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MicroRNA Microarray Analysis of Human-Induced Pluripotent Stem Cell-Derived Neurons and Cardiomyocytes following Exposure to the Organophosphate Nerve Agents Soman and VX

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14. ABSTRACT

Chemical warfare nerve agents (CWNAs) are potent cholinesterase inhibitors that may also have non-cholinesterase effects. Several in vivo studies have shown that exposure to CWNA compounds induces damage in the brain and heart. Underlying mechanisms of this damage are a critical area of research for the development of medical countermeasures. This study utilized microRNA (miRNA) analysis to evaluate potential direct cellular effects of the nerve agents GD (soman; O-Pinacolyl methylphosphonofluoridate) or VX (o-ethyl-s-[2 (diisopropylamino) ethyl]) on neurons derived from human-induced pluripotent stem cells (iPSCs) and iPSC-derived cardiomyocytes. This approach was taken since miRNA expression changes are stimulus specific and no previous studies of miRNA profiles have been conducted for CWNA exposure. Cells were exposed to GD or VX at concentrations of 0 µM, 0.1 µM or 100 µM for either 1 hour or 6 hours. Following exposure, isolated total RNA was processed for miRNA microarray analysis and analyzed for significant changes. GD- and VX-treated samples were analyzed separately. Changes in miRNA expression for both neurons and cardiomyocytes were modest. Nonetheless, expression profiles that were unique to either GD or VX exposure were observed with both neurons and cardiomyocytes. Further analysis of select miRNAs, namely, neuronal miR-941 and cardiomyocyte miR-576-3p, supports this observation that expression changes are dependent on the nerve agent.

15. SUBJECT TERMS

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ABSTRACT

Chemical warfare nerve agents (CWNAs) are potent cholinesterase inhibitors that may also have non-cholinesterase effects. Several *in vivo* studies have shown that exposure to CWNA compounds induces damage in the brain and heart. Underlying mechanisms of this damage are a critical area of research for the development of medical countermeasures. This study utilized microRNA (miRNA) analysis to evaluate potential direct cellular effects of the nerve agents GD (soman; O-Pinacolyl methylphosphonofluoridate) or VX (o-ethyl-s-[2 (diisopropylamino) ethyl]) on neurons derived from human-induced pluripotent stem cells (iPSCs) and iPSC-derived cardiomyocytes. This approach was taken since miRNA expression changes are stimulus specific and no previous studies of miRNA profiles have been conducted for CWNA exposure. Cells were exposed to GD or VX at concentrations of 0 µM, 0.1 µM or 100 µM for either 1 hour or 6 hours. Following exposure, isolated total RNA was processed for miRNA microarray analysis and analyzed for significant changes. GD- and VX-treated samples were analyzed separately. Changes in miRNA expression for both neurons and cardiomyocytes were modest. Nonetheless, expression profiles that were unique to either GD or VX exposure were observed with both neurons and cardiomyocytes. Further analysis of select miRNAs, namely, neuronal miR-941 and cardiomyocyte miR-576-3p, supports this observation that expression changes are dependent on the nerve agent.

INTRODUCTION

Central nervous system and cardiac homeostasis is maintained through the action of various molecular mechanisms. Recent studies indicate that microRNAs (miRNAs) play a central role during this process [1-2]. These non-coding RNAs are short nucleotides that regulate cellular signaling at the posttranscriptional level via translational repression, mRNA degradation and translational activation. miRNA expression occurs in a stimuli-, species-, tissue- and cell-specific manner. miRNA expression that is unique to specific sub-cellular compartments also occurs in some cells such as the neuronal axon [3]. Several studies suggest that these unique expression profiles within neurons facilitate the fine-tuning of signal transmission [4]. Heart-specific miRNAs have also been identified, and the term myomiRs is often used to describe these miRNAs that regulate cardiac homeostasis [1]. The condition-specific nature of miRNA expression has been utilized to develop miRNA disease signatures based on unique expression profiles. Despite major advances in the miRNA field, few studies have utilized this useful tool for the study of chemical warfare agents.

Exposure to the CWNAs GD and VX leads to acute systemic changes that are potentially fatal. The mechanism of action for organophosphorus nerve agents is well established. CWNAs covalently attach to the acetylcholine (ACh) binding site of the enzyme acetylcholinesterase (AChE). This in turn interferes with ACh hydrolysis, consequently inducing cholinergic overstimulation and subsequent tissue damage in the brain and heart [5-6]. Even though the mechanism of action and symptomatic outcomes are similar, the toxicity of V agents such as VX is higher than that of G agents, for example, GD [7-8].

This study utilized neurons derived from human-induced pluripotent stem cells (iPSCs) and iPSC-derived cardiomyocytes to evaluate potential direct cellular effects of the nerve agents GD and VX in neuronal and cardiac tissue *in vitro*. Human iPSC-derived neurons that were utilized for this study were a mixture of post-mitotic neural subtypes, comprised primarily of GABAergic and glutamatergic neurons, whereas iPSC-derived cardiomyocytes were a mixture of active atrial, nodal, and ventricular-like myocytes. Both cell types have extensively been characterized. Functional characterization using electrophysiological studies has confirmed the similarity between these iPSC-derived cells and human cells [9-10]. Furthermore, they display phenotypic features that are consistent with each cell type, for example, extension of axon processes with iPSC-derived neurons and synchronous beating with iPSC-derived cardiomyocytes. Due to the sensitivity of miRNAs to environmental factors, this pilot study utilized miRNA microarray analysis to evaluate the direct AChE-independent effects of GD and VX on these cells with the goal of developing *in vitro* cellular models for the assessment of nerve agent toxicity.

MATERIALS AND METHODS

Cell Culture: iPSC-derived neurons and cardiomyocytes (Cellular Dynamics International, Madison, WI) were utilized for our studies. Cells were plated and maintained according to the manufacturer's specifications. iPSC-derived neurons were seeded at a density of 2.0 $X10^6$ cells per well in 6-well plates, whereas cardiomyocytes were seeded at a density of 1.0 $X10^6$ per well. One week after plating, cells were exposed to either GD or VX (n=4 for each group) as described below.

Agent Exposure: Human iPSC-derived neurons and iPSC-derived cardiomyocytes were exposed to 0.1µM or 100 µM nerve agent (GD or VX) and incubated in a chemical surety fume hood at 37^{o}C with ambient air. Time-matched saline controls (0 µM) were included with the study. After 1 or 6 hours, samples were gently rinsed five times with Hanks' balanced salt

solution (HBSS), and cells were homogenized in trizol reagent. Total RNA was then isolated and processed for miRNA microarray analysis as described below.

RNA Isolation and Quality Assessment: Total RNA was isolated from exposed and control iPSC-derived cells using miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNase digestion was not included with our isolation. RNA quality and quantity were determined using an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA) and NanoDrop ND-1000 UV-vis 186 spectrophotometer (Thermo Scientific, Wilmington, DE) respectively.

miRNA Microarray Analysis: Total RNA was labeled using the RNA biotin labeling flashtag biotin HSR RNA labeling kit (Affymetrix, Santa Clara, CA) as per manufacturer's instructions. Labeled RNA was analyzed for miRNA profile changes using the Affymetrix GeneChip miRNA 2.0 microarrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. In brief, 500 ng total RNA was subjected to poly (A) tailing of the 3' end using an enzymatic reaction. Samples were then incubated at room temperature with the biotin-containing molecule 3DNA. A poly (T) sequence on this molecule facilitates ligation with the RNA molecule via the poly (A) tail. Labeled RNA was then hybridized onto miRNA GeneChips. This was followed by washing and staining, after which the GeneChips were scanned. Raw signal intensities were exported into Partek Genomic suite v6.6 (Partek Incorporated, St. Louis, MO) for miRNA expression statistical analysis.

Statistical Analysis: Analysis of variance (ANOVA) was used to evaluate miRNAs that were most significantly affected by nerve agent concentration, time or the interaction of both time and nerve agent concentration. GD- and VX-exposed samples were analyzed separately. No targets were identified based on stringent configurations (false discovery rate [FDR] adjusted p-value ≤0.05). Therefore, analysis was configured to include the unadjusted p-value of <0.05 for each of these factors.

RESULTS

GD and VX exposure does not induce neuronal and cardiomyocyte cell death in vitro: Earlier studies have shown that exposure to nerve agents rapidly induces seizures due to cholinergic overstimulation [11-12]. These seizures occur within minutes and can last for hours. Observable tissue damage occurs in both the brain and heart as a result of this exposure [11]. To examine the direct effect of the nerve agents GD and VX on neurons and cardiomyocytes, iPSC-derived neurons and iPSC-derived cardiomyocytes (Figure 1) were exposed to 0 µM, 0.1 µM or 100 µM nerve agent for 1 or 6 hours. In contrast to *in vivo* studies whereby observable neuronal and cardiac damage occurs, no phenotypic changes such as cell detachment were detected *in vitro* following GD or VX exposure with these cells at both 1 hr and 6 hrs post-exposure.

GD- and VX-induced miRNA expression changes in neurons: Even though no phenotypic changes were observed with neurons exposed to nerve agent, statistically significant miRNA changes were identified with various miRNA targets as determined by ANOVA analysis of miRNA microarray data. Analysis was based on nerve agent concentration, nerve agent exposure time or the interaction between nerve agent concentration and exposure time. Following exposure of iPSC-derived neurons to GD 157 targets were altered (Table 1), whereas exposing these cells to VX induced miRNA expression changes in 162 targets (Table 2). The highest rated targets were miR-572 and miR-877 for GD and VX exposure, respectively. Statistically significant time-dependent changes were identified in 70 targets in GD-exposed neurons, and 107 targets were altered based on the interaction between GD exposure and time as determined by the two-way ANOVA analysis (Table 1). Time-dependent variations in 88

targets were detected with VX-exposed iPSC-derived neurons, while 96 targets were altered as a function of the combined effect of VX exposure and time (Table 2).

GD- and VX-induced miRNA expression changes in cardiomyocytes: Similar to studies with iPSC-derived neurons, statistically significant miRNA expression changes were identified for various miRNA targets as determined by ANOVA in iPSC-derived cardiomyocytes. Analysis was based on agent exposure, time or the interaction between agent exposure and time. Following exposure of iPSC-derived cardiomyocytes to GD 62 targets were altered (Table 3), whereas exposing these cells to VX induced miRNA expression changes in 67 targets (Table 4). miR-554 and miR-576-3p were the highest rated targets that were altered as a result of GD and VX exposure, respectively, based on this analysis. Statistically significant time-dependent changes were identified in 61 targets in GD-exposed cardiomyocytes, and 32 targets were altered based on the interaction between GD exposure and time as determined by two-way ANOVA (Table 3). Time-dependent variations in 61 targets were detected with VX-exposed samples, while 32 targets were altered as a function of the combined effect of VX exposure and time (Table 4).

Differential nerve agent-dependent regulation of hsa-miR-941in iPSC-derived neurons and hsa-miR-576-3p in iPSC-derived cardiomyocytes: Signal intensities for select miRNAs were graphed to compare the individual effect of GD and VX on specific targets that were significantly affected. The targets miR-941 and miR-576-3p were assessed in iPSC-derived neurons and iPSC-derived cardiomyocytes, respectively. GD exposure induced a significant concentration-dependent decrease in neuronal miR-941 (Figure 2A). A modest decrease that was not significant was observed following exposure of these cells to 0.1µM VX, whereas 100 µM VX did not alter this target (Figure 2B). Cardiomyocyte miR-576-3p was unaltered in GD-exposed cardiomyocytes (Figure 3A). Significant changes were observed with this target in VX-exposed cardiomyocytes, primarily due to the effect of 0.1 uM VX, since expression levels from 100 uM VX-exposed cells were comparable with control unexposed cells (Figure 3B).

Table 1: GD-Exposed iPSC-Derived Neurons miRNA Microarray Data ANOVA

iPSC-Derived Neuron GD Exposure	One Way ANOVA- GD Conc.	One Way ANOVA- Time	Two Way ANOVA - GD Conc. and Time
Number of miRNA targets with significant expression changes	157	70	107
Top ten miRNAs significantly affected by condition (p value)	mir-572 (5.0×10^{-4}) miR-578 (5.2×10^{-4}) miR-328 (5.2×10^{-4}) miR-941 (5.3×10^{-4}) miR-516b (7.1×10^{-4}) mir-1306 (7.2×10^{-4}) miR-129-5p (1.2×10^{-3}) miR-1910 (1.2×10^{-3}) mir-875 (1.8×10^{-3}) miR-181b* (2.2×10^{-3})	miR-124* (3.8×10^{-4}) miR-1246 (1.0×10^{-3}) miR-615-5p (1.5×10^{-3}) miR-508-3p (2.3×10^{-3}) mir-30a (3.2×10^{-3}) miR-1308 (3.4×10^{-3}) mir-1324 (4.0×10^{-3}) mir-1252 (4.6×10^{-3}) miR-586 (6.8×10^{-3}) miR-212 (7.4×10^{-3})	miR-100 (8.3×10^{-5}) miR-181b (1.0×10^{-3}) miR-124* (1.1×10^{-3}) miR-1292 (1.6×10^{-3}) mir-875 (2.0×10^{-3}) miR-23a (2.4×10^{-3}) miR-106b (2.5×10^{-3}) mir-30a (3.0×10^{-3}) miR-551b (3.9×10^{-3}) miR-516b (5.0×10^{-3})

Table 2: VX-Exposed iPSC-Derived Neurons miRNA Microarray Data ANOVA

iPSC-Derived Neuron VX Exposure	One Way ANOVA- VX Conc.	One Way ANOVA- Time	Two Way ANOVA - VX Conc. and Time
Number of miRNA targets with significant expression changes	162	88	96
Top ten miRNAs significantly affected by condition (p value)	miR-877(5.7×10^{-4}) miR-378(6.7×10^{-4}) mir-302a(1.0×10^{-3}) miR-2110(1.1×10^{-3}) mir-2909(1.6×10^{-3}) miR-1301(2.2×10^{-3}) miR-138-2*(2.2×10^{-3}) miR-505*(2.2×10^{-3}) miR-542-3p (2.3×10^{-3}) miR-921(2.4×10^{-3})	miR-3159(3.6×10^{-4}) mir-548s(1.0×10^{-3}) mir-1255a (1.8×10^{-3}) mir-545(2.2×10^{-3}) mir-585(2.4×10^{-3}) mir-488(2.4×10^{-3}) miR-1308(3.2×10^{-3}) miR-124*(3.8×10^{-3}) miR-1246(4.2×10^{-3}) mir-3139 (4.7×10^{-3})	miR-100(6.3×10^{-4}) miR-551b*(8.2×10^{-4}) miR-1197(2.2×10^{-3}) miR-921(2.5×10^{-3}) miR-1308(2.5×10^{-3}) miR-302b*(2.5×10^{-3}) mir-302a(3.5×10^{-3}) mir-2909(4.0×10^{-3}) miR-1284(5.2×10^{-3}) miR-608(5.7×10^{-3})

Table 3: GD-Exposed iPSC-Derived Cardiomyocyte miRNA Microarray Data ANOVA

iPSC-Derived Cardiomyocytes GD Exposure	One Way ANOVA- GD Conc.	One Way ANOVA- Time	Two Way ANOVA - GD Conc. and Time
Number of miRNA targets with significant expression changes	62	61	32
Top ten miRNAs significantly affected by condition (p value)	mir-554 (5.2×10^{-6}) miR-323-5p (3.9×10^{-4}) mir-100 (9.7×10^{-4}) miR-1289t (9.8×10^{-4}) mir-3132 (1.5×10^{-3}) miR-518c-* (2.7×10^{-3}) miR-296-3p (3.7×10^{-3}) miR-3145 (4.8×10^{-3}) mir-548h (5.8×10^{-3}) miR-3129 (7.6×10^{-3})	miR-3178 (1.8×10^{-6}) miR-200c (2.9×10^{-4}) miR-1202 (7.9×10^{-4}) mir-4277 (1.4×10^{-3}) miR-1972 (1.9×10^{-3}) mir-495 (3.4×10^{-3}) mir-545(4.2×10^{-3}) miR-320c (4.8×10^{-3}) miR-744* (5.0×10^{-3}) mir-454 (5.6×10^{-3})	mir-554(3.9×10^{-5}) miR-3178 (7.2×10^{-5}) miR-200c (2.2×10^{-3}) miR-323-5p(5.9×10^{-3}) mir-548i-3(9.6×10^{-3}) miR-26a-1-*(1.3×10^{-3}) miR-126(1.4×10^{-3}) mir-4277 (1.5×10^{-3}) mir-10t (1.8×10^{-3}) miR-1289(1.8×10^{-3})

Table 4: VX-Exposed iPSC-Derived Cardiomyocyte miRNA Microarray Data ANOVA

iPSC-Derived Cardiomyocyte VX Exposure	One Way ANOVA- VX Conc.	One Way ANOVA- Time	Two Way ANOVA - VX Conc. and Time
Number of miRNA targets with significant expression changes	67	43	35
Top ten miRNAs significantly affected by condition (p value)	miR-576-3p (3.6×10^{-3}) miR-335 (6.2×10^{-3}) mir-520h (6.3×10^{-3}) miR-509-3-5p (6.9×10^{-3}) mir-518b (7.8×10^{-3}) miR-380 (8.2×10^{-3}) miR-99b (9.6×10^{-3}) mir-196b (1.1×10^{-3}) miR-519b-3p (1.1×10^{-3}) miR-4329 (1.1×10^{-3})	miR-3178 (1.5×10^{-6}) miR-1202 (1.5×10^{-4}) mir-149 (3.0×10^{-3}) miR-126 (3.1×10^{-3}) miR-139-5p (3.5×10^{-3}) miR-610 (3.8×10^{-3}) mir-450a-1 (5.4×10^{-3}) mir-1278 (5.5×10^{-3}) mir-656 (6.5×10^{-3}) miR-638 (8.3×10^{-3})	miR-3178 (2.6×10^{-4}) miR-1202 (2.6×10^{-3}) miR-509-3-5p (4.8×10^{-3}) miR-432 (5.5×10^{-3}) miR-26a-1* (6.2×10^{-3}) mir-365-2 (6.9×10^{-3}) miR-876-5p (7.3×10^{-3}) mir-519e_x (7.6×10^{-3}) mir-222 (9.2×10^{-3}) mir-371 (9.6×10^{-3})

Figure 1:

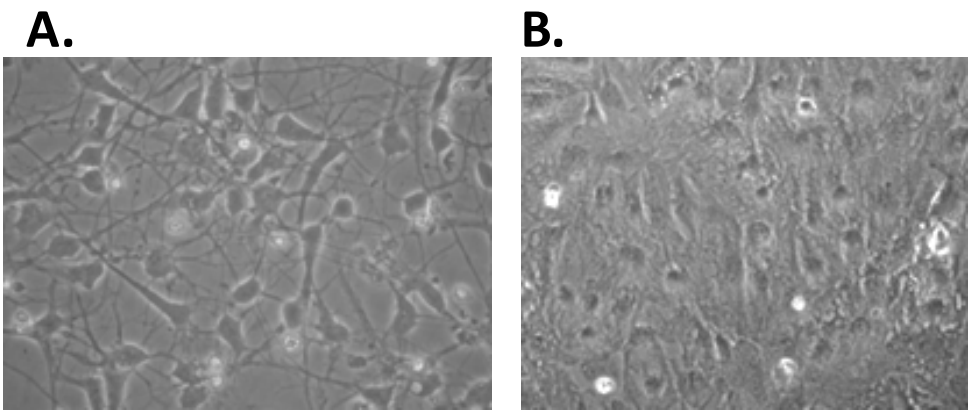

Figure 1: Phase contrast images of iPSC-derived neurons (A) and cardiomyocytes (B). Neurons and cardiomyocytes demonstrated features that typify these cell types including neuronal morphology for the neurons and synchronous rhythmic beating for the cardiomyocytes.

Figure 2:

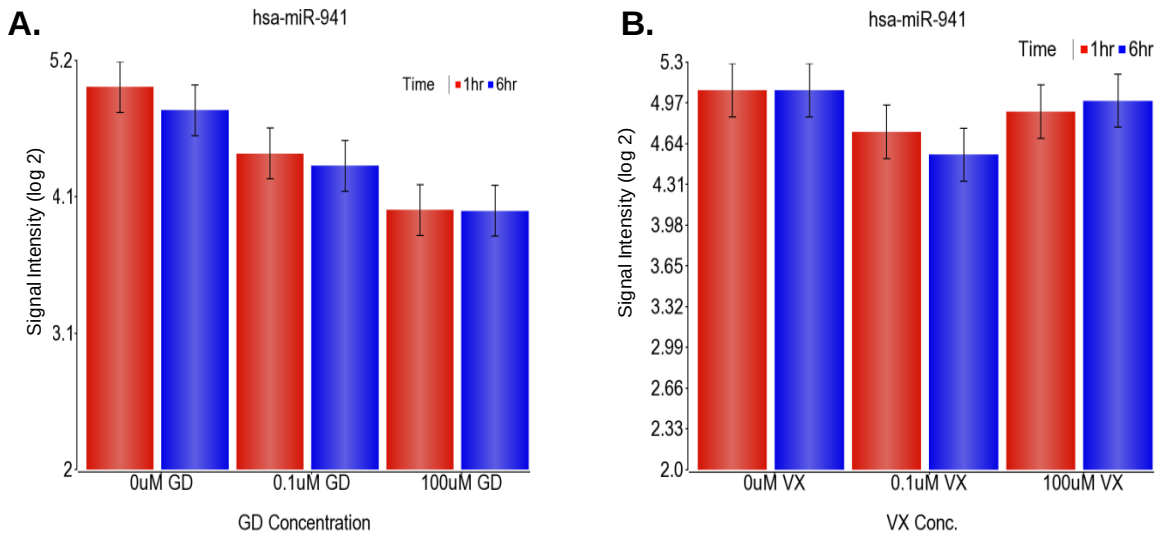

Figure 2: Neuronal miR-941 Expression Patterns following Exposure to the Nerve Agents GD and VX. Graphs based on signal intensities from microarray analysis following (A) GD and (B) VX exposure of iPSC-derived neurons were plotted for miR-941. This target was significantly altered based on one-way ANOVA. Changes in VX–induced miR-941 expression in neurons were not statistically significant.

Figure 3

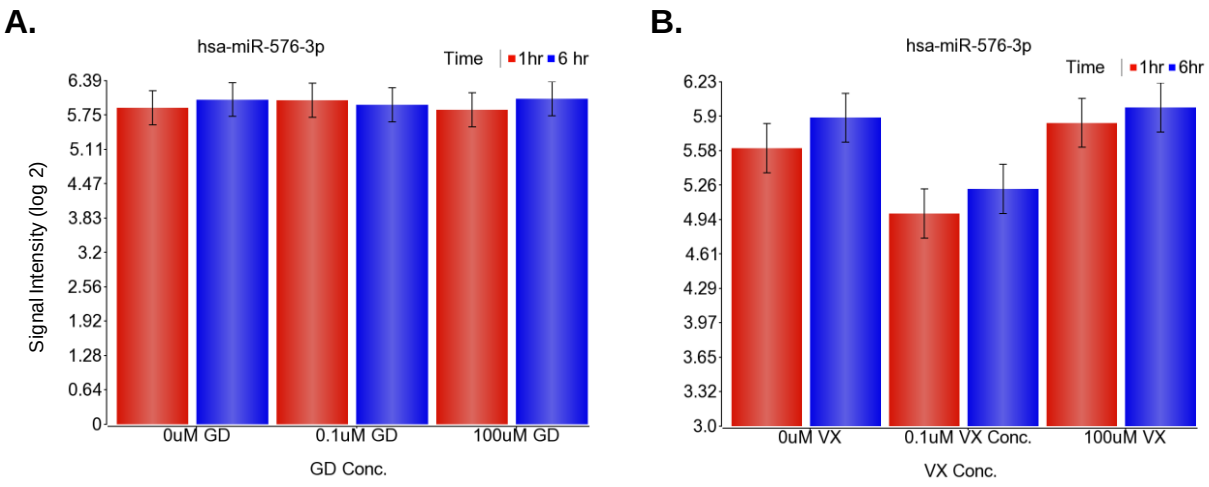

Figure 3: Cardiac miR-576-3p Expression Patterns following Exposure to the Nerve Agents GD and VX. Graphs based on signal intensities from microarray analysis following (A) GD and (B) VX exposure of iPSC-derived cardiomyocytes were plotted for miR-576-3p. This target was significantly altered based on one-way ANOVA analysis with VX as a factor. GD exposure did not induce statistically significant miR-576-3p expression changes.

DISCUSSION

This study utilized iPSC-derived neurons and iPSC-derived cardiomyocytes to evaluate GD- and VX-induced miRNA changes *in vitro.* Terminally differentiated neurons and cardiac cells from human adults are amitotic. Therefore, obtaining large amounts of homogenous tissue for *in vitro* studies is a major challenge. Human iPSC-derived cells provide an alternative source for adequate amounts of neurons and cardiomyocytes that are pure and homogenous. Previous studies have demonstrated electrical properties that are consistent with each cell type [9-10]. Furthermore, various features that typify terminally differentiated neurons or cardiomyocytes were observed with both cell types. Exposing these cells to GD and VX did not alter them phenotypically, and no observable detrimental effects, for example, cell detachment and death, were detected. This demonstrates that AChE inhibition and subsequent cholinergic overstimulation are necessary for the acute effects of these compounds. Although no observable morphological changes were observed following *in vitro* nerve agent exposure, several targets were significantly altered based on ANOVA ($p \leq 0.05$) analysis of the miRNA microarray data (Table 2-5). Nevertheless, most of these changes were modest since no targets were identified when stringent analysis conditions (p-value with FDR ≤0.05) were included with our analysis. Data presented are based on unadjusted p-value ≤0.05.

Select miRNAs from ANOVA analysis were further examined for patterns in nerve agent-induced miRNA expression changes. Concentration-dependent miR-941 decreases were observed in GD-exposed neurons, whereas decreased expression for this target was observed only with the lower concentration (0.1µM) in VX-exposed neurons (Figure 2). VX-induced changes were not statistically significant. miR-941 has been implicated in human evolution due to the exclusive presence of this miRNA in humans but not in non-human primates [13]. In addition, copy numbers directly correlate with migration patterns [13]. The direct correlation between GD concentration and miR-941 repression demonstrates the potential use of this target as a biomarker for evaluating GD exposure levels. Functionally, this target regulated genes that are involved in the hedgehog signaling pathway and insulin signaling pathway as determined by gene expression microarray studies following transfection of two human-derived kidney cell lines (HEK and 293T) and one human skin fibroblast cell line (HSF2) with miR-941[13]. It would be interesting to compare miR-941 levels between primary neurons and iPSC-derived neurons as this miRNA is highly expressed in pluripotent cells but diminished in differentiated cells [13].

Changes in miR-576-3p have previously been correlated with early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) [14]. miR-576-3p was one of six targets that was down regulated in a study that compared human subjects with ETP-ALL, non-ETP-ALL and healthy controls. Several targets are regulated by this miRNA as determined by TargetScan. These targets include cell cycle regulation proteins and RNA binding proteins. Results from our study demonstrate that miR-576-3p is differentially regulated by GD and VX exposure in cardiomyocytes (Figure 3). This miRNA is unaltered in GD-exposed cardiomyocytes (Figure 3A), whereas concentration-dependent changes were observed following exposure to VX (Figure 3B). Similar to the results from neuronal analysis this observation indicates that changes in miRNA expression within cardiomyocytes are nerve agent specific.

Although it is well established that damage from nerve agents results from AChE inhibition, others have demonstrated non-cholinergic consequences following exposure to these compounds [15-16]. Microarray studies that demonstrated gene expression changes in non-seizing animals following nerve agent exposure also support this observation [17]. In addition, the potential for non-cholinergic intervention for the treatment of nerve agent exposure [18] demonstrates the viability of therapies that target other pathways besides AChE. A comparison

of cardiac and neuronal pathology following nerve agent exposure and anticonvulsant treatment also supports the need to develop supplemental therapies since changes in cardiac pathology were not consistently associated with the effectiveness of anticonvulsants in reducing seizures [11].

Our studies that show alterations in miRNA expression profiles following neuronal and cardiomyocyte GD and VX exposure support previous reports that have documented nerve agent-induced molecular changes that are not dependent on cholinesterase signaling. Further studies on nerve agent-induced neuronal and cardiac miRNA expression changes *in vivo* would provide insight on the role of these nucleotides during nerve agent-induced damage. It would also be interesting to examine potential CWNA-induced miRNA profile changes in non-seizing animals. miRNA expression profile changes from seizing and non-seizing animal studies can additionally be compared with *in vitro* miRNA changes to identify targets that are unique to the cholinergic and non-cholinergic effects of nerve agents both *in vitro* and *in vivo*.

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