Downregulation of long noncoding RNA SNHG6 rescued propofol-induced cytotoxicity in human induced pluripotent stem cell-derived cardiomyocytes

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Background: Propofol (PPF) overdose is a rare but lethal condition, which may lead to severe cardiac failure. In this study, we established an in vitro PPF-induced cardiac cytotoxicity model, and investigate the functional role of long non-coding RNA (lncRNA) small nucleolar RNA host gene 6 (SNHG6).

Methods: Human induced pluripotent stem cell-derived cardiomyocytes (HiPSC-CMs) were exposed to PPF in vitro. PPF-induced cytotoxic effects were measured. PPF-induced SNHG6 expression change in HiPSC-CMs were monitored by qRT-PCR. SNHG6 was downregulated in HiPSC-CMs to examine its role in PPF-induced cardiac cytotoxicity. The expression of competing endogenous RNA (ceRNA) candidate of SNHG6, human microRNA-186-5p (hsa-miR-186-5p) was also investigated in PPF-exposed HiPSC-CMs. Functions of hsa-miR-186-5p were further investigated in PPF-exposed and SNHG6-downregulated HiPSC-CMs.

Results: PPF induced significant cytotoxicity, as well as SNHG6 upregulation in HiPSC-CMs. SNHG6 downregulation had rescuing effects on PPF-induced cardiac cytotoxicity. Dual-luciferase activity assay confirmed that hsa-miR-186-5p was the ceRNA candidate of SNHG6. QRT-PCR showed hsa-miR-186-5p expression was reversely correlated with SNHG6 in PPF-exposed HiPSC-CMs. Suppressing hsa-miR-186-5p reduced the rescuing effects of SNHG6-downregulation on PPF-induced cardiac cytotoxicity.

Conclusions: SNHG6/hsa-miR-186-5p can modulate PPF-induced cardiac cytotoxicity in HiPSC-CMs, and thus may be a future drug target to prevent PPF infusion syndrome.

Keywords: Propofol (PPF); cardiac cytotoxicity; long non-coding RNA (lncRNA); SNHG6; miRNA; hsa-miR-186-5p

Introduction

Propofol (PPF) is one of the widely used anesthetic reagents for non-procedural sedation in intensive care units. Although rarely occurring, PPF infusion syndrome (PRIS) is potentially lethal, may cause severe side effects, such as hyperlipidemia, rhabdomyolysis, renal and cardiac failures (1-3). Unfortunately, due to its nature of rarity, the underlying genetic mechanisms of PRIS are largely unknown.

During past decades, limited investigations had used animal models or human patient-derived samples to study PRIS (4,5). Among them, human induced pluripotent stem cells (iPSCs) had been showing significantly clinical potentials, as they may differentiate into various human...
cells or organs, such as cardiomyocytes (6,7). Studies also demonstrated that human iPSC-derived cardiomyocytes (HiPSC-CMs) possessed similar cellular and molecular characteristics as those in \textit{in situ} human cardiac cells (8,9). Specifically, a recent study showed that HiPSC-CMs responded to \textit{in vitro} PPF exposure with similar cytotoxic characteristics as observed in human patients with PRIS (10).

Long non-coding RNAs (lncRNAs) are groups of long-length (>200 nucleotides), non-protein-coding RNA transcriptomes, that were recently discovered to play critical roles in almost all phases of human health and diseases (11-13). In cardiomyocytes, various studies had demonstrated that lncRNAs are actively involved in many aspects of cardiac development and pathophysiology (14-16). For example, a lncRNA named as cardiac apoptosis-related lncRNA (CARL) was reported to modulate cardiomyocyte apoptosis and mitochondrial fission through miR-539 and Prohibitin 2 (PHB2) (17). In addition, lncRNA of Urothelial Cancer Associated 1 (UCA1) was found to induce cardiomyocyte hypertrophy through competitively binding of miR-184/HOXA9 epigenetic axis (18). Moreover, lncRNA of H19 was discovered to suppress cardiomyocyte autophagy in diabetic cardiomyopathy (19).

Of many of the lncRNAs associated with human health or diseases, small nucleolar RNA host gene 6 (SNHG6) belongs to the super lncRNA family of SNHG and was found to be an active epigenetic transcriptome in various types of human cancers (20-22). However, there are only limited reports demonstrating functional mechanisms of SNHG6 in the cardiovascular system. Among them, it was shown that SNHG6 is likely to be aberrantly expressed in patients with atherosclerosis, and SNHG6 may modulate the progression and pathology of atherosclerotic cardiac tissues (23-25). However, the possible biological function of SNHG6 involved in the process of PPF-induced cardiac cytotoxicity has never been elucidated.

In this study, we established an \textit{in vitro} PPF-induced cardiac cytotoxicity model by introducing PPF exposure to the \textit{in vitro} culture of HiPSC-CMs. Then, we investigate the expression and function of SNHG6 during this process. The goal of this study is to explore the epigenetic underlying mechanisms of PRIS.

\section*{Methods}

This study was approved by the Ethics Committee for Human & Clinical Research at the People’s Hospital of China Three Gorges University in Yichang, Hebei Province, China (Approval ID: CTGU20190455). All procedures were performed in accordance with the Declaration of Helsinki (2013 version). Informed consent was obtained from the patient for publication of this manuscript.

\subsection*{Induction of HiPSC-CMs}

Induction of \textit{in vitro} HiPSC-CMs was performed according to a previously published protocol with slight modification (26). A human embryonic stem cell (hESC) H7 cell line (Research Resource Identifier, RRID: CVCL_S799) was purchased from WiCell (WiCell Research Institute, USA) and maintained in 6-well tissue-culture plates in mTeSR medium (Stem Cell Technologies, Canada). For cardiac differentiation, H7 cells were moved into glass-bottomed dishes to form floating embryoid bodies in 48–72 h. Then, embryoid bodies were collected and re-plated in a 6-well tissue-culture plate in StemPro-34 medium (Thermo Fisher Scientific, USA) supplemented with 10 ng/mL BMP4 (MilliporeSigma, Shanghai, China), 2 mM glutamine (Thermo Fisher Scientific, USA), 4 mM monothioglycerol (MilliporeSigma, Shanghai, China), 50 μg/mL ascorbic acid (MilliporeSigma, Shanghai, China) and 3 ng/mL activin A (Thermo Fisher Scientific, USA) for 4 days. Then, 150 ng/mL DKK1 (Thermo Fisher Scientific) and 10 ng/mL VEGF were added into the culture for an additional 4 days. Finally, 5 ng/mL FGF-2 (MilliporeSigma, Shanghai, China) was added from day 9 and the medium was replenished every 3–4 days. From days 21 to 24, to purify HiPSC-CMs, beating cells were isolated and transferred to a new 6-well plate in RPMI medium (Thermo Fisher Scientific, USA) supplemented with non-essential amino acids (NEAA 1x, Thermo Fisher Scientific, USA), L-glutamine GlutaMAX (1x, Thermo Fisher Scientific, USA), antibiotic-antimycotic solution (1x, Thermo Fisher Scientific, USA) 4 mM lactic acid (MilliporeSigma, Shanghai, China) and 55 nM β-mercaptoethanol (Thermo Fisher, USA) for 7 days (27).

\subsection*{PPF treatment}

\textit{In vitro} treatment of PPF (MilliporeSigma, Shanghai, China) on HiPSC-CMs was conducted according to the method in a previously published study (10), with slight modification. Briefly, HiPSC-CMs were collected from 6-well plates and re-seeded in 96-well plates at approximately 3.5×10^3/well in culture medium supplemented with fetal bovine serum (FBS, 10%, MilliporeSigma, Shanghai, China) for 24 h. After that,
culture medium was freshly replenished with 2% FBS, with the addition of PPF (MilliporeSigma, Shanghai, China) at 0, 1, 2, 5, 10, 20, 50 or 100 μg/mL for 48 h.

**Cell viability assay**

Cell viability assay was performed using a PrestoBlue™ HS Cell Viability Assay (Thermo Fisher Scientific, USA) according to the manufacturer’s instruction. Relative viability was assessed by measuring fluorescence (Excitation/Emission, 560/605) using a Fluoroskan microplate fluorometer (Thermo Fisher Scientific, USA).

**Mitochondrial superoxide assay**

The production of mitochondrial superoxide was assessed by immunofluorescence staining. To identify superoxide-induced mitochondrial oxidation, a MitoSOX™ Red Mitochondrial Superoxide Indicator dye (Thermo Fisher Scientific, USA) was applied and recognized by a TexasRed/mCherry filter cube on an Evos FL Auto fluorescent microscope system (Thermo Fisher Scientific, USA). In addition, cell nuclei were identified by using a Hoechst 33342 antibody (Thermo Fisher Scientific, Cat. # R37605, RRID: AB_2651135, USA) and recognized by a DAPI filter cube on the Evos FL Auto fluorescent microscope.

**Mitochondrial membrane potential assay**

A TMRE-Mitochondrial Membrane Potential Assay Kit (Thermo Fisher Scientific, USA) was used, according to the manufacturer’s instruction, to assess mitochondrial membrane potential. Th relative TRME signal was measured by fluorescence (Excitation/Emission, 530/580) using a Fluoroskan microplate fluorometer (Thermo Fisher Scientific, USA).

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

RNA extraction and purification were conducted using a RNAqueous™-Midi Total RNA Isolation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. The first-strand cDNA was then generated from RNA product using a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Quantitative real-time PCR (qRT-PCR) was performed using an ABI Prism 7900 sequence detection system (Applied Biosystems, USA). For SNHG6, a pre-designed human TaqMan™ SNHG6 Non-coding RNA Assay (Invitrogen, USA) was used. For hsa-miR-185-5p, a pre-designed hsa-miR-186-5p TaqMan® Advanced miRNA Assay (Applied Biosystems, USA) was used. Relative gene expression levels were characterized using the 2^(-ΔΔCt) method.

**SNHG6 downregulation assay**

A lentiviral vector containing short-hairpin RNA (shRNA) specifically targeting human IncRNA SNHG6 (SNHG6_Sh), and another lentiviral vector containing a control non-specific shRNA (C_Sh) were designed and manufactured by GenePharma (GenePharma, Shanghai, China). Lentiviral packaging, transfection and supernatants collection/purification were also conducted by GenePharma (GenePharma, Shanghai, China). In the culture of H7 cells, cells were infected with lentiviral supernatants (SNHG6_Sh or C_Sh) for 48 h, followed by a selection process using G418 (0.5 mg/mL, MilliporeSigma, Shanghai, China) for 72 h. After 3–5 passages, cells were collected and examined by qRT-PCR to verify the downregulation of SNHG6. Then, these cells were differentiated toward HiPSC-CMs using the method described above.

**Dual-luciferase reporter assay**

The 3’-untranslated region (3’-UTR) of wild-type SNHG6, which includes a putative binding site of hsa-miR-186-5p was sub-cloned into pmiR-REPORT luciferase plasmid (Promega, USA) and named as SNHG6_Lu. In addition, the hsa-miR-186-5p binding site was mutated and the mutant SNHG6 3’-UTR was sub-cloned into pmiR-REPORT, named as SNHG6(MU)_Lu. In human HEK293T cells, SNHG6_Lu or SNHG6(MU)_Lu were co-transfected with hsa-miR-185-5p mimics (miR-186-5p, GenePharma, Shanghai, China) or a control non-specific human miRNA mimics (C_mmiC, GenePharma, Shanghai, China). Forty-eight hours later, a dual-luciferase reporter assay (Promega, USA) was performed. Relative Firefly/Renilla luciferase activities were then calculated and normalized to the luciferase activity in HEK293T cells cotransfected with SNHG_Lu and C_mmiC.

**Hsa-miR-186-5p downregulation assay**

A miRNA inhibitor specifically targeting hsa-miR-186-5p (miR186_In), and a control non-specific miRNA
inhibitor (C_In) were both purchased from GenePharma (GenePharma, Shanghai, China). In the culture of purified HiPSC-CMs, cells were collected and re-seeded into 6-well plates in fresh culture medium with the addition of 10% FBS for 12 h. Transient transfection of miR186_In or C_In was then conducted for 12 h using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, USA). After that, qRT-PCR was performed to verify the downregulation of hsa-miR-186-5p.

Statistical analysis

All experiments were independently repeated at least three times. Data were shown as means ± standard error of the mean (SEM). Statistical analyses were performed using an unpaired two-tail student's t-test on SPSS software (SPSS, IBM, RRID: SCR_002865, USA). The difference was termed as statistically significant if a P value was less than 5% (0.05).

Results

PPF induced cytotoxicity and upregulated SNHG6 in HiPSC-CMs

HiPSC-CMs were exposed to PPF for 48 h. After that, a viability assay indicated that, at concentrations between 2 and 100 μg/mL, PPF induced significant cell death (Figure 1A, *P<0.05). Also, an immunofluorescent assay showed that, 50 μg/mL PPF markedly increased mitochondrial superoxide production in HiPSC-CMs (Figure 1B). In addition, a TMRE assay demonstrated that mitochondrial membrane potentials in HiPSC-CMs were drastically decreased by 5–100 μg/mL PPF (Figure 1D, *P<0.05). Moreover, a qRT-PCR assay indicated that human lncRNA, SNHG6, was upregulated by 5–100 μg/mL PPF (Figure 1D, *P<0.05).

Thus, these data suggested that PPF induced significant cytotoxicity and upregulated SNHG6 in HiPSC-CMs.

SNHG6 downregulation rescued PPF-induced cytotoxicity in HiPSC-CMs

H7 cells were transduced with SNHG6-specific shRNA lentivirus (SNHG6_Sh) to knock down SNHG6 expression (Figure 2A, H7, *P<0.05). After cardiac differentiation, qRT-PCR assay confirmed that, SNHG6 was also downregulated in HiPSC-CMs differentiated from SNHG6_Sh-transduced H7 cells, as compared to those differentiated from H7 cells transduced with a control non-specific shRNA lentivirus (C_Sh) (Figure 2A, HiPSC-CMs, *P<0.05).

Then, transduced HiPSC-CMs were exposed to various concentrations of PPF (0–100 μg/mL) for 48 h, followed by a viability assay. The quantitative comparison demonstrated, while exposed to 20–100 μg/mL PPF, SNHG6_Sh-transduced HiPSC-CMs had much better surviving rates than C_Sh-transduced HiPSC-CMs (Figure 2B, *P<0.05). Also, after PPF treatment, transduced HiPSC-CMs were examined by the fluorescent mitochondrial superoxide assay. It demonstrated that, while exposed to 50 μg/mL PPF, SNHG6_Sh-transduced HiPSC-CMs had much less mitochondrial superoxide production than C_Sh-transduced HiPSC-CMs (Figure 2C).

In addition, a TMRE assay indicated that, while exposed to 50–100 μg/mL PPF, SNHG6_Sh-transduced HiPSC-CMs had much less mitochondrial membrane potential drop than C_Sh-transduced HiPSC-CMs (Figure 2D, *P<0.05).

Thus, these data suggested that SNHG6 downregulation could rescue PPF-induced cytotoxicity in HiPSC-CMs.

Hsa-miR-186-5p is reversely correlated with SNHG16 expression in HiPSC-CMs

Using an online epigenetic targeting algorithm, StarBase 3.0 (28,29), it was noticed that human microRNA-186-5p (hsa-miR-186-5p) may serve as the downstream competing target of SNHG6 (Figure 3A). We thus constructed two luciferase vectors, one containing the wild-type human SNHG6 3'-UTR which hosts a putative hsa-miR-186-5p binding site (SNHG6_Lu), and the other containing the mutant SNHG6 3'-UTR with modified DNA sequences on the putative hsa-miR-186-5p binding site (SNHG6(MU)_Lu). Then, a dual-luciferase activity assay confirmed our hypothesis, indicating that SNHG6 could bind hsa-miR-186-5p (Figure 3B, *P<0.05, ∆P>0.05).

Also, we used qRT-PCR assay to investigate the expression pattern of hsa-miR-186-5p in HiPSC-CMs. It showed that, in HiPSC-CMs exposed to 50 μg/mL PPF, hsa-miR-186-5p expression was significantly lower than in control HiPSC-CMs (Figure 3C, *P<0.05). In addition, in lentiviral-transduced HiPSC-CMs, qRT-PCR indicated that SNHG6 downregulation inversely upregulated hsa-miR-186-5p (Figure 3D, *P<0.05).

Thus, these results suggested that hsa-miR-186-5p is the downstream competing target of SNHG6, thus reversely
Figure 1 The cytotoxic effects of propofol (PPF) on human-induced pluripotent stem cell-derived cardiomyocytes (HiPSC-CMs). HiPSC-CMs were exposed to 0, 1, 2, 5, 10, 20, 50 or 100 μg/mL PPF for 48 h. (A) A cell viability was conducted (*, P<0.05, vs. 0 μg/mL PPF). (B) A mitochondrial superoxide assay was conducted for HiPSC-CMs exposed to 0 and 50 μg/mL PPF. Mitochondrial superoxide production was identified by MitSox staining (red). HiPSC-CM nuclei were identified by Hoechst 33342 staining (blue). (C) A TMRE-mitochondrial membrane potential assay was conducted (*, P<0.05, vs. 0 μg/mL PPF). (D) A qRT-PCR assay was conducted to measure expression levels of human lncRNA SNHG6 in PPF-exposed HiPSC-CMs (*, P<0.05).

Figure 2 Functions of SNHG6 suppression on PPF-induced cytotoxicity in HiPSC-CMs. (A) H7 cells were transduced with lentiviruses of C_Sh or SNHG6_Sh. After transduction was stabilized, a qRT-PCR assay was conducted to compare expression levels of SNHG6 in lentiviral-transduced HiPSC-CMs (H7, *, P<0.05). Those H7 cells then underwent cardiac induction and purification. The resulted HiPSC-CMs were also examined by a qRT-PCR to compare their SNHG6 expression levels (HiPSC-CMs, *, P<0.05). (B) Lentiviral-transduced HiPSC-CMs were exposed to 0, 1, 2, 5, 10, 20, 50 or 100 μg/mL PPF for 48 h. After that, cell viability was compared between C_Sh- and SNHG6_Sh-transduced HiPSC-CMs (*, P<0.05). (C) Mitochondrial superoxide production was immunostained and compared between C_Sh- and SNHG6_Sh-transduced HiPSC-CMs which were exposed to 50 μg/mL PPF. (D) Mitochondrial membrane potentials were measured and compared between C_Sh- and SNHG6_Sh-transduced HiPSC-CMs (*, P<0.05).
correlated with SNHG6 expression in HiPSC-CMs.

**Hsa-miR-186-5p inhibition reversed the rescuing effect of SNHG6 downregulation on PPF-induced cytotoxicity in HiPSC-CMs**

Lentiviral-transduced HiPSC-CMs were transiently transfected with hsa-miR-186-5p-targeted miRNA inhibitor (miR186_In) to knock down endogenous hsa-miR-186-5p expression (Figure 4A, *P<0.05). Along with those transfected with a control miRNA inhibitor (C_In), double-infected HiPSC-CMs were exposed to various concentrations of PPF (0–100 μg/mL) for 48 h. After that, the quantitative comparison demonstrated, while exposed to 5–50 μg/mL PPF, HiPSC-CMs double-infected with SNHG6_Sh/miR186_In had much higher death rates than HiPSC-CMs double-infected with SNHG6_Sh/C_In (Figure 4B, *P<0.05).

Also, the immunohistochemical assay showed that, while exposed to 50 μg/mL PPF, HiPSC-CMs double-infected with SNHG6/Sh/miR186_In had much more mitochondrial superoxide production than HiPSC-CMs double-infected with SNHG6/Sh/C_In (Figure 4C, *P<0.05).

Furthermore, a TMRE assay demonstrated that, while exposed to 20–100 μg/mL PPF, HiPSC-CMs double-infected with SNHG6/Sh/miR186_In had more mitochondrial membrane potential drops than HiPSC-
CMs double-infected with SNHG6_Sh/C_In (Figure 4D, *P<0.05).

Therefore, these results indicated that hsa-miR-186-5p inhibition reversed the rescuing effect of SNHG6 downregulation on PPF-induced cytotoxicity in HiPSC-CMs.

Discussion

It was reported that, in order to prevent PRIS, the dosage of in vivo induction of PPF in human patients shall not be above 4 mg/kg/h for a 48-h time duration (1,2). In this study, we mimicked PPF-induced cardiac cytotoxicity in an in vitro model HiPSC-CMs. We discovered that cell viability was reduced and mitochondrial functions were damaged in HiPSC-CMs exposed to PPF approximately at a concentration of 5 μg/mL or higher. These findings are in line with the results in a previous study [10], and in accordance with the prediction that the non-toxic plasma concentration PPF was around 2.5 μg/mL (30).

Also in this study, we discovered SNHG6 was upregulated in PPF-exposed HiPSC-CMs. In a previous study, it was demonstrated that SNHG6 was aberrantly upregulated in murine fetal cardiac tissues with ventricular septal defect, and forced overexpression of SNHG6 inhibited proliferation and induced apoptosis in cardiomyocyte cell line P19 cells (23). In addition, it was noted SNHG6 was upregulated in cardiac tissues in patients with atherosclerosis, and knockdown of SNHG6 alleviated oxidative-stress induced cardiac apoptosis (24,25). Thus, our results are in line with those in previous studies, suggesting that aberrant expression (mostly likely upregulation/overexpression) of SNHG6 is very likely associated with pathological conditions in cardiovascular diseases.

The most important findings of our study were that, after we knocked down SNHG6 expression in HiPSC, the downregulation pattern of SNHG6 can be maintained until cardiac differentiation and we were able to show the rescuing effects of SNHG6 downregulation on PPF-induced cytotoxicity in HiPSC-CMs. Within the superfamily of SNHG IncRNAs, newly emerged evidence suggested that, other members than SNHG6, such as SNHG1 and SNHG16 are also actively involved in the development and pathology of cardiomyocytes (31,32). Our preliminary data also showed gene expression changes of other SNHG super-family than SNHG6 (data not shown). However, after gene modification at stem cell stage, downregulating or upregulating patterns of other SNHG...
family members could not be maintained in HiPSC-CM stage. Thus, future investigations, possibly utilizing other in vivo or in vitro models with much feasible gene-editing capabilities, would further our understanding on the biological functions of SNHG family in regulating PPF-induced cardiac cytotoxicity.

Also, in our study, we explored the intrinsic signaling network of SNHG6 responsible for rescuing PPF-induced cytotoxicity in HiPSC-CMs. We demonstrated that hsa-miR-186-5p was an endogenous ceRNA candidate of SNHG6 (using dual-luciferase activity assay), and their expressions were inversely correlated in PPF-exposed HiPSC-CMs (using qRT-PCR assay). More importantly, we verified hsa-miR-186-5p was the functional downstream competing target of SNHG6, as suppressing hsa-miR-186-5p reversed the rescuing effects of SNHG6 downregulation on PPF-induced cytotoxicity in HiPSC-CMs. It is worth noting that, during the processes of SNHG6 regulating other human diseases (such as human cancers), SNHG6 was reported to be associated with various ceRNA candidates, probably depending on the sites or organs of diseases (22,33). Thus, more studies are required, in order to fully understand the associated signaling pathways of SNHG6 during the process of PRIS.

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Conflicts in Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/cdt-20-443). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee for Human & Clinical Research at the People’s Hospital of China Three Gorges University in Yichang, Hebei Province, China (approval ID: CTGU20190455). All procedures were performed in accordance with the Declaration of Helsinki (2013 version). Informed consent was obtained from the patient for publication of this manuscript.

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