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Sensitivity of hiPS-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage

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ABSTRACT

Pyrroloquinoline quinone (PQQ) is a factor influencing on the mitochondrial biogenesis. In this study the PQQ effect on viability, total cell number, antioxidant capacity, mitochondrial biogenesis and differentiation potential was investigated in human induced Pluripotent Stem Cells (iPSC) - derived: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP). Here we demonstrated that sensitivity to PQQ is dependent upon its dose and neural stage of development. Induction of the mitochondrial biogenesis by PQQ at three stages of neural differentiation was evaluated at mtDNA, mRNA and protein level. Changes in *NRF1*, *TFAM* and *PPARGC1A* gene expression were observed at all developmental stages, but only at eNP were correlated with the statistically significant increase in the mtDNA copy numbers and enhancement of SDHA, COX-1 protein level. Thus, the "developmental window" of eNP for PQQ-evoked mitochondrial biogenesis is proposed. This effect was independent of high antioxidant capacity of PQQ, which was confirmed in all tested cell populations, regardless of the stage of hiPSC neural differentiation. Furthermore, a strong induction of *GFAP*, with down regulation of *MAP2* gene expression upon PQQ treatment was observed. This indicates a possibility of shifting the balance of cell differentiation in the favor of astroglia, but more research is needed at this point.

1. Introduction

Differentiation ability and the therapeutic potential for personalized diagnosis and treatment are the main qualities of human induced pluripotent stem cells (hiPSC) (Takahashi et al., 2007; Szablowska-Gadomska et al., 2012). hiPSC can generate neural stem cells, as well as neural and glial progenitors (Choi et al., 2014). Neural stem cells (NSC) and neural progenitors (NP) can subsequence be differentiated into neurons, astrocytes and oligodendrocytes. Neural stem cells can be obtained from hiPSC by direct differentiation under adherent conditions (Chambers et al., 2010), or indirectly, through the stage of three-dimensional aggregates formed by hiPSC (Embryoid Bodies) in a suspension culture (Karumbayaram et al., 2009; Szablowska-Gadomska et al., 2012). The latter method is considered currently as the closest model of *in vitro* embryo neurogenesis.

Mitochondria play a central role in cell metabolism by controlling the cellular respiration and energy production. Somatic cells reprogramming into iPSC is associated with decrease in mitochondria content and activity, and with metabolic shift toward glycolysis. The opposite occurs during differentiation (Wanet et al., 2015). In 2010 Prigione with colleagues, introduced a "metabolic state hypothesis" linking mitochondrial state and cellular metabolism to the stage of differentiation. The differentiation process is an inverse process to the reprogramming and is associated with increase the mtDNA copy number, ATP levels and oxidative phosphorylation intensity, while the decreasing production of lactic acid. Mitochondria take a part in the stem cells differentiation decisions and development of stem cells by the level of free radicals (ROS) modulation and intensity of oxidative phosphorylation (Xu et al., 2013), but also by the increase of mitochondrial mass, which is proportional to the neuronal mass growth (Zheng et al., 2016). However, the mechanisms linking mitochondrial biogenesis to the neural stem cell differentiation are still unknown.

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Substances which induce mitochondrial biogenesis may play a role in neural stem cells (NSC) development. One of them is Pyrroloquinoline quinone, a well-studied compound modulating mitochondria DNA content and positively influencing the biogenesis of mitochondria (Bauerly et al., 2006; Chowanadisai et al., 2010). PQQ is responsible for the increase of the amount of mitochondria by modulation in *CREB* (cAMP-response-element-binding protein) phosphorylation and subsequent *PPARGC1A* (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)) directed up-regulation of *NRF-1* (nuclear respiratory factor-1), *NRF2* (nuclear factor, erythroid 2-like 2) and *TFAM* (transcription factor A, mitochondrial) expression (Chowanadisai et al., 2010).

Despite the plethora of studies on PQQ, its activity on different stages of neural differentiation has not been evaluated. We present here our studies on the response of neural stem cells generated from hiPSC to Pyrroloquinoline quinone treatment. We were investigating whether sensitivity to different concentrations of PQQ and induction of mitochondrial biogenesis depend on the stage of differentiation of NSC. For that purpose we generated from hiPSC three cell populations at different developmental stages: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP). NSC, eNP and NP phenotype has been confirmed with qualitative and quantitative analysis of protein expression (immunocytochemistry) and gene expression (RT-PCR and RNA-seq). Subsequently, we tested the influence of PQQ on cell viability, total cell number, ROS level, mitochondrial membrane potential and differentiation capacity. The biogenesis of mitochondria has been checked by analysis: succinate dehydrogenase (SDHA) and cyclooxygenase isoenzymes (COX-1) protein level, mtDNA copy number (qPCR) and *NRF-1*, *TFAM* and *PPARGC1A* genes expression (RT-qPCR). The influence of PQQ on neural differentiation capacity of hiPSC was evaluated by the analysis of the expression of genes related to neuronal (*MAP2*) and astrocytic (*GFAP*) differentiation.

2. Materials and methods

2.1. Cell culture and neural differentiation

The induced pluripotent stem cells (iPS) were feeder free cell line derived from CD34 + fraction of human cord blood cells by transfection with EBNA1-based episomal system comprising of seven factors: *SOX2*, *OCT4*, *KLF4*, *MYC*, *NANOG*, *LIN28*, *SV40L T* antigen (The Gibco® Human Episomal iPSC Line, Life Technologies, Thermo Fisher Scientific). The cells were grown on recombinant human Vitronectin (Thermo Fisher Scientific), maintained in culture in Essential 8 Medium (Thermo Fisher Scientific). The neural commitment to the stage of NSC was performed according to Yan et al., 2013 with some modifications, while further differentiation was proceeded with our protocol implementing neural differentiation medium type I and type II. Briefly, at the 80% of confluence Essential E8 Medium was changed to the neural induction medium (Gibco® PSC Neural Induction Medium, Thermo Fisher Scientific) and cells were cultured for 7 days with medium changed every second day. On the 7th day of culture cells were plated in 96 or 6 well plates (Nunc) coated by 1:30 Matrigel:DMEM/F12 ratio (BD Matrigel™ Basement Membrane Matrix, BD Biosciences) in Neural Expansion Medium (Neural Induction Supplement 1:50, Neurobasal, DMEM/F12, 1:1) to obtain neural stem cell (NSC) population. NSCs at passage 6 were used for derivation of early neural progenitors (eNP). To obtain eNP stage of development, cells were grown for 14 days in neural differentiation medium type I: Neurobasal, DMEM/F12 [1:1], N2 supplement 1%, B27 supplement 1%, EGF [20 ng/ml], bFGF [20 ng/ml]. The third tested cell population – neural progenitors (NP), was obtained by withdrawing of EGF and bFGF from the above mentioned medium (neural differentiation medium type II) and culturing for the following 14 days. NSC, eNP and NP phenotype has been confirmed with RT-PCR, RNA-seq (mRNA copy number) and immunocytochemistry staining (protein expression), as described below.

2.2. Immunofluorescence staining

After fixation with 4% of PFA (15 min) cells were washed with PBS, permeabilized with 0,1% Triton X-100 and blocked with 10% goat serum. The following primary antibodies were applied overnight: NESTIN (1:500 Millipore), β-TUBULIN III (1:1000, Sigma-Aldrich), DCX (1:500, Cell Signaling Technology), MAP-2 (1:500, Sigma-Aldrich), Ki67 (1:500, Novocastra), NF200 (1:200, Sigma-Aldrich), GFAP (1:500, Dako). After washing with PBS, appropriate secondary antibodies (Alexa Fluor 488 and 546, 1:1000, Thermo Fisher Scientific) were applied for 1 h, and cell nuclei were contra stained with Hoechst 33,258 (Sigma-Aldrich). The measurement of fluorescence was performed by calculating the % of the area of selected positive marker staining per image. Images and calculations were obtained using Confocal Laser Microscope LSM 510 (Zeiss) and ZEN 2012 blue edition software in the Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Centre, Polish Academy of Sciences.

2.3. PQQ treatment

Cells were seeded at a 96-well or 6-well plate covered with the solution of Matrigel:DMEM/F12, 1:30 ratio (BD Matrigel™ Basement Membrane Matrix, BD Biosciences) at density 5×10^5 cells/cm^2 in Neural Expansion Medium for Neural Stem Cells (NSC) population; 2) in neural differentiation medium (type I differentiation medium) for Early Neural Progenitors (eNP) population; 3) in neural differentiation medium (type II differentiation medium) for Neural Progenitors (NP) population. After 24 h the medium was replaced with fresh medium supplemented with PQQ (Sigma) at concentrations 0,5 μM; 0,25 μM; 0,125 μM; 0 μM.

2.4. Alamar blue cell viability assay

Cell viability was measured by Alamar blue viability assay (Sigma-Aldrich) after 5 days of incubation with PQQ at various concentrations (0–0,5 μM). Untreated NSC, eNP and NP cell populations were used as the controls. Alamar Blue (0,1 mg/ml Sigma) was added to the culture medium (1:10) for 3 h in 37 °C. The fluorescence intensity of resorufin was measured by Fluoroscan Ascent (FL, Labsystems) plate reader at 544 nm and 590 nm wavelengths for excitation and emission, respectively. The results were normalized to the whole cell number with Janus green normalization staining (Abcam) according to the manufacturer's protocol. Final data are presented as % of the untreated control.

2.5. Reactive oxygen species detection

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay (Sigma-Aldrich) was used to measure the ROS level at the experimental and control cell populations. The DCFHDA reagent at a concentration of 1 μM was added to the cell cultures then incubated for 3 h. After the fat-soluble DCFH-DA enters the cell, it is hydrolyzed by lipase into DCFH, then the non-fluorescent DCFH is oxidized by intracellular ROS to become fluorescent DCF, detected by quantitative fluorescent measurements. The fluorescence of DCF was measured at a wavelength: 485–538 nm on plate reader (Fluoroscan Ascent FL (Labsystems). The normalization of the fluorescent measurements to the whole cell number for each sample was performed with Janus green normalization staining (Abcam) according to the manufacturer's protocol. Final data are presented as % of untreated control.

2.6. Mitochondrial membrane potential assay

Changes in the mitochondrial membrane potential were evaluated at three different stages of hiPSC neural differentiation (NSC, eNP and NP) in control cells and after 5 day of cell exposition to PQQ (0–0,5 μM), using the mitochondrial membrane potential (Δψm)-