Hungarian University of Agriculture and Life Sciences

Doctoral School of Animal Biotechnology and Animal Science

IMPACT OF ENVIRONMENTAL EXPOSURES ON EARLY HUMAN CARDIAC DIFFERENTIATION OF INDUCED PLURIPOTENT STEM CELLS

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1 INTRODUCTION & AIMS

The hypothesis known as Developmental Origins of Health and Disease (DOHaD) states that, during early embryonic development, the foetus can be predisposed to stress conditions leading to the onset of non-communicable diseases (NCDs) after birth and/or later in life. Exposure to environmental factors, such as chemicals, can negatively affect the embryo and organ development.

Bisphenol A (BPA), an endocrine-disrupting compound (EDC), is frequently used in the production of plastics and epoxy resins. Due to its widespread use, BPA is pervasive within the environment and potentially harmful for humans even in low concentrations, increasing the risk of metabolic disorders, cancer, cardiovascular and neurological diseases. The BPA daily intake for humans is estimated between 1-5 µg/kg/day. However, according to the Endocrine Society, assumptions regarding a safe daily intake are highly questionable, because BPA responses are typically non-monotonic, a trait of EDCs. Numerous effects of BPA on the heart and its development are documented. Nevertheless, the effects of BPA toxicity on human foetal heart development are still largely unknown, as the current research mostly comes from animal models, which have significant limitations, including inter-species physiological differences with humans.

Recently, *in vitro* models have attracted considerable attention for the study of developmental toxicity, representing a valid tool for regulators to characterise and integrate new strategies for risk assessment of environmental chemicals. Because of this, the use of human induced pluripotent stem cells (hiPSCs) as a New Approach Methodology (NAM) for studying cardiac toxicity and development has gained considerable attention. The differentiation of hiPSCs into cardiomyocytes (hiPSC-CMs) provides a unique platform for cardiotoxicity studies *in vitro*, with a broad variety of chemicals. However, a vast majority of studies focus on the immediate effects of toxicants after acute treatment, while chronic exposure (repeated for several days to weeks) has only been investigated recently.

BPA toxicity *in vitro* has been documented mainly after acute exposure, showing the effects of higher doses than those that are typically observed in an environmental pollution context. Nevertheless, even acute exposure to environmentally relevant concentrations of BPA caused significant functional alterations (e.g., delayed repolarization, prolonged action potential, inhibition of ion channels). However, little is known about the extent of cardiac damage when compared to a single (acute) BPA exposure. For this reason, this study aimed to investigate the effects of BPA after a repeated, and environmentally relevant exposure during early human cardiomyocyte development. Potential chronic effects that occur overtime are not usually considered

with *in vitro* experiments of short durations. Thus, developing new model systems to mimic human chronic exposure and its effects is of the utmost importance to make *in vitro* methods more broadly useful in risk assessment. Moreover, co-exposure with other endogenous and exogenous agents, which could easily occur during daily life and early development, is an additional confounding factor in understanding the impact of BPA on human health. For example, *in vitro* and *in vivo* studies investigated the interactions between BPA and factors such as dietary compounds, therapeutics, and other environmental compounds, or well-known stressors such as hypoxia. For this reason, the integration of other stress factors can provide more robust study designs to evaluate BPA's toxicity.

In the present study, we established an *in vitro* human-based iPSC-CM model to assess early cardiac developmental toxicity of DOHaD-related chemical exposure. To increase the relevance of toxicity assessment *in vitro*, we mimicked, for the first time, a chronic exposure scenario to investigate the effects of environmental doses of BPA during human cardiomyocyte differentiation. Our model provided information of disturbances caused by BPA on functionality, molecular features, and cellular surrounding environment during early phases of human foetal cardiomyocyte development. Additionally, quantitative proteomics together with network mapping revealed BPA-induced molecular alterations that could be linked to hiPSC-CMs phenotypical alterations, and CVDs associations. Furthermore, the toxicity raised after BPA treatment was investigated exposing the hiPSC-CMs to a hypoxia-reoxygenation (H/R) insult. Thus, our *in vitro* cardiotoxicity study can provide valuable supplementary information on environmental chemical risk assessment for regulators.

Objectives of this study

The overall aim of this study was to find answers to the following scientific questions:

- Do hiPSC-CMs represent a valid model to study chemical exposures during early cardiomyocyte development *in vitro*?
- Do low doses of BPA affect the efficiency of cardiomyocyte differentiation and their viability over time? Does the chemical cause alterations to cellular proliferation and DNA lesions?
- Is the functionality altered in hiPSC-CMs after 21-day treatment with BPA?
- Is the proteome profile different in hiPSC-CM treated with BPA? Does the BPA treatment reveal early biomarkers which can be linked to cardiomyocyte alterations and/or heart diseases?
- What impact does the repeated treatment with BPA have on the hiPSC-CMs, if exposed to additional stress such as hypoxia-reoxygenation? Are the hiPSC-CMs more vulnerable?

Specific objectives of the research:

- Establishment and maintenance of hiPSC-CM cultures and their characterisation by the expression of cardiac markers and their spontaneous beating activity.
- Validation of the hiPSC-CMs as a NAM to study chemical exposures *in vitro*.
- Evaluation of the effects of BPA during early stages of cardiomyocyte differentiation.
- Protein analysis of the hiPSC-CMs treated with environmentally relevant doses of BPA.
- Investigation of altered biomarkers and diseases association study.
- Evaluation of the effects of hypoxia-reoxygenation stress on the hiPSC-CMs treated with BPA.

2 MATERIALS AND METHODS

2.1 hiPSC line

The human iPS cell line (hiPSC; SBAD2 clone) used in this study originated from Normal Adult Human Dermal Fibroblasts (NHDF-Ad) cells (51-year-old Caucasian male, Lonza #CC-2511), reprogrammed using non-integrative Sendai virus transduction. The cell line was provided during the IMI-funded StemBANCC project (Morrison et al., 2015). BD Matrigel (BD Biosciences #354277) was used for plate coating and cells were cultured with mTeSR™1 medium (Stem Cell Technologies #85870) supplemented with 1% Penicillin-Streptomycin. Cells were maintained in vitro at 37°C in a humidified atmosphere containing 5% CO_2. For routine passages, Versene® (EDTA) (0.02%, Lonza #BE17-711E) was used according to the manufacturer's protocol. Routine screening for mycoplasma was carried out using the Venor®GeM-Advance (Minerva Biolabs #11-7024) Mycoplasma Detection Kit, according to the manufacturer's instructions.

2.2 Pluripotency test

After single cell dissociation with Accutase® (Thermo Fisher #A6964), cells were cultured in suspension for five days in mTeSR™1. They formed embryoid bodies (EBs), which were plated on 0.1% gelatin (Merck) coated surface in differentiation medium (DMEM, 20% FBS, 1% MEM Non-Essential Amino Acid Solution (100×), 0.1 mM β-mercaptoethanol, 1% Pen/Strep). After 14 days of spontaneous differentiation, the cells were fixed with 4% paraformaldehyde (PFA) solution and evaluated for the 3 germ layer markers by immunocytochemistry.

2.3 Cardiomyocyte differentiation from hiPSC

For cardiomyocyte differentiation, the PSC Cardiomyocyte Differentiation Kit (Thermo Fisher #A2921201) was used according to the manufacturer's protocol. Plates were coated with Geltrex™ (Thermo Fisher #A14133) and the hiPSC were plated after Accutase® induced single-cell dissociation. Cells were seeded at a density of 1.25 x 10^4 cells/cm^2 in mTeSR™1 medium supplemented with RevitaCell™ (Thermo Fisher #A2644501). When the hiPSC confluency reached 70-80%, the mTeSR™1 medium was aspirated and slowly replaced with Medium A. Two days later, the medium was substituted with Medium B, and two days after the medium was replaced with Maintenance Medium. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C, and the Maintenance Medium was replaced every other day. On sampling days, cells were collected after dissociation with TrypLE™ Select Enzyme (10X) (Thermo Fisher #A1217702).

2.4 Immunocytochemistry

Cells on glass coverslips were fixed with 4% PFA at room temperature (RT) for 15 minutes. Then, the samples were rinsed with DPBS and permeabilized using 0.2% Triton X-100 (Merck) in DPBS. Afterwards, the cells were blocked in a buffer solution containing 5% bovine serum albumin in DPBS for 1 h at RT, then stained with primary antibodies, and incubated overnight at 4 °C. The following day, cells were washed with DPBS and incubated for 2 hours in the dark with secondary antibodies in 1% BSA solution. Next, cells on coverslips were mounted with ProLong™ Diamond Antifade Mountant (Thermo Fisher #P36961) containing DAPI. The slides were analysed with a fluorescence microscope (Axio Imager system with ApoTome; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) controlled by AxioVision 4.8.1 software (Carl Zeiss MicroImaging GmbH).

2.5 Reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from hiPSC and hiPSC-CMs through digestion with Proteinase K (QIAGEN #19131) using the RNeasy Plus Mini Kit (QIAGEN #74134) following the manufacturer's instructions. One microgram of RNA was used for cDNA synthesis through the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher #K1641). The amplification was carried out in a total of 15 µL using SYBR Green JumpStart Taq ReadyMix (Thermo Fisher #S4438). Oligonucleotide primers were designed with Primer 3 software (Untergasser et al., 2012) and blasted on the human reference genome on Ensembl (Cunningham et al., 2022). All RT-qPCR reactions were normalized using *GAPDH* as a housekeeping gene and analysed using the comparative $2^{-\Delta\Delta Ct}$ method by normalising hiPSC-CMs and hiPSC to human adult heart reference RNA (Takara #636532).

2.6 Cellular viability assay

The hiPSC-CMs were seeded into a 96-well plate with a density of $2x10^5$ cell/cm^2. After three days, the cells were exposed to increasing concentrations of compounds for different time periods (24-96 hours). CellTiter-Glo® Luminescent Cell Viability Assay (Promega #G7570) was used to perform the ATP viability assay following the manufacturer's protocol. The luminescent signal was recorded using the Varioskan Flash Multimode Reader (Thermo Fisher). Cell survival was calculated by assigning 100% viability for untreated controls and 0% viability for cells killed by exposure to water.

2.7 Oxidative stress detection

To measure reactive oxygen species (ROS) in hiPSC-CM cultures, the cells were treated with 5 mM CellROX™ Deep Red (Thermo Fisher #C10422) for 1h at 37°C.

Cells were then fixed with 4% PFA and mounted with ProLong™ Diamond Antifade Mountant. The images were acquired with a fluorescence microscope (Axio Imager system with ApoTome; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) controlled by AxioVision 4.8.1 software (Carl Zeiss MicroImaging GmbH). The signal quantification was performed using ImageJ software (Schneider et al., 2012).

2.8 Bisphenol A repeated treatment

Bisphenol A (Merck, #239658, CAS No. 80-05-7) stocks were prepared by dissolving the compound in DMSO to a final concentration of 100 mM and stored at -20 °C. BPA was further diluted to the chosen concentrations selected based on typical levels of human environmental exposure (Murata & Kang, 2018). For the repeated treatment, cells were exposed to 0.1% DMSO (vehicle) or BPA at selected concentrations (0.01 μM, 0.1 μM, 1 μM), starting from day 0 until day 21 of cardiomyocyte differentiation. For this process, a fresh differentiation medium containing BPA at the designated treatment concentrations was added every two days after completely removing the spent medium.

2.9 Flow Cytometry

The collected cells were washed with DPBS and moved into flow cytometry tubes (Beckman Coulter, #2523749) for staining. Firstly, the cells were stained with the Fixable Viability Dye eFluorTM 660 (eBioscienceTM #65-0864) for 30 minutes at 2-8 °C, protected from light. Then, the samples were rinsed with 1% BSA solution and centrifuged at 300-400xg at RT for 10 minutes. The True-Nuclear™ Transcription Factor Buffer Set (BioLegend #424401) was used for staining, following the manufacturer's instructions, with the chosen conjugated antibodies. Samples were processed using the Flow Cytometer Cytomics FC 500 (Beckman Coulter), and data analysis was conducted with FlowJo (BD Bioscience, V10.8.1).

2.10 Contractility assay

The contractile motion of hiPSC-CMs was recorded through phase contrast video-microscopy using the Olympus IX71 microscope equipped with a DP21 camera (Olympus) and its CellSens software (Olympus; V1.11). Videos lasting a minimum of 10 seconds and running at 12 frames/second were analysed using the Pulse analysis platform (Curibio) (Maddah et al., 2015).

2.11 Quantitative proteomics: sample preparation

Using a Sonopuls HD3200 (Bandelin, Berlin, Germany), cells were lysed (18 cycles of 10 s) in 8M urea/0.5 M NH_4HCO_3. Protein quantification was performed with the Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). A total

of 20 µg of protein was processed for digestion with porcine trypsin (Promega, Madison, WI, USA) for 16 hours at 37°C (1:50 enzyme to protein ratio.

2.12 Nano-liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis and statistics

The sample (1 µg) was injected into an UltiMate 3000 nano-LC system connected online to a Q-Exactive HF-X mass spectrometer operated in data-dependent acquisition mode. Then, peptides were transferred to a PepMap 100 C18 trap column (100 µm×2 cm, 5 µM particles) and separated using a PepMap RSLC C18 analytical column (75 µm×50 cm, 2 µm particles) at a flow rate of 250 nl/min, with a gradient of 5-20% of solvent B used for 80 minutes followed by a 9-minute increase to 40%. Formic acid 0.1% in water and acetonitrile were used as solvents A and B, respectively. Raw data were processed using MaxQuant (version 1.6.7.0) (Tyanova et al., 2016). The human SwissProt reference proteome (downloaded in October 2022) was used for all searches. The dataset has been submitted to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2022) with the dataset identifier PXD042046. Statistics and data visualization were performed in R using custom scripts. Differentially abundant proteins were detected using MS-EmpiRe pipeline (Ammar et al., 2019) as described previously (Flenkenthaler et al., 2021). Briefly, proteins were quantified with at least two peptides with a minimum of three replicate measurements in each condition. Peptides with complete measurements in one condition but insufficient measurements in the other condition were inputed from a normal distribution (downshift = 1.8, scale = 0.3). Proteins with a Benjamini-Hochberg-adjusted p-value < 0.05 and a fold change above 1.3 were considered as significantly changed. The ComplexHeatmap R package (Gu et al., 2016) generated the heatmap. WebGestaltR package (Liao et al., 2019) and the functional category 'GO Biological Process nonRedundant' were used to perform over-representation analysis.

2.13 Human Protein-Protein Interactions (PPI) network construction and analysis

The human PPI network was constructed using publicly available resources (Alanis-Lobato et al., 2017; Luck et al., 2020; Menche et al., 2015), resulting in 18,816 proteins and 478,353 physical interactions. Proteins with a fold change (|FC|)>1.5 were mapped onto this network, and their connectivity was analysed by computing a z-score of the largest connected component (lcc) size for each protein group compared with 10,000 randomly selected protein sets of identical size. The global perturbation of each condition and the proteins that were up- and down-regulated were analysed. To create a connected core from the sparse network, a random walk with restart algorithm was used. The algorithm used a restarting parameter (alpha) of

0.9 to keep the propagation closer to the initial seed genes. Furthermore, only nodes with high expression levels in the heart were included at each step. The Human Protein Atlas (HPA) was used to extract this information. The resulting list contained 36 tissue enriched proteins (|FC|heart>4*|FC|any tissue), 129 group enriched proteins (|FC|heart>4*|FC|any tissue-4), and 257 tissue enhanced proteins (|FC|heart>4*|FC|average(all tissues)), totalling 452 proteins.

2.14 Protein enrichment analysis

The biological characterization of the core and expanded modules was carried out via enrichment analyses for the three main branches of the Gene Ontology (GO) (Ashburner et al., 2000): biological processes (BP), molecular functions (MF), and cellular components (CC), as well as KEGG pathway (Kanehisa & Goto, 2000) using GSEApy (Fang et al., 2023).

2.15 Disease relationship

Diseases-gene associations (GDA) were acquired from DisGeNet (Piñero et al., 2015). To retrieve data for 11,099 disorders, we only chose relationships having a GDA score >0.3. To determine the relationship between each differentially abundant protein set (s1) and set of disease proteins (s2), two methods were applied. Firstly, the Jaccard index (intersection (s1,s2)/union(s1,s2)) was calculated, and secondly, network proximity was used, which compares the distance between the two protein sets against 10,000 random sets with similar topological features (Guney et al., 2016). This enabled the correction for interactome biases, including the heavy-tail degree distribution and the discretization of other common network distances. Moreover, only heart diseases with a false discovery rate (FDR)<0.01 (n=1096) were considered and grouped into 9 phenotypic categories. The created a network incorporated the shortest molecular path and the shortest hub-preferential path between the BPA-unified core and those heart diseases, based on heart transcriptomic protein expression. To achieve this, a random walk with a restart algorithm was applied to the 452 proteins elevated in the heart, obtained from HPA database, to obtain a heart-specific interactome. This network was then used to determine both the shortest and the hub-preferential path between the given gene sets.

2.16 Hypoxia and reoxygenation model

The hypoxia/reoxygenation (H/R) model was established according to the previously published method (Häkli et al., 2021), and using a HeraCellTM Vios 250i CO_2 incubator (Thermo Fisher). Briefly, one day prior to hypoxia treatment, hiPSC-CMs were cultured in serum- and glucose-free medium, containing 1% MEM NEAA, 1% GlutaMAX and 0.5% Penicillin-Streptomycin. The following day, hiPSC-CMs were

subjected to a hypoxic environment of 1% O_2 and 5% CO_2 gas for a duration of 8 hours. After this period, hiPSC-CMs were supplied with Maintenance Medium (containing glucose) and cultured in the standard cell culture environment to simulate the reperfusion phase for 15 hours. For control conditions, cells were cultured in serum- and glucose-free medium followed by Maintenance Medium in the standard culture environment (referred to as "normoxia").

2.17 Analysis of apoptosis and mitochondrial activity

To monitor apoptosis and active mitochondria of hiPSC-CMs, Caspase 3/7 Green Dye (Incucyte®, Sartorius # 4440) and MitoTracker™ Deep Red (Thermo Fisher #M22426) were used, respectively, according to the recommended protocols. The fluorescent signal was assessed using the IncuCyte S3 Live-Cell Analysis Instrument (Sartorius AG). The images were analysed using the IncuCyte Basic Software (Sartorius), with the acquisition times for the green and red channels being 300ms and 100ms, respectively.

2.18 Statistical analysis

All data were analysed using Prism 8 (GraphPad Software, La Jolla, CA, USA) and handled in Microsoft Excel (Redmond, WA, USA) unless otherwise specified. The data analysis is presented as the mean ± standard error of the mean (SEM). The significance of the data was determined using one-way ANOVA or two-way ANOVA with Dunnett's post hoc test.

3 RESULTS

3.1 Characterisation of hiPSC-derived cardiomyocytes

To assess the suitability of the 2D cardiac induction *in vitro*, hiPSCs were differentiated in cardiomyocytes (hiPSC-CMs) and deeply characterised. The expression levels of key cardiac transcripts were monitored throughout crucial time points of cardiomyocyte differentiation. While the pluripotency marker *OCT3/4* was rapidly downregulated, cells expressed key genes of mesodermal commitment and cardiac progenitors, starting from the second day of differentiation (*TBXT*, *HAND2*, *GATA4*, *NKX2.5*). Spontaneous beating activity was observed after one week of differentiation, together with the increased expression of myofilament genes (*TNNT2*, *TNNI1*, *MYH6*, *MYL7*). Key foetal features of hiPSC-CMs were observed by higher mRNA expression of foetal troponin I (*TNNI1*) compared to the adult isoform (*TNNI3*), together with higher levels of *MYH6* and *MYL7*, compared to *MYH7* and *MYL2*, respectively. Accordingly, immunocytochemistry labelling revealed that hiPSC-CMs exhibit misaligned localization of myofilaments. After 21 days of differentiation, about 80% of the population was represented by hiPSC-CMs. Moreover, the proteome profile of hiPSC-CMs has been characterised, comparing the hiPSC stage (D0) and the hiPSC-CMs after 21 days of differentiation (D21). About 73 % of the identified proteins were significantly altered at D21 compared to D0. While proteins being part of heart morphogenesis and cardiomyocyte differentiation were increased at D21, defining characteristics of stemness, such as cell cycle checkpoint and DNA replication, were decreased. Additionally, typical cardiomyocyte structural markers, such as multiple myosin isoforms (e.g., MYL7, MYL3 and MYL4) and troponins (e.g., TNNT2 and TNNI1), and heart metabolic markers (e.g., FABP3) were significantly increased in abundance in differentiated cells.

3.2 Validation of hiPSC-CMs as a cardiotoxicity model

To assess the suitability of our hiPSC-CM model for investigating the toxicity of environmental chemicals on foetal cardiomyocytes *in vitro*, ATP-release based viability assay was performed to generate dose-response curves of doxorubicin, menadione and acrylamide. The cytotoxicity model produced repeatable dose-responses, identifying specific half maximal effective concentration (EC50) for each compound, according to the treatment duration. Additionally, a qualitative and quantitative detection of ROS was performed, observing a positive fluorescent signal that increased in a dose-dependent manner.

3.3 Experimental set up for Bisphenol A treatment

Once the model was tested and validated on well-known toxic compounds, the experimental set up for the repeated treatment with BPA was designed. Concentrations between 0.01 μM and 1 μM represent the target range of this study, corresponding to the TDI of ≤ 4 μg/kg/day agreed after 2015 (University of Hertfordshire, 2021). Firstly, we assessed the potential cytotoxicity effects of BPA in a wider concentration range (0.01–100 μM) on 21-day differentiated hiPSC-CMs for 24 and 72 hours. The viability of hiPSC-CMs was affected at concentrations above 0.25 μM, even if the cellular mortality was not particularly extended, and the highest value of cellular death (35%) was observed at 100 μM BPA after 72-hour exposure. When three physiologically relevant concentrations of the chemical were selected (0.01 μM, 0.1 μM, 1 μM), the cells were exposed to BPA every second day, starting from the pluripotency stage at day 0. Flow cytometry analysis showed that the viability of the whole cell population was not affected by BPA treatment over the duration of the differentiation. According to the cardiomyocyte commitment, the proportion of hiPSC-CMs increased during the first two weeks of differentiation. However, the percentage of cardiac troponin T-positive (cTnT+) cells and their viability was mostly unchanged upon BPA treatment.

3.4 Bisphenol A altered the contraction properties of hiPSC-CMs

After 21 days of BPA treatment, the contraction properties of hiPSC-CMs were investigated. As the interplay of contraction's parameters and their temporal relationship are essential for the proper functioning of hiPSC-CMs, we assessed the BPA effects on the cellular spontaneous contraction using the software platform Pulse (Curibio). The beat rate of hiPSC-CMs treated with BPA showed a remarkable slowdown in a concentration-dependent manner, with a 70% reduction of beats per minute at the highest dose of BPA. The treatment with BPA also led to a significant increase in the beat duration. Accordingly, the contraction and relaxation times were prolonged in a concentration-dependent manner. Finally, BPA treatment significantly decreased the beating velocity of hiPSC-CMs, with the highest effect observed in the 1 μM treated group.

3.5 Proteome alterations in hiPSC-CMs treated with Bisphenol A

To investigate the effect of BPA exposure on hiPSC-CM proteome remodelling in a comprehensive and unbiased manner, we performed a label-free LC-MS/MS of hiPSC-CMs at day 21 exposed to repeated doses of 0 μM (vehicle), 0.01 μM, 0.1 μM and 1 μM BPA for the duration of cardiac differentiation. Compared to the vehicle group, 81, 26 and 16 proteins were changed in abundance in 0.01 μM, 0.1 μM and 1 μM BPA-treated groups, respectively. While twenty-one proteins were altered in at

least two groups, six of them were increased in abundance in all three BPA concentrations. These proteins are COL4A1, COL4A2, LAMC1 and NID2, components of the basement membrane (BM), (Boland et al., 2021), TNNC1, pivotal for muscle contraction (Li & Hwang, 2015) and SERBP1, involved in complex translational processes (Brown et al., 2018; Yan et al., 2021). The largest number of differentially abundant BM proteins, together with LAMB1, LAMA1 and HSPG2, was identified in 1 μM BPA-treated group.

3.6 Disease network analysis of hiPSC-CMs treated with Bisphenol A

To investigate the correlations of the differentially abundant proteins with molecular pathways and diseases, firstly we compared them to a set of expert-curated gene-BPA annotations in humans from The Comparative Toxicogenomic Database (CTD) (Davis et al., 2023), observing a statistically significant overlap (p: 6e-05, Fisher's exact test). Thereafter, we mapped them in a human protein-protein interaction (PPI) network, observing that the number of interactions was mainly driven by the upregulated proteins. In the BPA-treated groups, we identified a core interacting module (BPA-upregulated core) consisting of four proteins (COL4A2, COL4A1, LAMC1, NID2) (p: 6e-39), and associated with the extracellular matrix (ECM) receptor-interaction (FDR: 2e-06). Thereafter, we investigated the underlying interactions of the BPA-upregulated core with SERBP1 and TNNC1, through highly expressed proteins in the heart. We obtained an extended unified core of 24 proteins (BPA-unified core), which was enriched in the ECM-receptor interaction and in pathological heart conditions, such as hypertrophic and dilated cardiomyopathy. Afterwards, to implement topological network traits on the gene sets overlap, we used the network proximity measure (Guney et al., 2016), which identified 1806 diseases associated with the BPA-unified core, including "Acute Coronary Syndrome" (FDR: 1e-19), "Heart Failure, Right-sided" (FDR: 4e-11), and "Cardiomyopathies" (FDR: 4e-09). We curated nine phenotypical groups, gathering them based on their common physiopathology. In this regard, we identified the closest interacting proteins that link the BPA-unified core to these diseases. The enrichment analysis of this network showed alterations in the ECM organization/interaction, and in the response to reactive oxygen species (ROS).

3.7 Effects of Bisphenol A upon hypoxia-reoxygenation challenge

Since alterations to ROS is a contributing factor affecting the antioxidant defences, we asked if hiPSC-CMs treated with BPA could be more vulnerable to a significant increase of ROS. After 21 days of BPA treatment, the hiPSC-CMs were subjected to a hypoxia/reoxygenation (H/R) challenge. The hiPSC-CMs were first exposed to 8 hours of hypoxia, followed by 15 hours of reoxygenation. The standard culture conditions are referred to as "normoxia". A significant increase of cellular death was

observed in hiPSC-CMs subjected to the sequential periods of H/R, compared to hiPSC-CMs in normoxia. Notably, after H/R induction, higher caspase activity in BPA-treated groups than in the vehicle control was observed, especially in the groups treated with two highest concentrations (0.1 µM, $p < 0.01$**; 1 µM, $p < 0.05$* by the end of the reoxygenation period). In normoxia, and regardless of the BPA treatment, the mitochondrial activity remained stable. However, the overall effect of the H/R induction was a 25% reduction in the mitochondrial fluorescent signal across all groups. After H/R, the mitochondrial activity decreased mildly in 0.1 µM BPA ($p < 0.05$*), compared to the vehicle control.

4 NEW SCIENTIFIC RESULTS

In this research, the effects of a repeated-dose BPA exposure during the cardiomyocyte differentiation were investigated. Firstly, a 2D *in vitro* differentiation system to obtain cardiomyocytes from hiPSCs was established and characterised to provide a suitable model for the early stages of cardiac development. The model was suitable for cardiotoxicity investigations, providing reproducible dose-response effects from different DOHaD-related compounds, BPA included. Subsequently, functional, and molecular investigations were performed to identify the effects of environmentally relevant concentrations of BPA on hiPSC-CMs, and the potential implications with cardiovascular diseases. The research allowed the use of hiPSC-CMs to provide the following new scientific findings:

1. For the first time, I have provided a human *in vitro* model to investigate the effect of 21-day repeated-dose exposure to BPA. Of note, the BPA treatment was performed from the hiPSC stage to hiPSC-CMs, offering a new perspective of the BPA toxicity at the earliest stage of cardiomyocyte development.
2. I have presented data showing a significant change in functional features of hiPSC-CMs treated with BPA, such as the decreased contraction frequency and beating velocity, and the increased contraction and relaxation time, demonstrating that low doses of BPA significantly affect the function of hiPSC-CMs.
3. I have performed, for the first time, a proteomics-based analysis on hiPSC-CM treated with BPA for 21 days, and, together with network analysis, identified links between BPA-perturbed proteins and several cardiovascular alterations. Of note, the accumulation of BM components (COL4A2, COL4A1, LAMC1, NID2), TNNC1, and SERBP1 are potentially associated with the altered cellular functionality observed, and CVDs, such as heart failure and cardiomyopathies.
4. Finally, I have provided evidence that BPA potentially renders the hiPSC-CMs more vulnerable to additional challenges, such as the hypoxia-reoxygenation insult.

5 DISCUSSION AND FUTURE PERSPECTIVES

In the present study, we used a human iPSC-derived model to investigate the effects of repeated and low-dose BPA exposure on early developing cardiomyocytes. The model consisted of human cardiomyocytes, which provide a well-established and suitable *in vitro* modelling system for studying cardiac toxicity mechanisms (Narkar et al., 2022).

Most of the currently available data on BPA toxicity originates from research done using animal models. While the resulting knowledge is extremely valuable and paved the way for further investigation in the field, our work with hiPSC-CMs represents a valuable contribution, extending the work on animal models to the human cellular system. Current advances with hiPSC-CM-based approaches showed the relevance of *in vitro* NAMs for accurately capturing the unique aspects of environmental chemicals on human cardiovascular health. Therefore, as well as a paucity of data regarding BPA's toxicity on the developing human heart, in this work hiPSC-CMs were used to contribute to the current urgency of BPA's regulatory safety assessment (EFSA CEF Panel, 2015; EFSA CEP Panel, 2023).

In this study, we demonstrated that our *in vitro* model was effective for the differentiation of hiPSCs to cardiomyocytes. We obtained ~80% $cTnT^+$ hiPSC-CMs, expressing key cardiac genes. Together with the distinctive contraction ability of hiPSC-CMs, the robust generation of cardiomyocytes was confirmed by proteomic-based analysis, showing a net increase of well-known cardiomyocyte markers. The generated hiPSC-CMs exhibited foetal phenotype, according to their small size and disorganised structures, and markers predominantly expressed in the sarcomeres of the foetal heart and in early neonatal life (e.g., *TNNI1*, *MYL2* and *MYH6*). Since the goal of this study was to investigate the toxicity of BPA during the early stages of cardiac differentiation, the foetal phenotype of hiPSC-CMs represented an important aspect of this research. However, further research will be needed to evaluate how BPA effects on foetal cardiomyocytes are reflected on the matured counterpart.

The generated cells provided a robust and reproducible model to validate the cytotoxicity of well-known and DOHaD-related compounds, thus suitable for further *in vitro* cardiotoxicity assessments. Therefore, to investigate the effects of environmentally relevant concentrations of BPA (0.01 µM, 0.1 µM, 1 µM), a chronic exposure scenario was mimicked, with the goal to replicate a real-life scenario in an *in vitro* setting. This allowed, for the first time, the examination of the BPA effects over the early human cardiac differentiation. Although low doses of BPA did not elicit severe alterations on crucial differentiation features, such as cellular viability, and efficiency of differentiation, we observed significant changes in the functionality

of the hiPSC-CMs. In this work, BPA treatment significantly decreased the beat rate of hiPSC-CMs, and longer contraction and relaxation times were observed. These results suggest that repeated treatment with BPA elicits a chronotropic effect, previously observed in rodent models (Pant et al., 2011; Posnack et al., 2014; Ramadan et al., 2018), although after acute BPA exposure. Additionally, the contraction displacement, and the beating velocity showed that both the speed and the range of cellular elongation-shortening were affected by BPA treatment.

To deeply investigate which molecular alterations could affect the cellular functionality, we investigated proteome-wide changes in hiPSC-CMs treated with BPA. We focused on the examination of six proteins significantly upregulated in all BPA-treated groups, compared to the untreated control. Notably, a higher abundance of TNNC1 was revealed. This is a key regulatory protein which binds calcium ions to initiate muscle contraction (Li & Hwang, 2015). Since BPA has a negative impact on ion channel activity and calcium handling, affecting the excitation-contraction coupling and the Ca^{2+} release/reuptake kinetics of the sarcoplasmic reticulum (Eisner et al., 2017; Hyun et al., 2021; Liang et al., 2014; Prudencio et al., 2021), these alterations may have an impact on the activation and deactivation rates of the myofilaments, which are mainly, but not exclusively, caused by altered Ca^{2+} binding and unbinding to TNNC1 (Chung et al., 2016). This provides a potential explanation for the higher levels of TNNC1 protein observed in hiPSC-CMs treated with BPA.

Next, increased levels of BM components (COL4A1, COL4A2, LAMC1, NID2) were observed in hiPSC-CMs treated with BPA. The BM has a wide range of biological functions, among which it provides structural and mechanical support to tissues (Boland et al., 2021). A pathological remodelling and deposition of the BM have been related to structural anomalies, such as fibrosis, either in the myocardium of animal models (Bahey et al., 2019; García-Arévalo et al., 2021; Rasdi et al., 2020) and in humans (Díez et al., 2002; Disertori et al., 2017; Heymans et al., 2005; Hinderer & Schenke-Layland, 2019). An excessive fibrosis can lead to detrimental effects on myocardial function, for example, anomalies in the contraction activity (Münch & Abdelilah-Seyfried, 2021), which is in line with the slow-down of the contraction frequency observed after BPA treatment. In this context, a rigid environment can increase the contraction force and restrain the cell shortening distance, as observed in our model, in line with observations of a heart wall stiffening model using hESC-CMs (Ribeiro et al., 2020). At the same time, the stiff environment perturbs the beating frequency (Engler et al., 2008; Heras-Bautista et al., 2019), in accordance with the decreased cellular beat rate observed in this study. Interestingly, to counteract the stiffness of the niche, one of the possible adaptive responses of cardiomyocytes might be the increased production of contractile

sarcomere proteins (Grossman & Paulus, 2013). This would explain the upregulation of proteins related to muscle contraction, such as TNNC1.

Moreover, higher rigidity of the cardiomyocyte niche has been linked to changes in the regulation of translational processes and protein synthesis (Simpson et al., 2020; Wu et al., 2020). Notably, in this study, proteins related to translational regulation were consistently changed in abundance in hiPSC-CMs after BPA treatment, such as the up- and down-regulation of ribosomal subunit components in at least two BPA concentrations. In all BPA-treated groups, we observed the upregulation of SERBP1. These results suggest that the ability to modulate global translation is required to maintain cardiomyocyte function and survival under cellular stress (Simpson et al., 2020).

Next, we explored if the molecular alterations of interest (BM components, TNNC1 and SERBP1) were involved in the pathophysiology of CVDs. We have identified hub genes which, upon interaction with BPA-perturbed genes, could have different roles in heart pathologies, such as heart failure, hypertrophic cardiomyopathy, or acute coronary syndrome. Notably, we found hub genes that may play a role in reactive oxygen species (ROS) response (e.g., JUN, TPM1, APOE, SOD2, MMP9, EGFR, SOD1).

Since alterations to ROS is a contributing factor affecting the antioxidant defences, we asked if hiPSC-CMs treated with BPA could be more vulnerable to a significant increase of ROS. Therefore, we exposed the hiPSC-CMs to a hypoxia-reoxygenation (H/R) insult. Over the hypoxic stress, the apoptotic events increased in hiPSC-CMs treated with BPA, alluding to an alteration of the cellular response. One possible explanation could be the altered deposition of BM components. Perturbations in the BM network may deprive cardiomyocytes of crucial molecular signals that promote cardiomyocyte survival and function (Heras-Bautista et al., 2019; Sekiguchi & Yamada, 2018). Otherwise, BPA has been previously linked to the destabilization of antioxidant defences *in vitro*, mainly through HIF-1α degradation (Kubo et al., 2004). Indeed, studies on zebrafish indicated that early cardiovascular development may be more susceptible to hypoxia under BPA exposure (Cypher et al., 2015, 2018). Nevertheless, further investigations are necessary to identify the apoptotic pathway involved. A good avenue for future experiments could be the examination of matrikines released from the BM remodelling/degradation, and the Fas ligand-mediated apoptosis, a known target for BM matrikines (Panka & Mier, 2003; Verma et al., 2013).

Despite the results obtained, which add a valuable contribution to the current knowledge regarding the toxicity of BPA, the model used in this study does not lack of limitations. Firstly, the use of one cell line limits the evaluation of BPA effects in

a wider spectrum, and further studies will be needed to validate and expand our findings using other cell line models. Secondly, stress exposure *in vitro*, for practical reasons, is different from the *in vivo* stress exposures, occurring over a longer period and in variable doses. Even if hiPSCs can be an effective model to reduce the use of experimental animals, using hiPSCs instead of animals is still a controversial issue, because it is difficult to predict the *in vivo* results with only *in vitro* data. For this reason, developing a more sophisticated *in vitro* model system, might help to overcome the current limitations. However, even if the features of hiPSC-CMs are improving due to numerous protocol developments, differentiation methods still need further improvement to reach the desired degree of maturity. This represents a major difficulty to recapitulate the adult phenotype and, therefore, an adult disease modelling observed *in vivo*. On the other hand, the use of hiPSC from the pluripotency stage represents a unique opportunity to evaluate the disease progression from early stages of development to the adult tissue and to understand late-onset changes, as well.

In conclusion, we investigated, for the first time, the effects of a repeated-dose exposure with BPA for 21 days on early developing cardiomyocytes using an *in vitro* human-relevant iPSC-derived model, as a NAM for cardiotoxicity evaluation (Parish et al., 2020; Stucki et al., 2022; Zink et al., 2020). The results showed a significant reduction of the contraction frequency and beating velocity, as well as an increase of the contraction and relaxation times, concluding that BPA clearly affects the function of hiPSC-CMs. The proteome changes of hiPSC-CMs treated with BPA revealed the accumulation of BM components, the upregulation of contractility proteins (e.g. TNNC1), and markers related to protein translational processes (e.g. SERBP1), which are additional factors of hiPSC-CMs functional alteration also later in the development. Accordingly, network analysis revealed the association of BPA-perturbed genes with CVDs, such as heart failure and hypertrophic cardiomyopathy. Finally, BPA potentially renders the hiPSC-CMs vulnerable to additional challenges, such as the hypoxia-reoxygenation insult. Indeed, hiPSC-CMs treated with BPA were more prone to apoptotic events, suggesting that their functional decline might be even more extended. In line with the DOHaD field, the above results add valuable insights to the current investigation of early biomarkers of cardiovascular alterations that can arise due to early-life chemical exposure.

6 PUBLICATIONS

International paper publications:

- **Lamberto, F**., Peral-Sanchez, I., Muenthaisong, S., Zana, M., Willaime-Morawek, S., Dinnyés, A. (2021). *Environmental Alterations during Embryonic Development: Studying the Impact of Stressors on Pluripotent Stem Cell-Derived Cardiomyocytes*. Genes, 12, 1564. doi:10.3390/genes12101564.
- Mitrečić, D., Hribljan, V., Jagečić, D., Isaković, J., **Lamberto, F.**, Horánszky, A., Zana, M., Foldes, G., Zavan, B., Pivoriūnas, A., Martinez, S., Mazzini, L., Radenovic, L., Milasin, J., Chachques, J. C., Buzanska, L., Song, M. S., & Dinnyés, A. (2022). *Regenerative Neurology and Regenerative Cardiology: Shared Hurdles and Achievements*. International Journal of Molecular Sciences, 23(2). doi:10.3390/ijms23020855
- Kistamás, K., Müller, A., Muenthaisong, S., **Lamberto, F.**, Zana, M., Dulac, M., Leal, F., Maziz, A., Costa, P., Bernotiene, E., Bergaud, C., & Dinnyés, A. (2023). *Multifactorial approaches to enhance maturation of human iPSC-derived cardiomyocytes*. Journal of Molecular Liquids, 387, 122668. doi:10.1016/j.molliq.2023.122668.
- **Lamberto, F.**, Shashikadze, B., Elkhateib, R., Lombardo, S. D., Horánszky, A., Balogh, A., Kistamás, K., Zana, M., Menche, J., Fröhlich, T., & Dinnyés, A. (2023). *Low-dose Bisphenol A exposure alters the functionality and cellular environment in a human cardiomyocyte model*. Environmental Pollution, 335. doi:10.1016/j.envpol.2023.122359.
- Horánszky, A., Shashikadze, B., Elkhateib, R., Lombardo, S. D., **Lamberto, F.**, Zana, M., Menche, J., Fröhlich, T., & Dinnyés, A. (2023). *Proteomics and disease network associations evaluation of environmentally relevant Bisphenol A concentrations in a human 3D neural stem cell model*. Frontiers in Cell and Developmental Biology, 11. doi:10.3389/fcell.2023.1236243.

International abstract and poster presentations:

- **Lamberto, F.,** Zana M., Dinnyés, A. Impact of environmental exposures on cardiac differentiation of pluripotent stem cells. The 2nd conference of the Visegrád Group Society for developmental biology, September 2021, Szeged, Hungary.
- **Lamberto, F.,** Zana M., Dinnyés, A. Evaluation of the effects of Bisphenol A levels on cardiomyocytes differentiation of human iPSCs. Microsymposium on RNA Biology, April 2022, Wien, Austria.

- **Lamberto, F.,** Zana M., Dinnyés, A. Evaluation of the effects of Bisphenol A levels on cardiomyocytes differentiation of human iPSCs. Fiatal Biotechnológusok V. Országos Konferenciája (FIBOK) - 5th National Conference of Young Biotechnologists, April 2022, Gödöllő, Hungary.
- **Lamberto, F.**, Shashikadze, B., Lombardo, S. D., Horánszky, A., Balogh, A., Kistamás, K., Zana, M., Menche, J., Fröhlich, T., & Dinnyés, A. Repeated low-dose Bisphenol A exposure induced alterations in human induced pluripotent stem cell-derived cardiomyocytes. The 57th Congress of the European Toxicologists and European Societies of Toxicology (EUROTOX), September 2023, Ljubljana, Slovenia.