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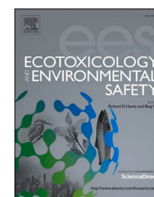

Oxygen as an important factor modulating *in vitro* MeHgCl toxicity associated with mitochondrial genes in hiPSCs

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ABSTRACT

Mitochondria are energy factories of cells and important targets for methylmercury chloride (MgHgCl). Methylmercury (MeHg) is a well-known environmental toxicant that bioaccumulates in fish and shellfish. It readily crosses the placental barrier, making it a threat to correct fetal development. Despite being comprehensively investigated for years, this compound has not been assessed for its *in vitro* mitochondrial toxicity under different oxygen conditions. In this study, human induced pluripotent stem cells (hiPSCs) were used to evaluate the dependence of the expression of genes associated with pluripotency and mitochondria on atmospheric (21% O_2) and low (5% O_2) oxygen concentrations upon MeHgCl treatment. We showed that the toxicity of MeHgCl was strongly related to an increased mtDNA copy number and downregulation of the expression of an mtDNA replication and damage repair-associated gene *POLG1* (*Mitochondrial Polymerase Gamma Catalytic Subunit*) in both tested oxygen conditions. In addition, the viability and mitochondrial membrane potential of hiPSCs were significantly lowered by MeHgCl regardless of the oxygen concentration. However, reactive oxygen species accumulation significantly increased only under atmospheric oxygen conditions; what was associated with increased expression of *TFAM* (*Transcription Factor A, Mitochondrial*) and *NRF1* (*Nuclear Respiratory Factor 1*) and downregulation of *PARK2* (*Parkin RBR E3 Ubiquitin Protein Ligase*). Taken together, our results demonstrated that MeHgCl could induce *in vitro* toxicity in hiPSCs through altering mitochondria-associated genes in an oxygen level-dependent manner. Thus, our work suggests that oxygen should be considered a factor was modulating the *in vitro* toxicity of environmental pollutants. Typical atmospheric conditions of *in vitro* culture significantly lower the predictive value of studies of such toxicity.

1. Introduction

The atmospheric concentration of oxygen represents a hyperoxic non-physiological condition to stem cells compared with their *in vivo* niche microenvironment. In the stem cell niche, the oxygen concentration is at the level of 2% O_2 –8% O_2, limiting inherent oxygen toxicity. It has been shown that these cells cannot properly defend themselves against the toxic influence of high oxygen (Keeley and Mann, 2019). High oxygen concentrations can increase reactive oxygen species (ROS) accumulation, which can damage lipids, proteins, and DNA (Busuttil et al., 2003; Hansen et al., 2007), as well as altering metabolic turnover and influencing stem cell differentiation *via* the loss of stemness and

Abbreviations: ATP, adenosine triphosphate; ATG13, Autophagy Related 13; DCF, dichlorofluorescein; DCFH-DA, 2′,7′ Dichloro-dihydro-fluorescein diacetate; ETC, Electron transport chain; hESCs, human embryonic stem cells; hESCs, NPs, human embryonic stem cells derived neuronal precursors; HIF1α, Hypoxia-inducible factor 1; HIF2α, Hypoxia-inducible factor 2; HIF3α, Hypoxia-inducible factor 3; Hg, mercury; HUCB-NSCs, Human Umbilical Cord Blood Neural Stem Cells; hiPSCs, human induced Pluripotent Stem Cell; ki67, Marker of proliferation Ki-67; MeHg, Methylmercury; MeHgCl, Methylmercury chloride; NANOG, Nanog Homeobox, pluripotency marker; ND1, Mitochondrially Encoded NADH: Ubiquinone Oxidoreductase Core Subunit 1; ND5, Mitochondrially Encoded NADH: Ubiquinone Oxidoreductase Core Subunit 5; NRF1, Nuclear Respiratory Factor 1, mitochondrial biogenesis marker; OCT3/4, Protein encoded by POU5F1 gene, pluripotency marker; PARK2, Parkin RBR E3 Ubiquitin Protein Ligase, a marker of mitophagy; PINK, PTEN Induced Kinase 1; POLG1, Mitochondrial Polymerase Gamma Catalytic Subunit, a marker of mtDNA replication and damage repair; POU5F1, POU Class 5 Homeobox 1, pluripotency marker; ROS, Reactive Oxygen Species; SERPINA 1, Serpin Family A Member 1; SLCO2B1, Solute carrier organic anion transporter family, member 2B1; TFAM, Transcription Factor A, Mitochondrial, mitochondrial biogenesis marker; TWI, Tolerable Weekly Intake; Δψm, Mitochondrial membrane potential.

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reduced proliferation (Mas-Bargues et al., 2019). When cells are cultured at a low oxygen concentration, the activity of prolyl hydroxylases is inhibited, and hypoxia-inducible factors (HIF's), which regulate the activity of many genes involved in different cellular functions, are activated (Semenza et al., 1991; Wang et al., 1995; Maxwell et al., 1999; Ivan et al., 2001; Jaakkola et al., 2001; Bell and Chandel, 2007; Panchision, 2009; Silván et al., 2009; Eliasson and Jönsson, 2010; Mas-Bargues et al., 2019). One of the important functions of HIF-2α is direct upstream regulation of Oct-4, Sox2, and Nanog, transcription factors essential for maintaining pluripotency (Covello et al., 2006; Forristal et al., 2010). Low oxygen concentration (2% O_2 – 6% O_2) has been shown to activate signaling pathways in multiple stem cell systems that appear to regulate two important modulators of stemness: Oct4 and Notch (Simon and Keith, 2008; Mohyeldin et al., 2010). Moreover, it caused the downregulation of ROS accumulation and down-regulation of DNA damage (Mas-Bargues et al., 2019).

Mercury occurs naturally in the earth's crust. The extraction and combustion of fossil fuels, mercury, and gold mining have largely contributed to mercury pollution of the environment (Camacho-dela-Cruz et al., 2021; Zhang et al., 2021; Dziok et al., 2021; Liu et al., 2021). Methylmercury (MeHg) easily bioaccumulates in fish and shellfish (Ursinyova al, 2019; Jinadasa et al., 2021; Médieu et al., 2022; Blanchfield et al., 2022), but it can also be accumulated in plants (Qian et al., 2021; Velásquez Ramírez et al., 2021). Mercury-containing pesticides are an important source of plant contamination (Schneider, 2021). In order to reduce high global mercury emissions, the Minamata Convention was introduced (Minamata Convention on Mercury: A Contemporary Reminder, 2017). Maximum levels of mercury in foodstuffs have been regulated (Regulation (EC) No. 1881/2006), and fish meat followed by fish products are the main source of exposure to methylmercury (EFSA, 2012). The established TWI (Tolerable Weekly Intake) is 1.3 μg/kg b.w. expressed as mercury and women in pregnancy are considered in the group of high and frequent consumers of fish where TWI could be up till 6-fold (EFSA, 2012). Chronic exposure to MeHg can induce disturbances of cognitive thinking, memory, attention, language, and fine motor and visuospatial skills (Rice et al., 2014). MeHg readily crosses the blood-brain and placental barriers and is concentrated in umbilical cord blood (Grandjean et al., 2010; Ceccatelli et al., 2013). Exposure to MeHg early in life is potentially associated with brain damage at levels much lower than those affecting the mature brain (Spurgeon, 2006; Stringari et al., 2008). MeHg has neurodevelopmental effects, causing the death of neurons by disrupting microtubule assembly (Sager et al., 1983; Go et al., 2018; Pan et al., 2022), as well as inducing oxidative stress, abnormalities in intracellular calcium level, and interaction with sulfhydryl groups of proteins (Tamm et al., 2006; Farina et al., 2011b;), which leads to modifications in the structure and function of proteins (Castoldi et al., 2001). Many studies have shown that an increase in ROS accumulation upon MeHg treatment causing oxidative stress and disturbance in the antioxidant defense system, which enhances the neurotoxicity of ROS (Carvalho et al., 2008; Stringari et al., 2008; Farina et al., 2011a, 2011b; Antunes dos Santos et al., 2016; Ferreira et al., 2018;). MeHg can change mitochondrial membrane potential, perturb the electron transport chain (ETC), and influence the production of adenosine triphosphate (ATP) by reacting with proteins in the mitochondrial membrane (Wang et al., 2016a, 2016b). Other effects of the exposure of cells to MeHg are related to oxidative damage of macromolecules, such as lipids and DNA (Joshi et al., 2014). Mitochondrial dysfunction caused by MeHg can induce cell death by apoptosis or necrosis (Farina et al., 2011a; Roos et al., 2012). MeHg may contribute to alterations in the abundance of mitochondria, mitochondrial DNA (mtDNA) integrity, and mtDNA copy number (Wang et al., 2016a, 2016b). In cells exposed to MeHg, upregulation of the expression of genes controlling mitochondrial biogenesis mediated by ROS accumulation was observed, which increased the mtDNA copy number (Wang et al., 2016b, 2016a).

Methylmercury chloride (MeHgCl) is the most commonly used source of methylmercury (MeHg) for *in vitro* culture. A high-throughput embryonic stem cell test showed strong embryotoxicity of MeHgCl *in vitro* (Peters et al., 2008); however, the role of oxygen in this mechanism was not evaluated. MeHgCl caused abnormalities in the structural development of the brain and noted the sensitivity of human embryonic stem cells derived neuronal precursors (hESCs-NPs) to MeHgCl in a manner dependent on the developmental stage (Stummann et al., 2009). Similar sensitivity was noted in a human stem-cell-based *in vitro* model, human umbilical cord blood neural stem cells (HUCB-NSCs) (Buzanska et al., 2009; Zychowicz et al., 2014). However, in these previous studies, attention was not paid to the role of oxygen in the *in vitro* prediction of MeHgCl toxicity.

The above findings have suggested that oxygen should be considered as a factor modifying toxicity when planning *in vitro* tests on environmental toxicants since the typical atmospheric conditions of *in vitro* culture significantly lower the predictive value of these studies. Therefore, we decided to investigate the effect of oxygen levels on MeHgCl toxicity in a human induced pluripotent stem cells (hiPSCs) *in vitro* model. The main aim of this study was to assess the influence and unravel the mode of action of oxygen levels on the *in vitro* mitochondrial toxicity of MeHgCl in hiPSCs.

2. Materials and methods

2.1. hiPSCs culture and treatment with MeHgCl

hiPSCs (Gibco® Human Episomal iPSCs Line, Thermo Fisher Scientific, Waltham, MA, USA) at the undifferentiated stage of development were cultured in Essential 8 Medium (Thermo Fisher Scientific, Waltham, MA, USA) on a six-well plate covered with rh-Vitronectin (Thermo Fisher Scientific, Waltham, MA, USA) under two different oxygen conditions: 5% and 21% O_2 concentrations, 5% CO_2 and 37 °C for more than 2 weeks before MeHgCl (Sigma-Aldrich(Merck SA), Darmstadt, Germany) exposure. Then, the hiPSCs were seeded on Nunc 6- or 96- well plates (Thermo Fisher Scientific, Waltham, MA, USA) covered with Corning Matrigel Matrix (Corning Inc., Corning, NY, USA) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Thermo Fisher Scientific, Waltham, MA, USA) solution at a ratio of 1:30. Next, the hiPSCs cultured on Corning Matrigel Matrix under the different oxygen conditions were treated with MeHgCl (Sigma-Aldrich(Merck SA), Darmstadt, Germany) at a concentration from 0 to 1 μM for 5 days. Concentrations of MeHgCl higher than 1 μM were cytotoxic to all hiPSCs populations under both oxygen conditions, so they were not used in the experiments.

2.2. Immunocytochemical staining

For immunostaining, hiPSCs were seeded in Nunc 24-well plates contained gloves covered with rh-Vitronectin and cultured under atmospheric and low oxygen concentrations for 5 days before staining. After fixing with 4% PFA for 5 min, hiPSCs were permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum. The primary antibodies (Table 1, Supplementary Material) were applied for the overnight incubation at 4 °C. After washing with PBS, the following secondary antibodies were used for 60 min at room temperature: goat anti-mouse IgG1 for HIF1α and anti-mitochondrial goat anti-mouse IgG2b for Oct-3/4, and goat anti-rabbit IgG (H+L) for HIF2α, HIF3α, and Ki67. All secondary antibodies were conjugated to either Alexa Fluor 546 or Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA, USA) and used at a dilution of 1:1000. Cell nuclei were stained with 5 μM Hoechst 33258 (Sigma-Aldrich (Merck SA), Darmstadt, Germany) for 15 min. After the final wash, the slides were mounted in Fluorescent Mounting Medium (Dako) (Agilent, Santa Clara, CA, USA). As a control, the first antibodies were omitted during immunocytochemical staining. A confocal laser scanning microscope (LSM 780/Elyra PS.1; Zeiss, Oberkochen, Germany) was used to obtain detailed images of the cells.

Following image acquisition, the images were processed using ZEN 2012 SP5 software (Zeiss, Oberkochen, Germany). All microscopy images were obtained at the Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Institute, Polish Academy of Sciences.

2.3. Alamar Blue cell viability assay

Cell viability was measured for hiPSCs grown under 21% or 5% oxygen conditions after 5 days of incubation with various concentrations (0–1 μM) of MeHgCl. hiPSCs were cultured in Essential 8 Medium (Thermo Fisher Scientific, Waltham, MA, USA) on Corning Matrigel Matrix (Corning Inc., Corning, NY, USA). Cell viability was determined by the Alamar Blue viability assay (Sigma-Aldrich(Merck SA, Darmstadt, Germany). Fluorescence was measured at wavelengths of 544–590 nm (Labsystems, Philadelphia, PA, USA) after 3 h of incubation with Alamar Blue (0.1 mg/ml;) diluted in culture medium (1:10). The final data are presented as percentages of the untreated control.

2.4. ROS accumulation assay

The ROS accumulation was measured in hiPSCs grown in 21% O_2 or 5% O_2 after 5 days of incubation with MeHgCl at various concentrations (0 –1 μM). hiPSCs were cultured in Essential E8 Medium (Thermo Fisher Scientific, Waltham, MA, USA) on Corning Matrigel Matrix (Corning Inc., Corning, NY, USA). The 2′,7′ dichloro-dihydro-fluorescein diacetate (DCFH-DA; Sigma-Aldrich(Merck SA), Darmstadt, Germany) reagent at a concentration of 1 μM was added to the hiPSCs for 3 h. After this period, the fluorescence of 2,7-dichlorofluorescein (DCF) was measured at wavelengths of 485–538 nm by a plate reader (Labsystems, Philadelphia, PA, USA). The final data are presented as percentages of the untreated control.

2.5. Mitochondrial membrane potential assay

Mitochondrial membrane potential (Δψm) was measured in hiPSCs grown under 21% or 5% oxygen conditions after 5 days of incubation with various concentrations (0–1 μM) of MeHgCl. hiPSCs were cultured in Essential E8 Medium (Thermo Fisher Scientific, Waltham, MA, USA) on Corning Matrigel Matrix (Corning Inc., Corning, NY, USA). Specifically, this variable was determined using MitoTracker Red CMXRos (Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence intensity was detected (544–590 nm) on a plate reader Fluoroskan Ascent (Labsystems, Philadelphia, PA, USA). The level of Δψm represented by the intensity of red fluorescence was measured 4 h after the administration of MitoTracker Red CMXRos (50 nM). The final data are presented as percentages of the untreated control.

2.6. qPCR

DNA was isolated from hiPSCs grown under 21% or 5% oxygen conditions after 5 days of exposure to MeHgCl at concentration of 0.5 μM. Following the manufacturer's protocol, DNA was isolated from hiPSCs with ZR-Duet™ DNA/RNA Mini-Prep Kit (Zymo Research, Irvine, CA, USA). The quantity of DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was performed with 10 ng of DNA template in a reaction mixture of 25 μl containing 12.5 μl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 0.25 μM each primer, and LightCycler 96 (Roche Molecular Systems, Inc., Pleasanton, CA, USA). qPCR reactions were subjected to a hot start at 95 °C for 3 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The ratio of mtDNA to nuclear DNA was calculated for two pairs of genes: MT-ND5 with *SERPINA1* and *MT-ND1* with *SLCO2B1*. mtDNA copy number was calculated as the average ratios of mtDNA copy numbers: *MT-ND5/SERPINA1* and *MT-ND1/SLCO2B1*. The sequences of the primers (Table 2, Supplementary Material) used for qPCR matched those used in our earlier studies (Augustyniak et al., 2017a, 2017b, 2019a).

2.7. qRT-PCR

RNA was isolated from hiPSCs grown under conditions with an oxygen concentration of 21% O_2 or 5% O_2 after 5 days of exposure to 0.5 μM MeHgCl. In accordance with the manufacturer's protocol, total RNA was isolated from cells using ZR-Duet™ DNA/RNA Mini-Prep Kit (Zymo Research, Irvine, CA, USA). Clean-Up RNA Concentrator kit (A&A Biotechnology, Gdynia, Poland) was used to eliminate genomic DNA from the RNA samples. The concentration of RNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA for qPCR was obtained by reverse-transcription polymerase chain reaction (RT-PCR) with High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, Waltham, MA, USA). For qRT-PCR, 10 ng of cDNA was loaded with 0.25 μM of forward and reverse primers, and 12.5 μl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), LightCycler 96 (Roche Molecular Systems, Inc., Pleasanton, CA, USA) and the following steps were performed: initial denaturation at 95 °C for 3 min, and then 45 cycles of denaturation at 95 °C for 10 s and annealing/extension at 58 °C for 1 min. Validation of reference genes was performed in NormFinder software (Andersen et al., 2004). *EID2* was chosen as a housekeeping gene. Analysis of relative gene expression results was determined using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). The primer sequences (Table 3, Supplementary Material) used to validate the reference genes were from a previous study (Augustyniak et al., 2019b). The results were presented as 1) expression of genes of interest in hiPSCs growth in 5% O_2 relative to hiPSCs growth in 21%O_2 (Fig. 1) and 2) fold change (log2) expression of genes of interest in hiPSCs treated with MeHgCl, relative to the gene expression in hiPSC untreated with MeHgCl in 5% and 21% O_2 (Fig. 3).

2.8. Statistical analysis

The results ($n = 3$) were analyzed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA; USA) using the following statistical tests: 1) t-test or Mann-Whitney U test; 2) one-way ANOVA, *post hoc* Tukey test, or Kruskal-Wallis test; or 3) two-way ANOVA, with Bonferroni multiple comparison test. Figures present data as mean with SD. The significance of the obtained results are presented as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Characterization of hiPSCs population cultured under 21% and 5% oxygen conditions

hiPSCs cultured under conditions with oxygen concentrations of 21% and 5% were tested for the expression and cellular localization of the pluripotency markers OCT3/4, proliferation marker Ki67, hypoxia-inducible factors (HIF1α, HIF2α, and HIF3α), and mitochondrial marker using confocal microscopy. In hiPSCs grown under both oxygen conditions, pluripotency marker OCT3/4 was expressed, but it was expressed more strongly under the low oxygen concentration. *POU5F1* gene encodes a transcription factor containing a POU homeodomain (OCT3/4) that plays a key role in embryonic development and stem cell pluripotency was stronger expressed at low oxygen conditions. Upregulation of *POU5F1* and *NANOG* gene expression and upregulation of OCT3/4 proteins was related to upregulation of the proliferation marker ki67 in 5% of oxygen concentration. The protein encoded by *NANOG* is a DNA binding homeobox transcription factor involved in embryonic stem (ES) cell proliferation, renewal, and pluripotency. *POU5F1* and *NANOG* gene expression is critical for pluripotency maintenance. We also identified some differences between the oxygen concentration groups related

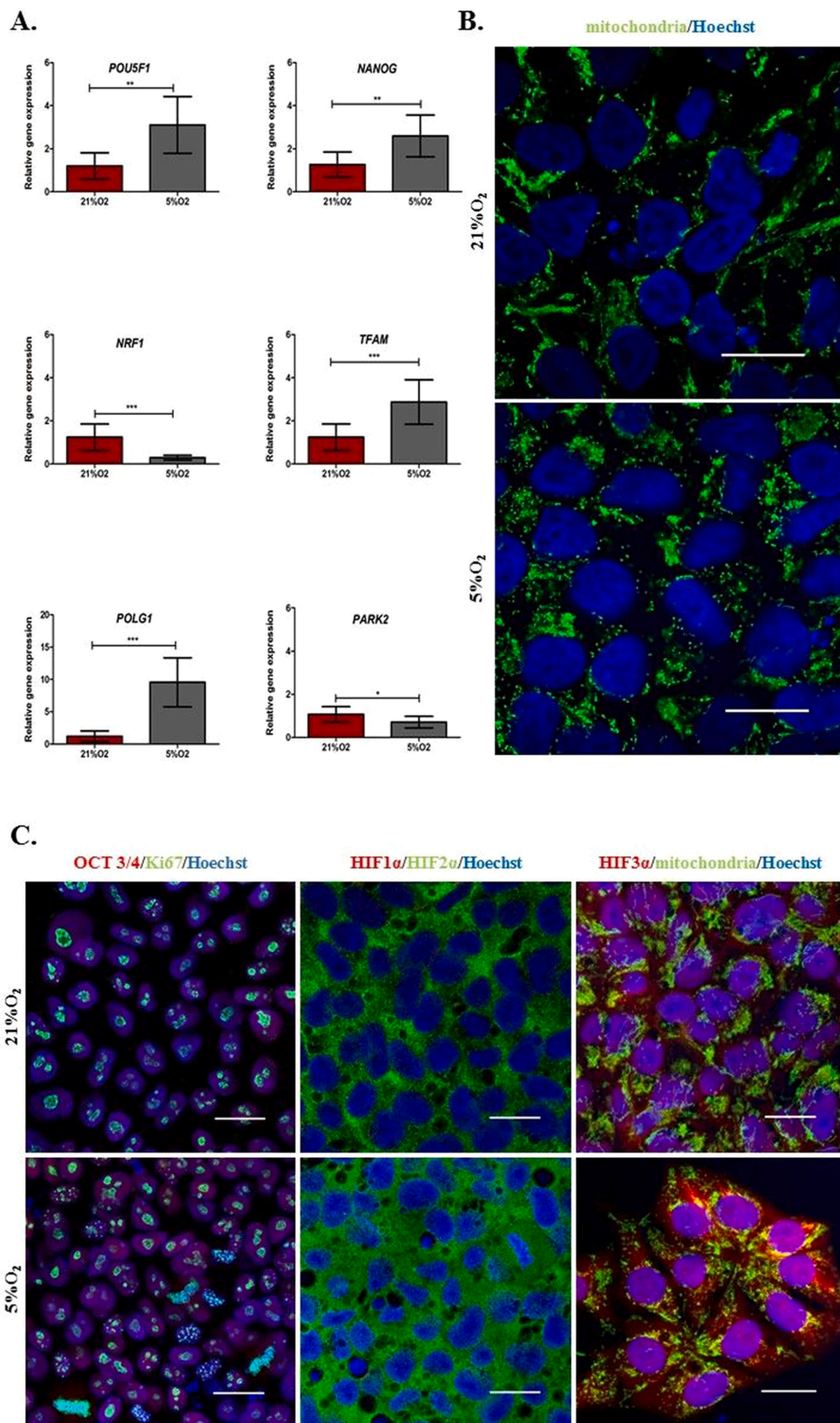

Fig. 1. The effects of oxygen concentration (21% O_2, 5% O_2) on expression of genes of interest in hiPSCs growth in 5% O_2 relative to hiPSCs growth in 21% O_2 and protein expression in hiPSCs cultured in different oxygen conditions. A. The impact of oxygen concentration (21% O_2, 5% O_2) on the expression of genes associated with pluripotency (*POU5F1*, *NANOG*), mitochondrial biogenesis (*TFAM*, *NRF1*; *NRF1* is also involved in antioxidant defense), mitophagy (*PARK2*), and mtDNA replication and damage repair (*POLG1*) in hiPSCs. Results are presented as mean (± SD). Brackets show statistically significant differences between hiPSCs cultured in 21% and 5% oxygen concentrations as determined by t-test or Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). B. Analysis of the shape of mitochondria in hiPSCs in 21% and 5% oxygen. Scale bar = 20 μm. C. Expression of OCT3/4, HIF1α, HIF2α, HIF3α, and ki67 in hiPSCs at atmospheric (21%) and low (5%) oxygen levels. Scale bar = 20 μm.

to the expression and cellular localization of HIF's, as indicated in Fig. 1C. At the low oxygen concentration, HIF2α and HIF3α were expressed in the cytoplasm and at low levels in the nucleus, while under atmospheric oxygen conditions, in the cytoplasm only. Meanwhile, HIF1α was not expressed under either condition, which is typical for cells cultured in low oxygen for more than 48 h. In hiPSCs cultured in 21% or 5% oxygen, numerous mitochondria were detected, but their shape depended on the oxygen level (Fig. 1B). In the 21% oxygen group, the mitochondria were elongated, while in the group with low oxygen, more rounded mitochondria were detected.

The expression of key genes controlling pluripotency (*POU5F1*, *NANOG*), and genes involved in mitochondrial biogenesis (*NRF1*, *TFAM*), antioxidant response (*NRF1*), mitophagy (*PARK2*), mtDNA replication, and damage repair (*POLG1*) was tested using qRT-PCR in hiPSCs cultured in 21% and 5% oxygen (Fig. 1A). In hiPSCs growth in low oxygen, the pluripotency-related genes *POU5F1* and *NANOG* were upregulated (3.10 ± 1.31 and 2.59 ± 0.97 fold changes, respectively) (both $p < 0.01$) compared with the levels in hiPSCs cultured in 21% oxygen. In addition, in hiPSCs in 5% oxygen, the expression of the *NRF1* associated with mitochondrial biogenesis and antioxidant defense was downregulated (0.29 ± 0.11 fold changes) ($p < 0.001$) compared with that in hiPSCs cultured in 21% oxygen. The second gene related to mitochondrial biogenesis *TFAM* exhibited increased expression in low oxygen (2.87 ± 1.03 fold changes, $p < 0.001$). The most significant upregulation in hiPSCs cultured in 5% oxygen was observed for the gene *POLG1* involved in mtDNA replication and damage repair (9.55 ± 3.79 fold changes, $p < 0.001$). Moreover, in hiPSCs grown in 5% oxygen, the important mitophagy gene *PARK2* was downregulated (0.71 ± 0.27 fold changes, $p < 0.05$). Changes in the expression of the studied genes and proteins were accompanied by changes in the shape of the mitochondria (Fig. 1B).

3.2. Cell viability

Human iPSC viability was tested after 5 days of exposure to MeHgCl at concentrations of 0–1 μM. At both low oxygen and atmospheric oxygen, we noted significant decreases in hiPSC viability. The strongest decrease in viability was associated with MeHgCl at 1 μM in both oxygen environments, compared with the untreated controls. In the atmospheric oxygen environment, 13.55% (± 4.29) of hiPSCs were viable, while in 5% oxygen 12.64% (± 2.012) of them were. There was a significant difference in viability between hiPSCs cultured in 21% and 5% oxygen at a MeHgCl concentration of 0.5 μM (Fig. 2A).

3.3. Mitochondrial membrane potential

Mitochondrial membrane potential was tested after 5 days of hiPSC treatment with MeHgCl at doses of 0–1 μM. We noted significant decreases of mitochondrial membrane potential for all tested concentrations of MeHgCl in hiPSC culture in 21% oxygen. For hiPSCs cultured in low oxygen, mitochondrial membrane potential was also decreased, but the effect was not significant for all concentrations of MeHgCl. The greatest decrease was observed for 1 μM. In atmospheric oxygen concentration, mitochondrial membrane potential for the concentration of 1 μM constituted 35.86% (± 3.96) of untreated control, while in low oxygen condition, 43.56% (± 5.58) of untreated control. The changes in mitochondrial membrane potential after MeHgCl treatment were independent of the oxygen concentration (Fig. 2B).

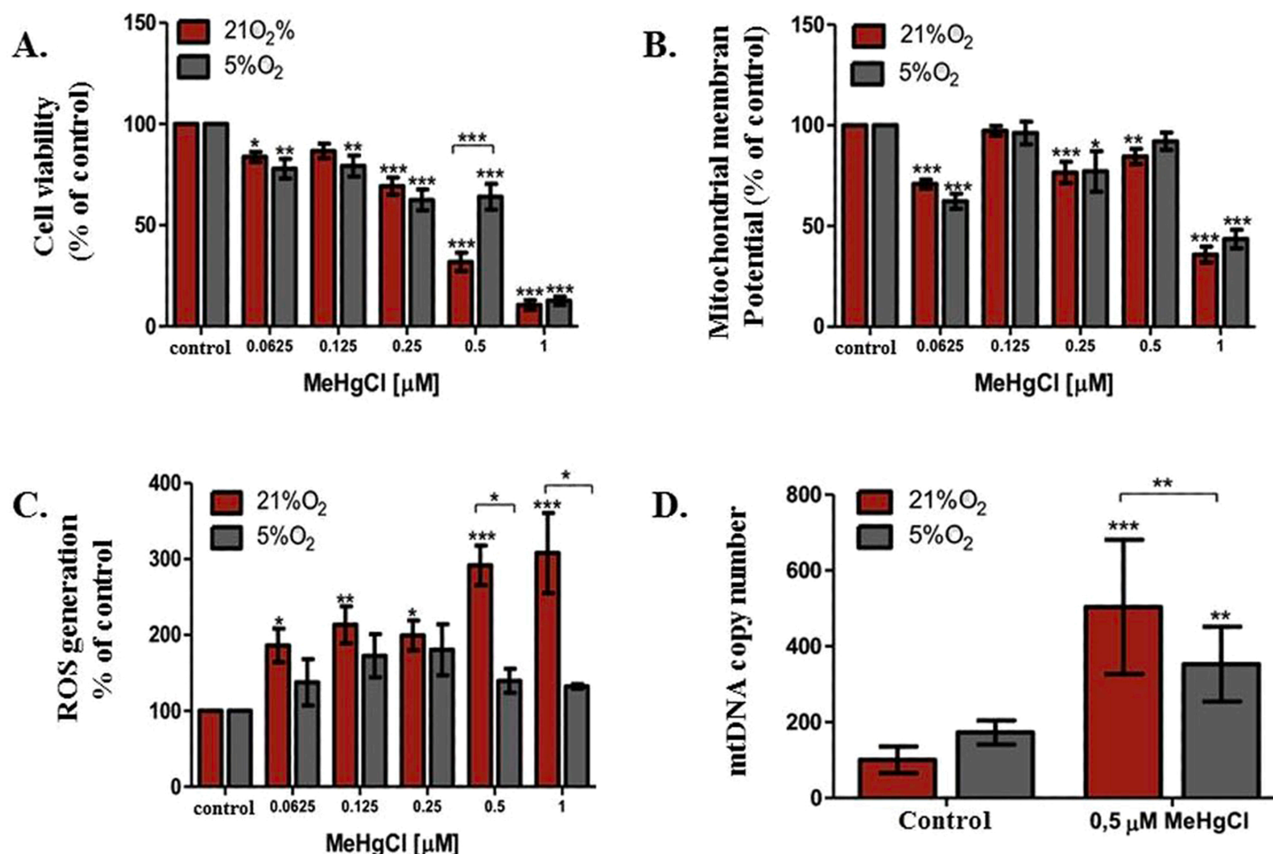

Fig. 2. The effect of oxygen concentration (21% O_2, 5% O_2) on hiPSCs after 5 days of exposure to MeHgCl (0.5 μM): A. Cell viability. B. Mitochondrial membrane potential (Δψm); C. ROS accumulation. D. The relative mtDNA copy number. Results are presented as mean (± SD). Brackets shows the statistical significance of differences between treated and untreated hiPSCs (t-test, Mann-Whitney U test); and between hiPSCs treated with MeHgCl under different oxygen conditions (two-way ANOVA, Bonferroni posttest): *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.4. ROS accumulation

After 5 days of growth of hiPSCs under 21% or 5% oxygen and treatment with MeHgCl, ROS accumulation was measured. In hiPSCs cultured in a 21% oxygen environment, MeHgCl induced significant upregulation of ROS accumulation compared with that in the untreated hiPSC controls, while in 5% O_2, this increase was not significant. In hiPSCs cultured in low oxygen, ROS accumulation was observed, but their growth was not statistically significant. However, significant differences in ROS accumulation were found between hiPSCs grown in 21% and 5% oxygen upon treatment with 0.5 and 1 μM MeHgCl. The increase of ROS accumulation in hiPSCs exposed to MeHgCl (0.5 and 1 μM) in 21% oxygen was about 3-fold relative to the level in the untreated hiPSC control (Fig. 2C).

3.5. Relative mtDNA copy number

Relative mtDNA copy number (Fig. 2D) was calculated as the averages of the *MT-ND1/SLCO2B1* ratio and *MT-ND5/SERPINA1* ratio, where *MT-ND1* and *MT-ND5* are encoded by the mitochondrial genome while *SLCO2B1* and *SERPINA1* are encoded by the nuclear genome. MeHgCl (0.5 μM) increased the mtDNA copy number in hiPSCs grown under atmospheric oxygen conditions from 49.11 (± 16.32) to 371.3 (± 102.1) ($p < 0.001$), while in hiPSCs grown under low oxygen conditions it increased from 97.44 (± 21.31) to 308.3 (± 116.5) ($p < 0.01$). We noted a significant difference in this variable between hiPSCs grown under the two different oxygen concentrations ($p < 0.01$).

3.6. Expression of genes involved in the regulation of pluripotency, mitochondrial biogenesis, mitophagy, mtDNA damage, and repair in hiPSCs exposed to MeHgCl under different oxygen conditions

Fold change (log2) expression of genes controlling pluripotency (*POU5F1*, *NANOG*), and genes involved in mitochondrial biogenesis (*NRF1*, *TFAM*), antioxidant defense (*NRF1*), mitophagy (*PARK2*), mtDNA replication, and damage repair (*POLG1*) in hiPSCs treated with MeHgCl, relative to the gene expression in hiPSC untreated with MeHgCl in 5% and 21% O_2 was shown in Fig. 3.

MeHgCl exposure resulted in the upregulation of *POU5F1* gene expression in hiPSCs growth at 21% O_2 and 5% O_2 concentration. After MeHgCl treatment, the *POU5F1* expression was enhanced (2.02 ± 0.26 fold changes) in hiPSC growth at 21% oxygen concentration and about 1.30 ± 0.72 fold changes in hiPSC at 5% oxygen concentration. There was also a significant difference in relative *POU5F1* gene expression in hiPSCs under 5% and 21% oxygen concentrations ($p < 0.05$) after MeHgCl treatment. Compared with the level in the untreated control, the level of *NANOG* expression was decreased (−2.27 ± 0.19 fold changes) in hiPSCs culture in 21% oxygen with exposure to MeHgCl. In

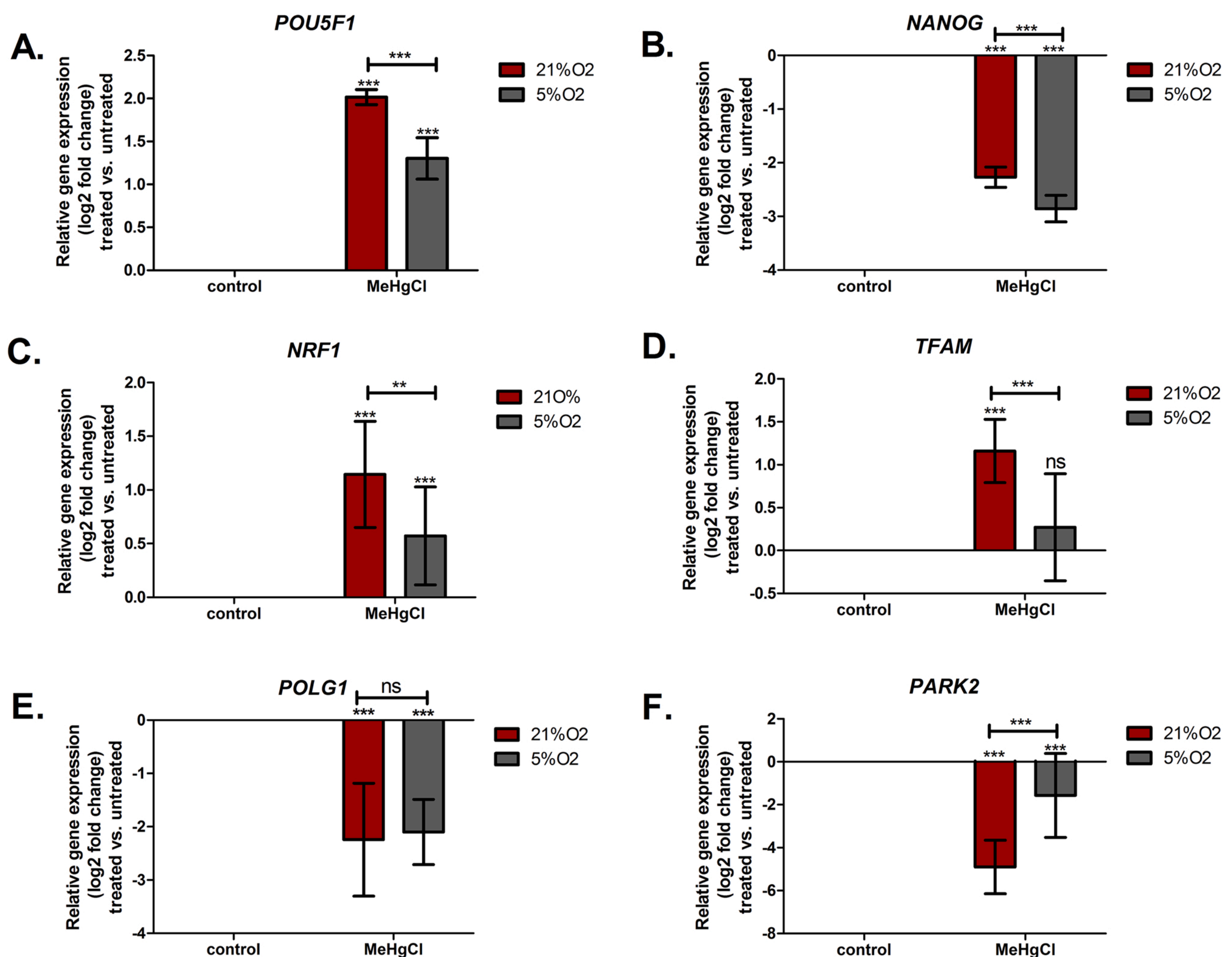

Fig. 3. Relative gene expression (log2 fold change) of genes of interest in hiPSCs treated with MeHgCl, relative to hiPSC untreated with MeHgCl in 5% and 21% O_2. hiPSCs were exposed to MeHgCl (0.5 μM) per 5 days. Results in brackets represent mean (± SD). The figure presents significant differences between: hiPSCs exposed to MeHgCl (0.5 μM) in 21% and 5% oxygen (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), as determined by two-way ANOVA, with Bonferroni multiple comparison test. Log2 fold change of control= 0.

hiPSCs cultured in 5% O_2, downregulation of *NANOG* (−2.86 ± 0.25 fold changes) was also observed. The significant difference in *NANOG* expression in hiPSCs cultured in both compared oxygen environments after MeHgCl exposure was improved ($p < 0.001$). *NRF1* gene expression was stronger upregulated (1.15 ± 0.49 fold changes) in hiPSCs growth in 21% oxygen concentration. In addition, *TFAM* expression was also upregulated in these oxygen concentrations (1.16 ± 0.37 fold changes). The *NRF1 ($p < 0.01$)* and *TFAM ($p < 0.001$)* expression level in hiPSCs after MeHgCl treatment have differed between 21% and 5% oxygen conditions. *POLG1* expression was downregulated upon MeHgCl treatment in 21% and 5% oxygen concentration (−2.24 ± 1.06 and −2.10 ± 0.61 fold changes, respectively). The difference in *POLG1* gene expression between the compared oxygen environments was not significant. Finally, the expression of the *PARK2* gene was reduced (−4.90 ± 1.25 fold changes) by MeHgCl (0.5 μM) treatment stronger in hiPSCs in 21% O_2 in compared to 5% O_2. A significant difference in the *PARK2* gene expression level was noted between hiPSCs cultured at 21% and 5% oxygen concentration after MeHgCl treatments ($p < 0.001$).

4. Discussion

In this study, we addressed whether the *in vitro* toxicity of MeHgCl on hiPSCs differs between 21% and 5% O_2 concentrations and to what extent changes in the expression of the main regulators of pluripotency and mitochondria are involved in the mechanism of this toxicity. hiPSCs were used as an ethical alternative to human embryonic stem cells (hESCs) to test the embryotoxicity.

The presence of HIF's in nucleus is required for their transcriptional activity and their ability to regulate the dependent from them expression of genes. We have demonstrated that, in low oxygen, HIF2α and HIF3α were mainly localized in the cytoplasm even if their nuclear localization was also detected. Their levels of expression in low oxygen were higher than under atmospheric oxygen conditions for both subcellular localizations. At the atmospheric oxygen concentration, HIF2α and HIF3α were expressed in cytoplasm. Meanwhile, HIF1α expression was not detected in hiPSCs in either oxygen condition. We postulated that the response to MeHgCl, under different oxygen conditions, may depend on HIF's and the results of our research support this hypothesis. Our data and a theoretical model of HIFs' influence on pluripotency-related and mitochondrial genes in the control groups and upon MeHgCl treatment are presented in Figs. 4 and 5 (Supplementary Material), respectively.

We think that upregulation of *POU5F1, NANOG* resulted from upregulation of HIF-2α in cytoplasm and nucleus in low oxygen conditions. HIF-2α is the direct upstream regulator essential for maintaining pluripotency transcription factors Oct-4, Sox2, and Nanog (Covello et al., 2006; Forristal et al., 2010). This is also why hypoxia increases reprogramming efficiency and promotes an undifferentiated cell state in iPSCs (Forristal et al., 2010). Under low oxygen conditions, we detected the upregulation of pluripotency markers (*POU5F1*, *NANOG*) and the main regulators of mitochondrial biogenesis (*TFAM*) and mtDNA replication/mtDNA repair (*POLG1*). hiPSCs cultured in 5% O_2 revealed significantly lower *NRF1* and *PARK2* gene expression than those in 21% O_2, which may indicate diminished mitochondrial biogenesis and mitophagy, respectively. Downregulation of these genes is associated with a decreased level of ROS accumulation in cells cultured in low oxygen (Mas-Bargues et al., 2019).

Contrary to the case in cells cultured in low oxygen, under atmospheric conditions, upregulation of *TFAM* and downregulation of *NRF1* can be explained by increased oxidative stress and a switch of metabolism. We also observed changes in mitochondria, as the shape of the mitochondria in 21% O_2 was elongated, suggesting a dependence on the O_2 concentration of the fission-to-fusion ratio. In contrast, the more rounded mitochondria in hiPSCs growing in low oxygen suggested increased mitochondrial fission.

In our studies the ROS accumulation increased significantly in 21% O_2 compared with that in 5%. As widely used in *in vitro* toxicology tests, 21% O_2 represents a hyperoxic non-physiological condition, impairing stem cell behavior by many mechanisms (Mas-Bargues et al., 2019). Taking into account the important role of low oxygen for stem cells, typical for their endogenous niche (Panchision, 2009; Silván et al., 2009; Eliasson and Jönsson, 2010), we assessed the effect of high oxygen levels on hiPSC response to MeHgCl, paying particular attention to mitochondrial biogenesis, mtDNA repair, and mitophagy.

Since the toxicity of MeHgCl to hiPSCs was related to the increase of mtDNA copy number in both oxygen conditions, to confirm the oxygen-independent toxicity of MeHgCl, we focused primarily on examining the mechanism responsible for the increased mtDNA copy number. Earlier research performed by Lee and Wei (2005) showed that oxidative stress increases mitochondrial abundance. In this study, MeHgCl toxicity was associated with increased ROS accumulation only under 21% O_2, which involved significant upregulation of *NRF1* and *TFAM*, and downregulation of *PARK2* (Table 1). This means that the increased mtDNA copy number in hiPSCs grown in 21% O_2 involved the production of new mitochondria generated by mitochondrial biogenesis along with damaged mitochondria not removed by mitophagy. In addition, in both oxygen conditions, we identified strong downregulation of *POLG1*, involved in mtDNA replication but also mtDNA damage repair after MeHgCl treatment. The differences in *POLG1* expression after MeHgCl exposure between hiPSCs grown under the different oxygen conditions were insignificant. However, we detected approximately eightfold higher *POLG1* expression (Fig. 1A) in the hiPSCs control (cells untreated with MeHgCl) population growth in low oxygen than in hiPSCs cultured under atmospheric conditions. The expression of *PARK2* in untreated hiPSCs in 5% O_2 was significantly lower than that in hiPSCs grown in 21% O_2. Thus MeHgCl induced toxicity in hiPSCs through altering mitochondrial function by suppressing *POLG1* involved in mtDNA damage repair and replication in both conditions. For this reason, we suggest *POLG1* as the main primary regulator of MeHgCl toxicity associated with mitochondria.

Our research suggests that this may be an effect of significantly increased ROS accumulation reported only in atmospheric oxygen conditions. At the same time, there were transparent relationships between ROS accumulation and *NRF1* and *TFAM* expression, although the relationship between ROS accumulation and *PARK2* gene expression requires further research. In both oxygen conditions after exposure to MeHgCl together with a decrease of hiPSC viability, we observed an increase of mtDNA copy number. However in 21% O_2, we observed a higher mtDNA copy number, which can be related to the increased expression of genes involved in mitochondrial biogenesis such as *NRF1* and *TFAM* and the down-regulation of *PARK2*. Thus the increased mtDNA copy number occurring in a manner dependent on oxygen concentration resulted from the *PARK2* downregulation in 21% O_2. hiPSCs grown under 21% oxygen conditions will have "new" mitochondria and "old damaged" mitochondria not removed in the process of mitophagy. In MeHg-treated BeWo cells, a reduction in mitophagy (*PINK* and *ATG13* genes down-regulation) has also been demonstrated

Table 1
Summary of the changes induced by MeHgCl (0.5 μM) in hiPSCs cultured under atmospheric and low oxygen concentrations ($p < 0.05$).

Factors		21% O_2	5% O_2	5% O_2 *vs.* 21% O_2
Viability		↓	↓	↑
Mitochondrial membrane potential		↓	ns	ns
ROS accumulation		↑	ns	↓
mtDNA copy number		↑	↑	↓
Pluripotency-related gene expression	*POU5F1*	↑	↑	↓
	NANOG	↓	↓	↓
Mitochondria-associated gene expression	*NRF1*	↑	↑	↓
	TFAM	↑	ns	↓
	POLG1	↓	↓	ns
	PARK2	↓	↓	↑

ns-not significant.

(Ganapathy et al., 2022). An increase in mtDNA copy number was also associated with a significant decrease of mitochondrial membrane potential in hiPSCs treated with MeHgCl under atmospheric oxygen conditions.

Meanwhile, in both conditions, the upregulation of *POU5F1 (*encoding the OCT3/4 transcription factors) and downregulation of *NANOG* was detected (Table 1). These genes are crucial for maintaining pluripotency, which is responsible for hiPSC self-renewal and differentiation potential. We did not observe disturbance in the morphology of hiPSCs treated with MeHgCl in both oxygen conditions; however, we think that strong suppression of one of the pluripotency markers, *NANOG*, can have a major influence on the differentiation potency of hiPSCs. It should be noted that hiPSCs obtain energy by glycolysis and are not dependent on mitochondria in this regard; therefore, mitochondrial disturbance allows undifferentiated hiPSCs to grow. MeHgCl does not preserve the self-renewal potential of hiPSCs but is likely to impair their ability to differentiate (downregulation of *NANOG*).

The results obtained under low oxygen conditions (physiological oxygen concentration) in the presence of MeHgCl can be a consequence of the transcriptional activity of HIF2α and HIF3α. The relationship between HIF2α and the expression of *POU5F1* and *NANOG* is clear, but other interactions are currently only speculative, so further study of them should be performed. We noted that *POLG1* downregulation could be regulated in an oxygen-independent manner. The mechanism behind the regulation of *POLG1* by MeHgCl may also differ between 21% and 5% O_2 conditions. This mechanism can depend on HIF1α and HIF2α at low oxygen levels, while it can depend on ROS accumulation in atmospheric conditions. A diagram presenting the potential mechanism of MeHgCl toxicity related to HIF's activity in hiPSCs culture at a low oxygen concentration is shown in Fig. 5 (Supplementary Material).

Our study showed that MeHgCl exposure led to reductions in viability and mitochondrial membrane potential while increasing ROS accumulation in a manner related to mtDNA copy number at a MeHgCl dose of 0.5 μM. In both tested oxygen conditions, hiPSC viability, mitochondrial membrane potential, and ROS accumulation were decreased in a MeHgCl concentration-dependent manner. Mitochondrial membrane potential was not significantly influenced in hiPSCs treated with MeHgCl at 21% and 5% oxygen conditions. Differences between atmospheric and low oxygen levels were noted for viability and ROS accumulation in hiPSCs treated with MeHgCl, but not at all concentrations. hiPSC viability was significantly lower, and ROS accumulation was increased in 21% oxygen starting from the dose of 0.5 μM MeHgCl. Meanwhile, at the low oxygen concentration, the increase in ROS accumulation was not statistically significant. Moreover, ROS accumulation at the atmospheric oxygen concentration showed a positive correlation with the mtDNA copy number, which was increased in both tested oxygen conditions (Table 1). Thus our data support the mode of action of MeHgCl, identified as a toxicant affecting human development, which is related to mitochondrial toxicity. Recently MeHg exposure was associated to mitochondrial damage in the spinal cord of chicken embryos disturbance of mitochondrial dynamics, and mitophagy (Ferreira et al., 2018). In an *in vitro* model of hESC-derived neural progenitors, low-dose methylmercury influenced mitochondrial biogenesis *via* miR-25, inducing developmental neurotoxicity (Wang et al., 2016b). It was also shown that MeHgCl induces ROS accumulation, leading to the upregulation of mitochondrial biogenesis (Wang et al., 2016a). POLG1 regulates mtDNA synthesis, replication, and mtDNA repair (Copeland and Longley, 2003).

Surprisingly, our results showed that an increase in mtDNA copy number correlates with the down-regulation of expression of *POLG1* gene, which is involved in mtDNA replication under both oxygen conditions. However, Liang and colleagues (Liang et al., 2020) proved that dysfunction of *POLG1* gene in human undifferentiated iPSCs did not affect mtDNA copy number level. Taking into account these results, we can hypothesize that in humans undifferentiated iPSCs mtDNA replication may occur independently of the *POLG1* gene. Therefore, the decrease in *POLG1* expression in iPS cells treated with MeHgCl did not cause the decrease in mtDNA copy number. Another function of *POLG1* is the rapid degradation of mtDNA fragments after double-strand breaks (DSBs) in the mitochondrial DNA (Nissanka et al., 2018). We hypothesized that this function of *POLG1* may be impaired and lead to the accumulation of defective mitochondria in our study.

Taken together, our data and discussion above, we think that the oxygen concentration play important role in response of hiPSCs to MeHgCl treatment. In addition, the suppression of *POLG1* is the main mechanism behind the toxicity of MeHgCl independent from oxygen level.

5. Conclusions

In this study, we observed significant differences between hiPSCs grown in atmospheric and lowered oxygen levels and a significant influence of the environmental oxygen concentration on the mitochondrial toxicity of MeHgCl. We showed that atmospheric oxygen increases the dose-dependent toxicity of methylmercury chloride by increasing ROS accumulation and decreasing hiPSC viability. In response to the increase in exogenous ROS, the expression of genes involved in mitochondrial biogenesis (*NRF1*, *TFAM*) and antioxidant defense (*NRF1*) was upregulated, and the repair of mtDNA damage was strongly inhibited (*POLG1*), as was the removal of damaged mitochondria by mitophagy (*PARK2*). Under reduced oxygen conditions, the increases in the expression of *TFAM* was not statistically significant. The increases in ROS accumulation and mtDNA copy number in low oxygen conditions after exposure of hiPSCs to MeHgCl were significantly lower than those in hiPSCs grown in 21% oxygen. Under both oxygen conditions, MeHgCl greatly influenced the expression of pluripotency-related genes by inhibiting the expression of *NANOG*, in contrast to the findings for *POU5F1,* which was up-regulated.

In summary, we showed the significant influence of oxygen conditions on the response of hiPSCs to MeHgCl treatment. We can conclude that oxygen conditions play an important role in the mechanism of methylmercury chloride toxicity. hiPSCs provide an important and ethical model for the testing of embryotoxicity *in vitro* as an alternative to human embryonic stem cells (hESCs), particularly important in countries where the use of hESCs is prohibited. However, the use of hiPSCs in toxicity studies of environmental toxicants, such as MeHgCl must take place at an oxygen biomimetic concentration that reflects the embryonic environment. Otherwise, the oxidative stress caused by atmospheric oxygen will mask the actual *in vitro* mechanism of toxicity of the analyzed environmental toxicants.

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CRediT authorship contribution statement

J. Augustyniak: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **G. Lipka:** Investigation. **H. Kozlowska:** Formal analysis, Investigation, Writing – review & editing. **F. Caloni:** Formal analysis, Writing – review & editing. **L. Buzanska:** Conceptualization, Formal analysis, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113737.

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