroid may be easier to handle and culture than the adult organ acting as a good model for fetal thyroid axis disruption. Here, we report a simple and inexpensive method for culturing gestation day (GD) 20.5 fetal rat thyroid glands. We describe experimental conditions feasible to establish in any laboratory, which allow the glands to remain viable for 24 h and respond to positive and negative modulators of thyroid function. We propose that this culture could be a suitable short term assay to study mechanisms of thyroid disruption.

2. Materials and methods

2.1. Experimental model

All procedures with experimental animals followed the Guide for the Care and Use of Laboratory Animals (NAS, 2011) and had the approval of the Faculty of Pharmacy Bioethics Committee (Universidad de Valparaíso, Chile). Sprague Dawley rats (250 ± 30 g) were kept under day/night cycle (12/12) and *ad libitum* access to a standard pellet diet (Lab Rat Diet, Champion, Chile) and tap water. One male was caged with three females from 17:00 PM to 9:00 AM. The detection of sperm in vaginal smears taken at the end of overnight caging (9:00 AM) was considered gestation day (GD) 0.5. Pregnant rats were put on a separate cage until GD 20.5 when they were euthanized by carbon dioxide asphyxiation for embryo collection by C-section. Embryos were then killed by decapitation and the thyroid and its adjacent tracheal segment was explanted following the procedure described in Delmarcelle et al. (2014).

2.2. Culture conditions

Thyroids (n = 10 per treament) were individually placed in 10 mL glass vials containing 7 mL of Hank's saline solution (HSS; 1.3 mM $CaCl_2$, 0.137 M NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 4.2 mM NaHCO3, 5.6 mM glucose, 0.25 mM Na_2HPO_4 and 0.44 mM KH_2PO_4). The vials were laid on a rotating plate at 60 rpm in an incubator at 37 °C. The glands were cultivated for 24 h. Prior to the culture, as well as at 12 h of incubation, the culture was oxygenated with a mixture of O_2:CO_2 in a proportion of 20:5.

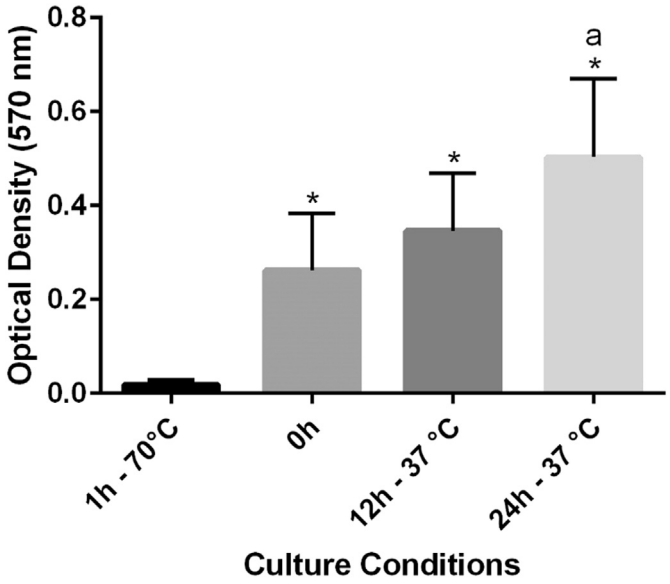

Fig. 1. Optical density of the formazan extracting solution from tissues cultured at different temperature and time. Results are expressed as mean ± standard deviation (n = 10). * denotes a statistically significant difference compared to heat shock treatment (p < 0,05). Letter (a) represents a statistically significant difference between different culture times (p < 0,05).

2.3. Viability of cultured thyroids

Viability of the explant was evaluated by the MTT assay using a technique modified from Nath, Babrekar, and Parthasarathy (2005). Once collected, glands were assigned to one of the following groups (10 glands per group) and viability assessed as described:

Viability at time 0: Explanted thyroids were immediately put into eppendorf tubes containing 75 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Vybrant® MTT Cell Proliferation Assay Kit) for 1 h at 37 °C and under agitation. At the end of incubation, formazan was solubilized with sodium dodecyl sulfate acid solution for 24 h. Absorbance of the solution was determined at 570 nm using a UV/VIS spectrophotometer (Perkin Elmer Lambda 25).
Viability at time 12 h: Explants were cultured for 12 h as described above and at the end of incubation time, viability was determined.
Viability at time 24 h: Explants were cultured for 24 h as described above except for oxygenation at 12 h. At the end of incubation time, viability was determined as described above.
Viability after heat-shock: Immediately after collection of the glands they were subjected to 70 °C for 1 h using a thermal bath. Viability was determined as described above.

2.4. Evaluation of positive and negative modulation of cultured thyroids

Thyroid response to positive and negative modulators in cultured conditions was assessed with morphometrical analysis of H & E stained gland sections and thyroxine expression was evaluated by immunohistochemistry.

2.4.1. Evaluation of positive modulation

Thyroids were cultured as described in 2.2. Glands were randomly assigned to one of four culture conditions consisting of HSS supplemented with either 0, 0.1 mU/mL, 1 mU/mL or 10 mU/mL of bovine thyrotropin (TSH; Sigma T8931-1VL). After incubation, gland tissues were fixed in Bouin's solution for 24 h and dehydrated through a series of alcohols before inclusion into paraffin wax for tissue sectioning. 5 µm sections were obtained using a rotatory microtome (Thermo Scientific,