Refinement of an in vitro testing battery for developmental neurotoxicity evaluation by integrating radial glia- and astrocyte-specific endpoints

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INTRODUCTION

Considering the resource-intensity and ethical aspects of the current in vivo test guidelines for developmental neurotoxicity (DNT), the need for a refinement of DNT testing attracted attention of the scientific community and regulatory authorities. The use of human-based in vitro new approach methodologies (NAMs) instead of animal studies can advance toxicity testing with regards to costs, testing throughput and predictivity of test results for humans. A DNT in vitro battery (DNT-IVB) has recently been assembled under guidance of the EFSA in collaboration with the Danish and U.S. EPA under the umbrella of the OECD and was challenged with 119 chemicals. Despite its broad coverage of neurodevelopmental key events, a gap analysis suggested that the implementation of test methods based on radial glia (RG), astrocytes (AC), and microglia (MG) could improve battery performance. Therefore, the aim of this study is to set up new test methods for measuring RG morphology and proliferation as well as for AC differentiation, maturation and function.

METHODOLOGY

For adding the additional endpoints to the DNT-IVB the Neurosphere Assay, which is a high-content assay based on primary human neural progenitor cells (hNPC), is used. This test system is already part of the current DNT-IVB used for studying hNPC proliferation and differentiation into neurons and oligodendrocytes. Addition of epidermal growth factor (EGF) and fibroblast growth factor (FGF) or bone morphogenic protein 2/4 (BMP2/4) and ciliary neurotrophic factor (CNTF) stimulates differentiation into proliferative RG and AC, respectively. In presence of a defined EGF/FGF ratio, RG proliferation, migration and morphology is assessed after 24-48 h of hNPC differentiation using immunocytochemical stainings with the proliferation marker Ki67 and RG-specific markers (e.g. nestin). AC differentiation is promoted by a combination of BMP2/4 and CNTF and assessed after 5 days of differentiation by immunocytochemical stainings for AC-specific markers (e.g. GFAP, AQP4) and qPCR analyses.

RESULTS

After 24h of hNPC differentiation in the absence of growth factors, the culture predominantly consisted of nestin-expressing cells displaying RG-like morphology. Additional exposure to EGF and FGF alone or in combination further accelerated RG migration and increased RG proliferation, as assessed by the total nuclei count and the number of Ki67-positive cells within the migration area. This nearly pure RG culture can be used to study chemical effects on RG homeostasis during foetal brain development. As a proof-of-principle, exposure of this RG test system to known DNT-positives, e.g. methyl mercury, impaired RG migration and altered RG morphology. The differentiation of hNPCs into AC was strongly enhanced by a combination of BMP2/4 and CNTF leading to an almost pure culture of cells positive for the AC-specific marker AQP4, which displayed the typical star-like morphology after 5 days of hNPC differentiation. Gene expression analyses confirmed that the differentiated culture expressed AC-specific markers. The data indicates a sufficient maturity of hNPC-derived AC.

DISCUSSION

Following the establishment and extensive characterisation of the new RG- and AC-based DNT test methods, the next steps will include the establishment of endpoint-specific controls and their challenge with known DNT positives and negative compounds as well as with false-positive (FP) and false-negative (FN) substances identified within the EFSA-coordinated DNT-IVB performance testing. Glutamine synthetase function needs to be studied as a readout for AC utility before test method establishment. We hypothesise that the FN rate of the classical DNT-IVB can be reduced by introducing RG- and AC-specific endpoints into the classical DNT-IVB. By enhancing the battery performance, we will increase the predictivity of testing results and thereby confidence in implementing DNT-IVB as the new gold-standard of in vitro DNT testing.