INHIBITION OF THYROXINE HORMONE SYNTHESIS WITH THYROID DISRUPTING CHEMICALS IN PRIMARY HUMAN THYROCYTES

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ABSTRACT

Perturbation of thyroid hormone synthesis by pesticides and other environmental agents are known to cause numerous developmental, metabolic and cognitive disorders in humans. Data collected from animal studies may or may not reflect the potency and duration of compound-induced effects due to species differences in thyroid stimulating hormone receptor (TSHR), sodium/iodide symporter (NIS) and thyroid peroxidase (TPO) sensitivity to exposure. Deisenroth et al. recently described a novel 3D human thyrocyte microtissue culture platform for identifying thyroid disrupting chemicals (TDCs) whose toxicity may occur through various modes of action (Toxicol. Sci. 2020;174:63-78). The aim of this study was to identify characteristics of qualified thyrocyte lots and further optimize assay conditions including plating format, seeding density, microtissue size and exposure period for compound testing. Cryopreserved human thyroid cells (passage 1) from healthy adult donors of both genders ≤55 y.o. and a body mass index of ≤35 were cryopreserved in a serum-free bio-preservation medium. Thyroid cells from multiple batches were thawed in human thyrocyte plating medium (HTPM) with an average yield per vial of ≤0.5x10^6 cells and an average viability of 80%.Thyroid cells were plated on Matrigel-coated 96-well culture plates at a seeding density between 7500 and 2500 cells/well and maintained in human thyrocyte culture medium containing 1mIU/mL bovine thyroid stimulating hormone (HTCM). 3D microtissue formation occurred over the initial 4 days after which a stable size (50-200 µm) was observed. Cultures were then treated with HTCM and media samples collected at day 7 and 14 for TG or T4 measurements, respectively, by ELISA. The results showed that thyroid microtissues seeded between 5000-7500 cells per well produced suitable levels of T4 with sufficient dynamic range for TDC screening (≥3.3 µg thyroglobulin/day/10^{6}cells on day 7 and ≥6.6 ng T4/day/10^6 cells on day 14). Some thyrocyte batches in 3D culture and all batches plated as 2D monolayers exhibited suitable TG production levels at day 7 but reduced or no T4 levels at day 14. Inhibition of T4 synthesis was determined after a 120-h treatment with TPO inhibitors methimazole and 6-propyl-2-thiouracil between days 9 and 14 (n=3). Half-maximal inhibitory concentrations (IC_{50}) for methimazole and 6-propyl-2-thiouracil were 0.4 µM and 0.75 µM, respectively. In conclusion, cryopreserved primary human thyrocytes may be a valuable tool to evaluate and prioritize potential TDCs for human health risk assessment.

INTRODUCTION

The purpose of this study was to validate cryopreserved primary human thyrocytes (PHT) which are isolated from normal adult donor tissues at LifeNet Health in a 96-well 3D microtissue format as an *in vitro* model to determine effects of potential TDCs on various T4-synthetic pathways. The focus on effects of TDCs has been increasing over the past 20 years and EPA urges the development of reliable and relevant non-animal, new approach methods. Due to extensive passage in culture and tumor specific origin, the current immortal thyroid cells typically do not retain the essential characteristics of native thyroid function and signaling pathways. Therefore, there is a need to develop an *in vitro* primary human thyrocyte model isolated from normal thyroids for TDC screening.

MATERIALS & METHODS

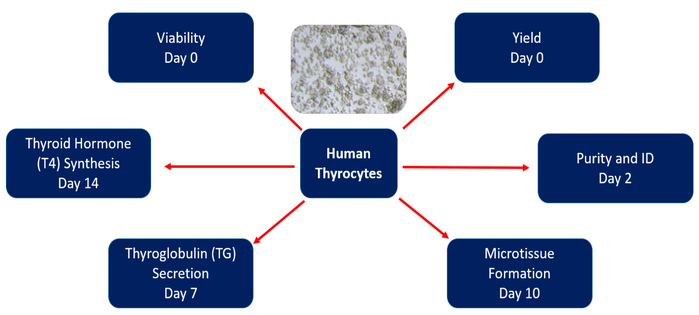


Figure 1. Schematic for characterization of thyrocyte lots.

Donor ID	Sex	Age	Race	BMI
THY2020525	M	22	C	29
THY2021159	M	43	C	23
THY2021598	M	43	AA	32
THY2024220	M	50	AA	22
THY2110448	M	44	AA	32
THY2111845	F	61	C	29
THY2120653	M	55	C	24
THY2211919	F	27	AA	30
THY2215425	F	51	C	28
THY2215668	M	59	C	33

Table 1. Donor specifications.

Isolation of Human Thyroid Follicular Epithelial Cells

Thyrocytes were isolated from healthy thyroid tissues as previously described[1] and cryopreserved using CryoMed controlled-rate freezers. Monolayer cultures and 3D thyroid microtissues were prepared with cryopreserved PHT on TC-treated and Matrigel® (Corning) coated 96-well plates respectively. The 3D microtissue morphology of each batch was assessed over a 14-day period.

Treatments and Assays

Expression of Keratin 7 (KRT7) and Thyroglobulin (TG) were assessed by immunocytochemistry with antibodies from Dako and Santa Cruz respectively. 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain was used to demonstrate DNA content and nuclei. The thyroid microtissues were treated with 0 or 1mIU/mL bovine thyroid stimulating hormone (TSH) (Sigma) from days 2-14. Cells were exposed to TPO inhibitors between days 9-14 in the presence of 1mIU/mL TSH. TG secretion and T4 synthesis were measured at select time points in collected media samples using ELISA kits from Invitrogen.

RESULTS

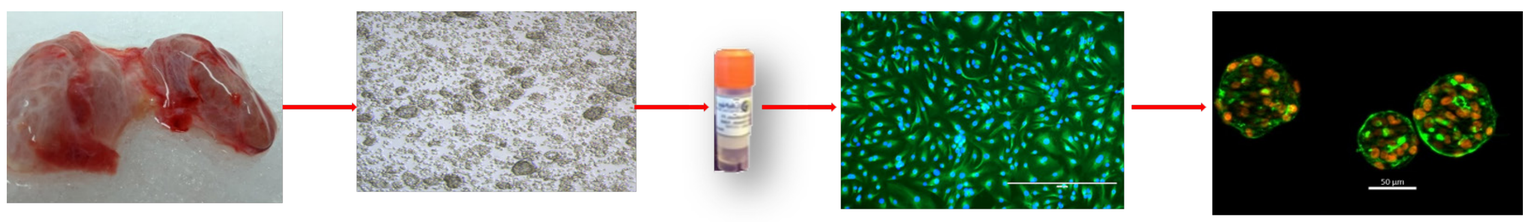


Figure 2. Flowchart for PHT isolation and QC process

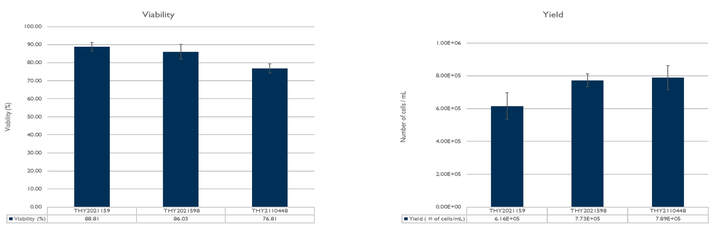


Figure 3. Post-thaw outcomes of three cryopreserved PHT batches. Cell viability and yield were determined with 0.4% trypan blue staining and an automated cell counting system (TC-20, BioRad)

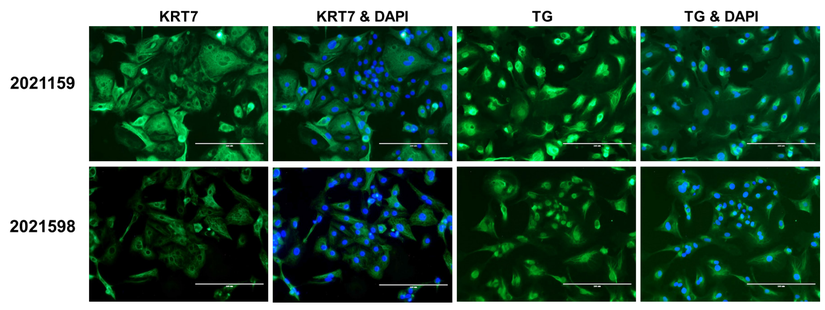


Figure 4. Immunocytochemistry of PHT monolayers in 96-well plates. Representative images of thyrocytes isolated from two separate donor tissues. ICC for KRT7and TG. Cell nuclei were stained with DAPI. Magnification: 20X

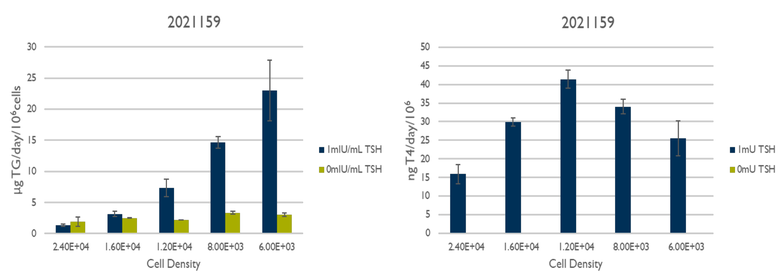


Figure 6.TSH-induced TG secretion at day 7 and T4 synthesis at day 14 from thyrocytes in 3D cultures seeded at five different seeding densities (from 2.4E+04 to 6.0E+03 cells per well). The microtissues were treated with 0 or 1mIU/mL TSH.

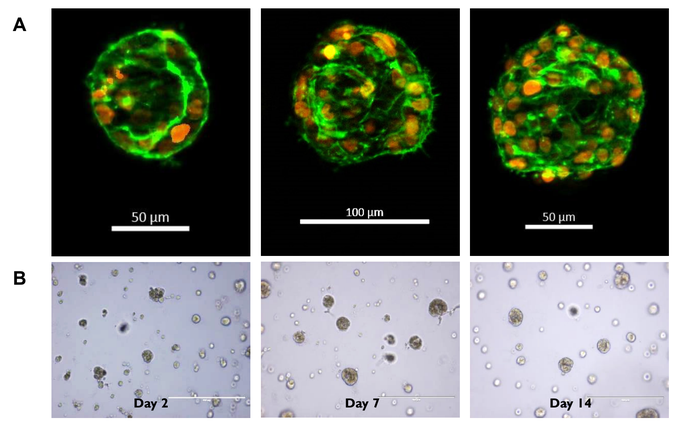
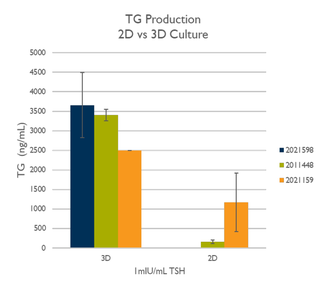


Figure 5. Morphology of 3D microtissues. **A.** Follicle-like morphology of 3D microtissues from three donors at day 9. Microtissues were stained with DAPI (orange) and phalloidin conjugated to Alexa Fluor 488 (green). **B.** Self assembly of 3D thyroid microtissues (Day 2-14). Magnification: 10X



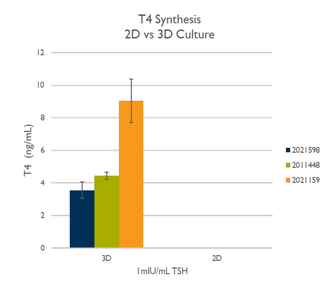


Figure 7.TG secretion (Day 7) and T4 synthesis (Day 14) in 2D and 3D cultures. The thyrocytes were treated with 1mIU/mL TSH starting day 2 (Seeding cell density ~1.2E+04 cells per well).

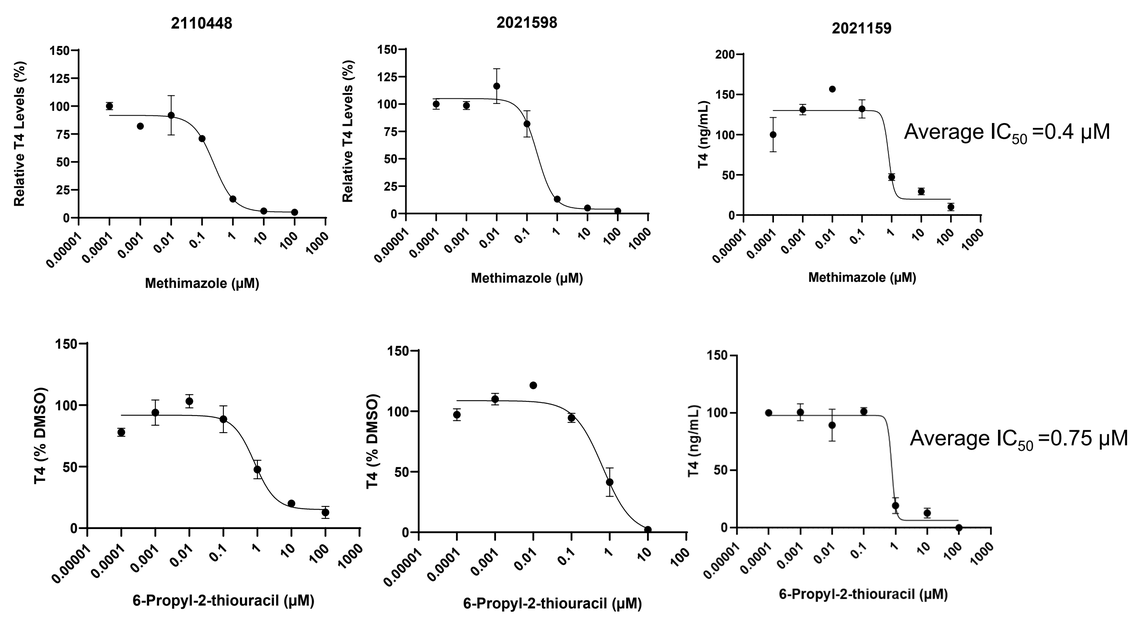


Figure 8. Inhibition of T4 synthesis by TPO inhibitors, methimazole and 6-propyl-2-thiouracil. Matured thyroid microtissues from three healthy donor tissues were exposed to TPO inhibitors (100pM to 100µM) from Day 9 to Day 14. Cytotoxicity was measured by ATP-based luminescence assay at Day 14.

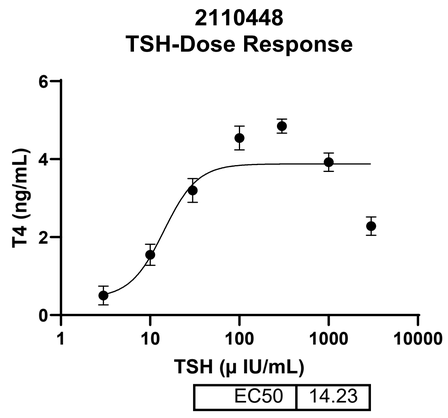


Figure 9. Dose-response curve for the effect of TSH on T4 synthesis. The thyroid microtissues were treated with dose-range of 30µIU-3mIU/mL TSH between days 2-14.

CONCLUSIONS

- ≥80% of the recovered PHT were viable at post-thaw.
- KRT7 and TG expression confirmed that cryopreserved human thyrocytes retain thyroid follicular epithelial cell markers.
- Cryopreserved thyrocytes formed 3D thyroid microtissues (diam: 50µm-150µm) at 14 days of culture.
- TSH promoted TG secretion and T4 synthesis.
- All qualified lots maintained native thyroid functions: TG secretion and T4 synthesis in 3D cultures.
- TPO inhibitors, methimazole and 6-propyl-2-thiouracil inhibited T4 synthesis of thyroid microtissues in a dose-dependent manner.
- Cryopreserved thyrocytes represent a useful *in vitro* tool for the testing of TDCs.

REFERENCES / ACKNOWLEDGEMENTS

1. Deisenroth C *et al.* (2019) Development of an *in vitro* human thyroid microtissue model for chemical screening. *Toxicol* Sci **174:** 63-78.
2. ECHA and EFSA, *et al. (2018)* Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009, EFSA Journal, 16 (2018), Article e05311.

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