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Novel scalable 3D cell based model for *in vitro* neurotoxicity testing: Combining human differentiated neurospheres with gene expression and functional endpoints

Ana Paula Terrasso[a,b], Catarina Pinto[a,b], Margarida Serra[a,b], Augusto Filipe[c], Susana Almeida[c], Ana Lúcia Ferreira[c], Pedro Pedroso[c], Catarina Brito[a,b,*], Paula Marques Alves[a,b]

[a] *iBET, Instituto de Biologia Experimental e Tecnológica, 2780-901 Oeiras, Portugal*
[b] *ITQB-UNL, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa Oeiras, 2780-157, Portugal*
[c] *Medical Department, Grupo Tecnimede Sintra, 2710-089, Portugal*

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ABSTRACT

There is an urgent need for new *in vitro* strategies to identify neurotoxic agents with speed, reliability and respect for animal welfare. Cell models should include distinct brain cell types and represent brain microenvironment to attain higher relevance. The main goal of this study was to develop and validate a human 3D neural model containing both neurons and glial cells, applicable for toxicity testing in high-throughput platforms. To achieve this, a scalable bioprocess for neural differentiation of human NTera2/cl.D1 cells in stirred culture systems was developed. Endpoints based on neuronal- and astrocytic-specific gene expression and functionality in 3D were implemented in multi-well format and used for toxicity assessment. The prototypical neurotoxicant acrylamide affected primarily neurons, impairing synaptic function; our results suggest that gene expression of the presynaptic marker synaptophysin can be used as sensitive endpoint. Chloramphenicol, described as neurotoxicant affected both cell types, with cytoskeleton markers' expression significantly reduced, particularly in astrocytes. In conclusion, a scalable and reproducible process for production of differentiated neurospheres enriched in mature neurons and functional astrocytes was obtained. This 3D approach allowed efficient production of large numbers of human differentiated neurospheres, which in combination with gene expression and functional endpoints are a powerful cell model to evaluate human neuronal and astrocytic toxicity.

1. Introduction

Human brain is a critical target organ of xenobiotics due to its developmental, structural and functional features that make it an extremely complex organ with highly elaborate physical, communicative and metabolic interactions between neurons and glial cells (Allen and Barres, 2009; Moors et al., 2009).

Given the insufficient information available on neurotoxicity and the growing number of chemicals to be tested, new testing strategies are required to identify neurotoxic agents with speed, reliability and respect for animal welfare. Moreover, generate in vitro tests with an increased high-throughput is needed to provide mechanistic data and evaluate more efficiently the toxics that may cause adverse effect in humans (Breier et al., 2010; Breslin and Driscoll, 2013; Tralau and Luch, 2012).

Numerous studies demonstrated that screens focusing solely on the direct effect of compounds on isolated neurons may not model consistently the basic Central Nervous System (CNS) functions and environment and will overlook potentially important neuroprotective and/or neurotoxic activities that act via astrocytes or other non-neuronal cells (Chen and Swanson, 2003; Gupta et al., 2012; Sofroniew and Vinters, 2010). Moreover, cell differentiation and tissue development and homeostasis *in vivo* are strongly dependent on cell spatial arrangement, essential for correct trafficking and communication between neurons and astrocytes and directional cues, so extracellular context profoundly affects cell behavior (Irons et al., 2008). Neurospheres, free-floating aggregates of neural progenitors (Conti and Cattaneo, 2010), allow formation of complex 3D network of cell-cell and cell-extracellular matrix interactions, being

* Corresponding author at: iBET, Apartado 12, 2780-901 Oeiras, Portugal. Tel.: +351 21 4469434; fax: +351 21 4421161.
E-mail address: anabrito@itqb.unl.pt (C. Brito).

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potentially relevant in the penetration and action of drugs (Breslin and Driscoll, 2013). Despite research with neurospheres largely focused on their application for neuroregeneration in CNS diseases, a few studies utilized neurospheres for in vitro developmental toxicity studies, providing support for their use in neurotoxics identification (Breier et al., 2010). Furthermore, neurospheres fabrication procedures have been described by our laboratory and others (Serra et al., 2009; Brito et al., 2012; Moors et al., 2009), using different cell sources, however, none of these systems generates neurospheres enriched in functional astrocytes.

Since the use of human origin tissues is limited due to availability issues, an insufficient potential to generate the necessary number of cells and ethical concerns, efforts should focus on creating affordable and sensitive methods to develop alternative human-cell based in vitro models that closely reconstructs the *in vivo* situation for investigation of neurotoxic effects in human neural cells (Hill et al., 2008; Moors et al., 2009).

A wide range of cell systems have been under investigation for their suitability for inclusion in neurotoxicity test batteries (Mori and Hara, 2013). In recent years, protocols have been developed for the isolation and in vitro culture of human neural stem cells (NSC) or neural progenitor cells as well as for the derivation of human neurons and astrocytes from pluripotent stem cells (hPSC), both embryonic (hESC) and induced (iPSC) (Molofsky et al., 2012; Mori and Hara, 2013). Although the use of latter holds great promise and remarkable advances have been made in expansion, differentiation and characterization, numerous challenges must be overcome before the use of this technology can be widespread (Mori and Hara, 2013; Serra et al., 2010).

Human pluripotent embryonic carcinoma cell lines, such as NTera-2/cloneD1 (NT2), represent an alternative that can provide an unlimited number of cells, with less time-consuming and well characterized neuronal differentiation protocols (Brito et al., 2007; Serra et al., 2009). NT2-derived neurons have been shown to be a valuable model for in vitro developmental neurotoxicity testing (Laurenza et al., 2013; Stern et al., 2013), demonstrating advantages regarding the identification of potential neurotoxicants. A few works described astrocytic differentiation of NT2 cell line, based on RA neural induction and increased differentiation times, in 2D culture systems (Bani-Yaghoub et al., 1999; Goodfellow et al., 2011).

The present work focused on the development of a robust process for generation of a human 3D cellular model, enriched in functional neurons and astrocytes and suitable to feed high-throughput neurotoxicity studies, using the human pluripotent NT2 cell line as cell source. Having as starting point a scalable process based on stirred suspension culture system previously developed by us for NT2 neuronal differentiation (Serra et al., 2007, 2009), we have extended differentiation in order to allow astrocytic differentiation and maturation. The feasibility of using these differentiated neurospheres as 3D human neural cell model for high-throughput neurotoxicity testing was attained by performing neurotoxicity assays in multi-well formats. The effect of neurotoxic compounds in differentiated neurospheres was evaluated by gene expression and functional endpoints, allowing distinguishing between neuronal and astrocytic toxicity.

2. Materials and methods

2.1. Cell culture and 2D differentiation

Undifferentiated NT2 cells from American Type Culture Collection (ATCC) were routinely cultivated in DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 1% (v/v) penicillin-streptomycin (P/S, Invitrogen), as previously described (Brito et al., 2007).

2.2. Stirred suspension culture

NT2 cells were cultured in stirred suspension culture systems in a humidified atmosphere of 5% CO_2 in air, at 37 °C. Undifferentiated NT2 cells were inoculated as single cell suspension in a silanized 125 mL spinner vessel (from Wheaton) equipped with ball impeller, at a density of 6.7×10^5 cell/mL in 75 mL DMEM, 10% FBS, 1% P/S. On day 2, 50 mL of fresh medium were added and cells allowed to aggregate for additional 2DIV (days in vitro). After this aggregation period, at day 3 differentiation induced by RA was initiated, by performing 50% medium exchange which was repeated every 2–3 days for 3 weeks (until 24DIV). The medium composition during this period was DMEM, 10% FBS, 1% P/S, 20 μM retinoic acid (RA, Fig. 1a). Following this period (from 24DIV onwards), the medium was composed by DMEM, 5% FBS, 1% P/S. Cultures were maintained up to 50DIV and applied in neurotoxicity assays (from 38 to 50 DIV). The agitation rate was increased along culture to avoid aggregate clumping and to control aggregate size (from initial 40 rpm, to 60 rpm during RA induction phase and up to 100 rpm by the end of this period).

2.3. Neurotoxicity assays

Neurospheres were collected between 38-50 DIV, distributed in 96 or 6 well plates and incubated in DMEM, 5% FBS, 1% P/S, supplemented with increasingly concentrations of test compounds, for 48–72 h, before carrying out the assay endpoints. Six wells were used per toxicant concentration and medium was used as untreated control.

The induced toxicity of tert-buthyl hydroperoxide (tBHP; 0.008–2 mM), acrylamide (Acr; 1 μM-1 mM) and chloramphenicol (39 μM–10 mM; all purchased from Sigma-Aldrich) was evaluated by performing dose-response curves using cell viability as endpoint, measured by the Presto blue viability assay (Invitrogen), based on resazurin reduction, following the manufacturer's instructions (calibration curves with neurospheres on Supplementary Fig. 1). Based on these results the concentrations of Acr and chloramphenicol for further analysis were selected.

2.4. Astrocytic functionality

Neurospheres were collected at 38DIV, distributed in 6-well plates and incubated with DMEM without glutamate (Glu) and glutamine (Gln; Invitrogen). Glu transporters and Gln synthetase (GS) activities were stimulated adding 5 mM Glu (Sigma) to culture medium. As control medium was added to empty wells and processed together with those containing neurospheres. Samples were collected at 0, 24, 48 at 72 h and Glu and Gln concentrations in culture medium were evaluated using an YSI 7100 MBS equipment (YSI Incorporated, USA).

2.5. Synaptic vesicle trafficking assay

Synaptic vesicles trafficking assay was based in Gaffield, 2006. Aggregates were plated in poly-D-lysine (PDL) – coated glass coverslips and incubated with 100 mM KCl buffer (5 mM HEPES-NaOH, pH 7.4; 10 mM glucose; 2.5 mM calcium chloride; 1 mM magnesium chloride; 100 mM potassium chloride; 37 mM sodium chloride) for 5 min. Afterwards, 100 mM KCl buffer was removed and aggregates incubated with 10 μM FM-1-43 dye (Invitrogen) in 5 mM KCl buffer (5 mM HEPES-NaOH, pH 7.4; 10 mM glucose; 2.5 mM calcium chloride; 1 mM magnesium chloride; 5 mM potassium chloride; 37 mM sodium chloride) for 15 min. Aggregates were washed for 1 min with 5 mM KCl buffer with ADVASEP-7 (Sigma), followed by three washes with 5 mM KCl buffer. Exocytosis was stimulated with 100 mM KCl buffer and samples were

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