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Development and characterization of a human embryonic stem cell-derived 3D neural tissue model for neurotoxicity testing

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ABSTRACT

Alternative models for more rapid compound safety testing are of increasing demand. With emerging techniques using human pluripotent stem cells, the possibility of generating human *in vitro* models has gained interest, as factors related to species differences could be potentially eliminated. When studying potential neurotoxic effects of a compound it is of crucial importance to have both neurons and glial cells. We have successfully developed a protocol for generating *in vitro* 3D human neural tissues, using neural progenitor cells derived from human embryonic stem cells. These 3D neural tissues can be maintained for two months and undergo progressive differentiation. We showed a gradual decreased expression of early neural lineage markers, paralleled by an increase in markers specific for mature neurons, astrocytes and oligodendrocytes. At the end of the two-month culture period the neural tissues not only displayed synapses and immature myelin sheaths around axons, but electrophysiological measurements also showed spontaneous activity. Neurotoxicity testing – comparing non-neurotoxic to known neurotoxic model compounds – showed an expected increase in the marker of astroglial reactivity after exposure to known neurotoxicants methylmercury and trimethyltin. Although further characterization and refinement of the model is required, these results indicate its potential usefulness for *in vitro* neurotoxicity testing.

1. Introduction

To date neurotoxicity (NT) and developmental neurotoxicity testing (DNT) rely on conventional *in vivo* methods, which are expensive and time-consuming (Bal-Price et al., 2015). With the increasing number of compounds that require testing according to REACH regulation (2007), alternative strategies are needed to rapidly identify neurotoxicity and developmental neurotoxicity of substances. Therefore, the development of *in vitro* and *in silico* approaches based on human material are seen as the future of toxicology (Leist et al., 2008b).

The brain is a complex target organ due to its developmental, structural and functional features, which are depending on multiple interactions between neurons and the different types of glial cells, astrocytes, oligodendrocytes and microglial cells. Therefore, *in vitro* models aiming at evaluating drug- and toxicant-induced neurotoxicity must take into account these multicellular interactions. Technologies are evolving from isolated neuronal cultures, or neuron-glia co-cultures on two-dimensional substrates to three-dimensional (3D) tissue-like structures, which better mimic brain complexity and its cell-cell interactions (Knight and Przyborski, 2015). Three-dimensional cell cultures can be prepared using synthetic or natural scaffolds, or can rely on the spontaneous synthesis of the extracellular matrix (ECM). ECM is necessary for structural organization and cell migration and its composition may modulate the sensitivity to neurotoxic compounds (Zychowicz et al., 2014). The 3D model prepared from re-aggregated fetal rat brain cells, first developed by Moscona (Moscona, 1961) fulfills most of these requirements: it contains all brain cell types that are synthesizing their own extracellular matrix. At the initiation of *in vitro* culturing, neurons are post-mitotic, while glial cells are still proliferating during the first two weeks. Over the course of culturing cells undergo extensive differentiation into a neural tissue-like structure, which can sustain long-term maintenance (Honegger et al., 2011; Honegger et al., 1979; Honegger and Zurich, 2011). This 3D system has been extensively characterized and used for DNT and NT (Monnet-Tschudi et al., 1993; Zurich et al., 2002; Zurich et al., 2004). In addition, the 3D aggregating rat brain cell model was involved in a pre-validation study, where specific neurotoxic readouts in this model was combined with a neutral red uptake assay performed in 3T3 cells for general cytotoxicity, and *in vitro* tests

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for blood-brain barrier passage. The combination of these three test-systems was proposed as a strategy improving the predictive capacity of *in vitro* toxicity testing (Prieto et al., 2013; Zurich et al., 2013).

Comparative DNT studies using rodent and human progenitor cells revealed species differences to several compounds tested for their early neurodevelopmental effects, such as effects on cell proliferation, migration and neuronal differentiation (Baumann et al., 2014; Gassmann et al., 2010). Therefore, the use of human cell based cultures obtained by differentiating human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs) or human neural progenitor cells (hNPCs) is currently seen as the future for *in vitro* toxicology (Leist et al., 2008a; Leist et al., 2008b; Singh et al., 2015). Several systems based on differentiation of hESCs or iPSCs into a population of NPC, recapitulating early neural development, do indeed exist and are used to predict DNT (Krug et al., 2013; Rovida et al., 2014; Schwartz et al., 2015; Shinde et al., 2015). But to study DNT beyond the NPC stage and to study NT, it is necessary to prolong culture and differentiation time. Numerous works have shown the benefits of 3D cell culturing with regards to increased cell survival, and thereby longevity of *in vitro* culturing, as well as augmented neuronal and glial differentiation (Alepee et al., 2014; Zhang et al., 2016; Zhou et al., 2016). Several protocols are available to prepare 3D cultures from human-derived cells: some maintain the cells and/or the 3D structures in static conditions using either a support matrix, Matrigel, or microporous membranes (Choi et al., 2014; Dubois-Dauphin et al., 2010; Lancaster et al., 2013; Li et al., 2012; Stoppini et al., 2016; Tieng et al., 2014); in others, cells are subjected to constant giratory agitation allowing the formation of the 3D structures, in which cells synthesize their own extracellular matrix (Hoelting et al., 2013; Hogberg et al., 2013; Pamies et al., 2014). Using the latter protocol, 3 D cultures cultures containing neurons and astrocytes in ongoing differentiation were prepared from hESC and hiPSC (Hoelting et al., 2013; Hogberg et al., 2013; Pamies et al., 2014). However, the presence of oligodendrocytes, which are appearing later in brain development (Gritti and Bonfanti, 2007), was not described.

The present study aimed at establishing a stable protocol for the generation of 3D histotypic neural cultures, where long-term culturing (up to two months) will allow, not only the development of mature neurons, but also the differentiation of mature astrocytes and a population of oligodendrocytes forming loose/immature myelin sheaths around axons. The progressive differentiation of neural cells was assessed both with changes in gene expression and by immunocytochemistry for specific markers of neurons, astro- and oligodendrocytes, as well as electron microscopy observation of synapses and myelin. Functionality of the neural tissue was evaluated with electrophysiological recordings. Finally, the effects of an acute exposure to three known neurotoxicants (methylmercury, trimethyl tin and paraquat) were tested on six week-old neural tissue using ibuprofen as negative control. As endpoints, cell viability was assessed by measuring the mRNA expression of actin, followed by analysis of extracellular lactate dehydrogenase (LDH) for the toxicants causing a decrease in actin expression. Increase in the mRNA expression of heme oxygenase (*HMOX1*) was used as marker of cellular and oxidative stress. Changes in the mRNA expression of synaptophysin monitored specific effects on neurons; whereas an increase in glial fibrillary acific protein (*GFAP*) expression allowed assessing astrocyte reactivity called astrogliosis.

2. Material and methods

2.1. Cell cultures

2.1.1. Culturing of human embryonic stem cell line CHES6

Human Embryonic Stem Cell (hESC) line CHES6: BAG-hES-GEW-005 was obtained from the laboratory of Prof. de Geyter (University Hospital of Basel, Switzerland). CHES6 cells were cultured on BD Matrigel™ in the mTeSR™2 feeder-free medium supplemented with 5 μM Rho- associated protein kinase (ROCK) inhibitor (Cellagen Technology) , used for single cell survival and attachment (Chen et al., 2012) and passaged every 4–5 days using StemPro® Accutase® according to the suppliers protocol (Thermo Fisher Scientific). Care was taken to not exceed 10 passages in order to avoid potential changes in the differentiation potential.

2.1.2. Neural precursor cell differentiation from CHES6 cell line

CHES6 cells of about 20% confluence, were dissociated using StemPro® Accutase® and re-plated on CELLstart coating (Thermo Fisher Scientific) for further culturing in mTeSR™2 medium supplemented with 5 μM ROCK inhibitor. The ROCK inhibitor was withdrawn the following day and neural differentiation was initiated with a medium composing equal proportions of DMEM/F12, 1% N2 supplementation and 2% Neurobasal-B27, supplemented with 10 nM TGF-β inhibitor (Tocris Bioscience), 10 nM Dorsomorphin (Tocris Bioscience) and 500 ng/mL Noggin (R&D Systems). After 6–8 days in culture, neural tube-like rosettes were observed, a signature of neural precursor cell (NPC) formation (Wilson and Stice, 2006). These culture conditions were maintained for 15–20 days, and medium was exchanged every 3 days. NPC rosettes were picked with a pipette under phase contrast microscope after gentle dissociation using StemPro Accutase. Picked NPCs (corresponding to 10–15 cells) were re-plated on Cellstart-coating and expanded in HB21 Proliferation Medium, composing of Knock-out DMEM/F12 supplemented with 2% Neural StemPro (Thermo Fisher Scientific), 20 ng/mL Fibroblast Growth Factor 8β (FGF8β) (Peprotech), and 20 ng/mL Epidermal Growth Factor (EGF) (Peprotech).

2.1.3. Generation of 3D neural spheres (Fig. 1A)

When confluent NPCs were dissociated with StemPro Accutase (as described above), a single cell suspension of 40.000 cells/mL was prepared in the HB21 Proliferation Medium. 2.5 mL/well NPC-suspension was distributed in 6-wells plates, giving a density of 100.000 cells/well. Plates were incubated at 37 °C (5% CO_2 and 95% air) on an orbital shaker at 80 rotations per minute (rpm). Free-floating three-dimensional structures (sphere) spontaneously formed within the first 24 h. Cultures were maintained under these conditions for 4 days, with medium replenishment every 2 days. At day 5 the HB21 Proliferation Medium was replaced by HB21 Differentiation Medium, which composes of equal parts (1:1:1) DMEM/F12 with 1% N2-supplementation, Neurobasal with 2% B27-supplementation, and Knock out DMEM/F12 with 2% StemPro-supplementation), this mix was further supplemented with 1% Fetal Bovine Serum (Thermo Fischer Scientific). Cultures were maintained under constant gyratory agitation (80 rpm) for 8 weeks with medium replenishment (80% fresh medium) every three days.

2.2. Toxicant exposure

Toxicant exposure was performed in 6-well plates with 10(±1) spheres per well. Treatment was started at day 42 of 3D culturing (week 6) and material was collected 72 h after a single exposure. Methylmercury chloride (Biochemicals, Aurora, USA) was dissolved in 10% ethanol, while trimethyl tin chloride (Aldrich), ibuprofen (Sigma-Aldrich) and Paraquat dichloride (Sigma-Aldrich) were diluted in ultrapure water in stock solutions of 1 μM to 5 mM concentrations. The single exposure was administrated from the stock solution in a 1:100 dilution and was added directly to the culture medium.

2.3. RT-PCR

Spheres were harvested once per week from the day of changing proliferation medium to differentiation medium and throughout the rest of the length of the culture period. Total RNA was extracted using the RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Thermo Fischer Scientific) with a

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