Dexmedetomidine protects PC12 cells from oxidative damage through regulation of miR-199a/HIF-1α

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ABSTRACT

Background: Although dexmedetomidine (Dex) has a significant neuroprotective effect in various nerve-damage models, the exact mechanism of which Dex protects cells from oxidative damage is not fully clear. This article recommended the protective effect of Dex on oxidative damage in PC12 cells.

Methods: The PC12 cells were incubated by hydrogen peroxide (H₂O₂) for 24 h and pre-treated by Dex for 30 min. Cell viability, apoptosis, HIF-1α expression and ROS level were detected by CCK-8, apoptosis assay, Western blot and ROS assay, respectively. The miR-199a expression was tested by qRT-PCR. Targeting relationship between miR-199a and HIF-1α was performed by dual luciferase activity assay. The activation of PI3K/AKT/mTOR and Wnt/β-catenin pathways was tested by western blot.

Results: Dex attenuated H₂O₂-induced oxidative damage, including the decline of cell viability, the raise of apoptosis and the generation of ROS in PC12 cells by down-regulating miR-199a expression. Moreover, Dex up-regulated HIF-1α expression via decreasing miR-199a level in PC12 cells and miR-199a targeted the 3'-UTR of HIF-1α. In addition, Dex activated PI3K/AKT/mTOR and Wnt/β-catenin pathways by declining miR-199a level.

Conclusions: This article illustrated the protective effect of Dex on oxidative damage in PC12 cells. Furthermore, Dex prevented PC12 cells from oxidative injury through the regulation of miR-199a/HIF-1α.

Introduction

It is well known that the neurodegenerative disease often occurs in middle-aged people in recent years, and the annual incidence is constantly increasing. The disease’s features are pathological changes in specific brain regions and denatured death of different neurons. The studies showed that oxidative damage and cell apoptosis played a significant role in the development of neurodegenerative diseases [1]. By the same token, a study showed that reactive oxygen species (ROS) was able to damage neurons [2]. Thus, it is fairly necessary to find a new drug to protect nerve cells from oxidative damage and thus act as a neuroprotective agent.

Dexmedetomidine (Dex) is a selective alpha 2 adrenergic receptor (α2AR) agonist with sedative, anxiolytic, antisympathetic and analgesic effects for intraoperative and postoperative management [3]. It has been widely used in perioperative anaesthesia and intensive care unit. In clinical researches, not only the protective effects of Dex are closely related to activate α2AR signalling pathway, but also other signalling pathways (such as PI3K/Akt) are involved in the protective impacts of Dex [4,5]. There was also increasing evidence illustrated that Dex had significant neuroprotective effects in a variety of neural injury models. For example, Dex could relieve neuroinflammation induced by LPS [6]. The study showed that Dex could protect neurons and astrocytes against β-amyloid (Aβ) [7]. Although countless studies have reported the neuroprotection of Dex, it is seldom informed that Dex plays the role of neuroprotection through antioxidative damage.

There have been studies reported that microRNAs (miRNAs) play a crucial significance in the improvement of neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD) [8–10]. For example, the activation of miR-3557/324 could regulate the CaMKs signalling pathway to decrease the incidence of PD [11]. The decline of miR-101a level accelerated the progress of AD [12]. Moreover, a study showed that the decline of miR-199a-5p, as well as miR-199a-3p levels, was involved in PD [13]. In addition, only a few studies have illustrated miR-199a is closely related to hypoxia-adaptation [14–17]. So, we speculated if the level of miR-199a expression participated in oxidative damage in neurodegenerative diseases.

The PC12 cells are derived from Rattus norvegicus adrenal pheochromocytoma (a tumour of the sympathetic nervous system) [18]. PC12 cell line is frequently used as a model system for neurobiological and neurochemical studies [19,20].
To sum up, the protective effects of Dex on oxidative damage were studied in PC12 cells. This article brought evidence to explain if Dex protected PC12 cells against oxidative injury via the regulation of miR-199a/HIF-1α.

Materials and methods

Cell culture and treatment

The PC12 cells were received from the Kunming Institute of Zoology (Kunming, China). The cells were brood in DMEM medium (Thermo Fisher, Waltham, MA) along with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (both from PAN Biotech, Aidenbach, Germany). Cells were seeded (1 × 10⁴ cells/mL). It was maintained in a chamber (Thermo Fisher, Waltham, MA, at 37°C embracing 5% CO₂.

The hydrogen peroxide (H₂O₂) solution was gained from Sigma-Aldrich (cat. number: 323381, St. Louis, MO). PC12 cells were incubated by various concentrations of H₂O₂ (0, 12.5, 50, 100 and 200 μM) for 24 h. The Dex was purchased from Hengrui Medicine Co., Ltd. (cat. number: 15030932, Lianyungang, China) and dissolved in DMEM medium. After the PC12 cells were pre-treated by Dex (1 nM) for 30 min, cells were incubated by 50 μM H₂O₂ for 24 h.

Cell transfection

The respective negative control (NC mimic) and miR-199a mimic were compounded by GenePharma Co. (Shanghai, China) and the transfection concentration of NC mimic and miR-199a mimic was 100 nM and 50 nM, respectively. The sequence of miR-199a mimic was 5'-CCCAGUGUUCAGACUACCUGUUC-3'. Transfection was conducted by adopting lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). The time of transfection was set as 48 h, in line with manufacturers’ contract.

Cell counting kit-8 (CCK-8) assay

Cells were celled as a density of 5000 cells per well in 96-well plates. After cells were treated by drugs, the CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD) was picked up into every well. After CCK-8 solution cultured cells for 1 h, the absorbance was computed by operating a Microplate Reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

Apoptosis assay

Cell apoptosis was completed by collecting propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining. After all the cells were treated with distinctive drugs, cells were washed by using phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). Then cells were coloured in 100 μL PI/FITC-Annexin V (Beijing Biosea Biotechnology, Beijing, China). Flow cytometry test was performed in agreement with the instruction of FACS can (Beckman Coulter, Fullerton, CA). FITC-positive and PI-negative cells were counted as apoptotic cells. All data were counted by FlowJo software (Treestar, San Carlos, CA).

ROS assay

PC12 cells were mixed and celled in six-well plates. After cells were incubated by different drugs, cells washed by PBS then were brood by medium without serum incorporating 10 μM DCFH-DA (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C. Subsequently, cells washed were suspended with 500 μL PBS again. Finally, the fluorescent degrees were calculated by applying a flow cytometer (Beckman Coulter, Fullerton, CA, USA). The excitation was set as 488 nm and the emission was set as 521 nm, respectively.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The entire RNA was isolated by utilizing Trizol reagent (Life Technologies Corporation, Carlsbad, CA). Single-step cDNA synthesis and miRNA quantitation were performed by using Mir-X™ miRNA First Strand Synthesis Kit and Mir-X™ miRNA qRT-PCR TB Green™ Kit (Takara, Dalian, China), respectively. A specific forward primer of miR-199a (5’-ACACTCCGACCTGGGCCCAGTGTTCAGACTAC-3’) and the reverse primer of miR-199a (5’-TGGTGCTGGAGACTGGTGAGTGC-3’) were used in this experiment. U6 (the forward primer: 5’-CAATTCGGTAAACCTTTTTCC-3!’ and the reverse primer: 5’-AACCGTTGAGTGC-3’) was thought as the control of miRNA. The data were analyzed by using 2-ΔΔCt method.

Dual luciferase activity assay

The 3'-UTR regain of HIF-1α was amplified by PCR and placed into pGL3 reporter vector to form a reporter plasmid comprising the wide type of HIF-1α 3'-UTR (HIF-1α-wt). The anticipated combining site of the 3'-UTR of HIF-1α which can bind with miR-199a was mutated and inserted to form a control plasmid of HIF-1α-mut. The plasmids were transfected together with miR-199a mimic or NC mimic into 293T cell (all from Promega, Madison, WI). Reporter assays were accomplished by performing the dual-luciferase assay system (Promega, Madison, WI) in consonance with the directive.

Western blot

Proteins in PC12 cells were obtained through collecting a protein extraction kit (Sigma-Aldrich, St. Louis, MO, USA). The concentration of those was counted by using BCA™ Protein Assay Kit (Pierce, Appleton, WI). A Bio-Rad Bis-Tris Gel system was used to separate proteins in consonance with manufacturer’s guidance. The membranes (Millipore, Billerica, MA, USA) were covered by 5% bovine serum albumin (BSA) (from Sigma-Aldrich, St. Louis, MO, USA). The experimental involved primary antibodies against Bcl-2 (ab185002, Abcam, Cambridge, UK), Bax (ab32503), pro-caspase-3 (ab238440), cleaved-caspase-3 (ab2302), HIF-1α (ab1), t-PI3K (ab86714), p-PI3K (ab182651), p-AKT (ab38449), t-AKT (ab8805), t-mTOR (ab2732), β-catenin (ab32572), Wnt3a (ab219412), β-actin (ab8227) and p-mTOR (#5536, Cell Signaling Technology, Beverly, MA, USA). Two kinds of second antibodies (goat
anti-rabbit (ab6940, Abcam, Cambridge, UK) and goat anti-mouse (ab97035) were used. Positive bands were developed by BeyoECL Moon (Beyotime, Shanghai, China). Different protein was estimated by Image Lab™ Software (Bio-Rad, Shanghai, China).

**Statistical analysis**

Three experiments were completed to repeat every result. All data are shown as mean ± SD. We adopted Graphpad statistical software 6.0 (Graphpad Prism, Chicago, IL) to analysis our data. Comparison between the two groups was tested by t test, and a comparison between three or more was adopted by one-way analysis of variance (ANOVA). We made a conclusion that *p < .05* was regarded as a meaningful result.

**Results**

**Dex attenuated H2O2-evoked oxidative damage in PC12 cells**

PC12 cells were incubated by distinctive concentrations of H2O2 (0, 12.5, 50, 100 and 200 μM) for 24 h. As described in Figure 1(A), cell viability showed the declined trends with the concentration of H2O2 increasing. When the concentration of H2O2 was 12.5 μM, cell viability was started to be lowered by one-way analysis of variance (ANOVA). We made a conclusion that *p < .05* was regarded as a meaningful result.

![Figure 1](image-url)

**Figure 1.** Dex attenuated H2O2-evoked oxidative damage in PC12 cells. Cells were incubated by different concentrations of H2O2 (0, 12.5, 50, 100 and 200 μM) for 24 h. (A) Cell viability was computed by CCK-8 assay. After pre-treatment by Dex (1 nM) for 30 min, PC12 cells were incubated by 50 μM H2O2 for 24 h. (B) Cell viability was tested by CCK-8, (C) the rate of cell apoptosis was counted by apoptosis assay, (D, E) the levels of Bcl-2, Bax and cleaved-caspase 3 protein expression were measured by western blot, (F) the level of ROS expression was detected by ROS assay. **p < .01 and ***p < .001 vs. control (Ctrl) group. #p < .01 and ###p < .001 vs. H2O2 group.**
As cell viability was about 50% when the concentration of H$_2$O$_2$ was 50 μM, 50 μM was used as following experimental concentration. After pre-treatment by Dex (1 nM) for 30 min, cells were incubated by 50 μM H$_2$O$_2$ for 24 h. As displayed in Figure 1(B), co-treatment of cells with Dex and H$_2$O$_2$ significantly increased cell viability as compared to treatment with H$_2$O$_2$ alone (p < .05). Rate of cell apoptosis was markedly raised in the H$_2$O$_2$ group, and the rising rate of cell apoptosis induced by H$_2$O$_2$ was partly reversed by Dex (both p < .05, Figure 1(C)). The Bcl-2 protein level was strikingly down-regulated, but the levels of Bax, as well as cleaved-caspase-3, were obviously raised by H$_2$O$_2$ (all p < .05, Figure 1(D,E)). The above three proteins’ changes induced by H$_2$O$_2$ were reversed by Dex (all p < .05, Figure 1(D,E)). The ROS level was significantly increased by H$_2$O$_2$, but Dex decreased ROS levels (both p < .05, Figure 1(F)). Above all results explained that Dex attenuated H$_2$O$_2$-evoked oxidative injury in PC12 cells.

**Dex induced the up-regulation of HIF-1α in PC12 cells**

HIF-1α is a transcription factor regulated by oxygen. Next, the downstream effectors of which Dex exerted its function were explored. As depicted in Figure 2(A), the level of HIF-1α was evidently up-regulated by H$_2$O$_2$, and H$_2$O$_2$-induced HIF-1α level was further raised by Dex in PC12 cells (both p < .05).

**Dex decreased miR-199a levels in PC12 cells**

As described in Figure 3, we could get a massage that miR-199a expression was obviously increased by H$_2$O$_2$, but Dex could decrease miR-199a express evoked by H$_2$O$_2$ (both p < .05).

**Dex attenuated H$_2$O$_2$-evoked oxidative damage by down-regulating miR-199a expression in PC12 cells**

The NC mimic and miR-199a mimic were transfected into PC12 cells. After transfaction, compared with the NC mimic transfected group, miR-199a level was significantly increased in the miR-199a mimic transfected group (p < .05, Figure 4(A)). MiR-199a mimic could decrease cell viability induced by Dex (p < .05, Figure 4(B)). MiR-199a mimic could reverse the decrease of cell apoptotic rate driven by Dex (p < .05, Figure 4(C)). The changes of Bcl-2, Bax and cleaved-caspase-3 protein level evoked by Dex were reversed by transfecting miR-199a mimic (all p < .05, Figure 4(D,E)). The Dex-induced ROS level was increased by up-regulating miR-199a expression (p < .05, Figure 4(F)).

**Dex up-regulated HIF-1α through declining miR-199a**

As shown in Figure 5(A,B), miR-199a mimic could down-regulate HIF-1α expression (p < .05). Moreover, as seen in Figure 5(C), with overexpression of miR-199a, the assays disclosed markedly decline of luciferase reporter gene activity for the HIF-1α-wt-3’UTR constructs (p < .05). To the contrary, luciferase reporter gene activity was not changed when applying the HIF-1α-Mut-3’UTR.

**Dex activated PI3K/AKT/mTOR and Wnt/β-catenin pathways by down-regulating miR-199a**

After miR-199a mimic and NC mimic were transfected, the simulation of PI3K/AKT/mTOR and Wnt/β-catenin pathways...
was tested by adopting western blot. The phosphorylation levels of PI3K, AKT and mTOR were decreased by H$_2$O$_2$ (all $p < .05$). The PI3K, AKT and mTOR phosphorylation levels were up-regulated by Dex (all $p < .05$). After transfection of miR-199a mimic, the increased phosphorylation levels of PI3K, AKT and mTOR induced by Dex were reversed (all $p < .05$, Figure 6(A,B)). Furthermore, the Wnt3a and β-catenin expression was declined by H$_2$O$_2$ (both $p < .05$). The above
two protein levels in H$_2$O$_2$-treated cells were increased by Dex (both $p < .05$). After transfection of miR-199a mimic, the two protein expression was decreased (both $p < .05$, Figure 6(C,D)). Above all results demonstrated Dex induced the simulation of the above two pathways in PC12 cells through down-regulating miR-199a expression.

**Discussion**

Oxidative stress could induce various diseases through oxidative modification of biomolecules and changes of signalling pathways including neurodegenerative diseases [21]. Thus, the protection of cells from oxidative damage is critical for the treatment of neurodegenerative diseases. In our studies, H$_2$O$_2$ decreased viability and promoted apoptosis in PC12 cells. But, Dex raised cell viability and repressed apoptosis in PC12 cells. Furthermore, we found that Dex further up-regulated HIF-1$\alpha$ expression induced by H$_2$O$_2$. In addition, Dex up-regulated HIF-1$\alpha$ expression via reducing miR-199a in PC12 cells. It was tested that Dex attenuated H$_2$O$_2$-induced oxidative damage and induced the simulation of PI3K/AKT/mTOR and Wnt/$\beta$-catenin signalling pathways in PC12 cells through down-regulating miR-199a expression.

Currently, countless researches have showed that Dex exerts neuroprotective effects through different mechanisms [3]. For instance, it was indicated that Dex could reverse neural apoptosis through anti-inflammatory and anti-apoptotic effects to protect neurons [22]. There was also a study showed that Dex inhibited excitatory neurotransmitter and decreased calcium entry into cells, which ultimately attenuated cell damage [23]. Besides, Dex reduced the release of glutamate and thus was considered to have neuroprotective effects [24]. In addition, it was generally reported that Dex had neuroprotective effects in different animal models due to its anti-oxidative damage effects [25–27]. This was similar to our results, in which Dex increased PC12 cell viability and decreased apoptosis, along with inhibited ROS formation to protect the cell from oxidative damage.

The HIF-1$\alpha$ controlled by oxygen was an ingredient that took part in the process of transcription and controlled the level of all kinds of genes [28], which were involved in cell growth and apoptosis, like CyclinD1, p62, Bax and Bcl-2. In our studies, H$_2$O$_2$ increased HIF-1$\alpha$ level in PC12 cells and Dex further promoted the increase of HIF-1$\alpha$ level, thereby protected cells from oxidative damage. What is more, the levels of different miRNAs can drive the stabilization of HIF-1$\alpha$. For example, miR-199a, miR-20b together with miR-17-92 destabilized HIF-1$\alpha$ [29,30]. But miR-210 along with miR-424 stabilized HIF-1$\alpha$ [31,32]. Next, the downstream effectors of which Dex exerted its function were explored. H$_2$O$_2$ also induced the increase of miR-199a expression and Dex inhibited the expression of miR-199a. Hence, we speculated that Dex regulated HIF-1$\alpha$ might be through miR-199a. Dual-luciferase activity assay results showed that Dex up-regulated HIF-1$\alpha$ expression via the decline of miR-199a in PC12 cells and 3’-UTR of HIF-1$\alpha$ was as a specific target of miR-199a-5p.

MiRNAs were a glass of critical factors that participated in cell proliferation as well as apoptosis [33]. A study has shown miR-199a-5p is strikingly declined in PD [13]. Moreover, a study explained that miR-199a suppressed HIF-1$\alpha$ level under hypoxic environment [34]. In addition to this, it was affirmed that the abnormal miR-199a expression was together with the generation of ROS [35,36]. Along with the deepened study gradually, a mass of researches released that miR-199a repressed cell viability and accelerated apoptosis [37].
above results of all researches were similar to our data. Although it was showed that miR-199a level took part in neurodegenerative disease, the specific neuroprotective mechanism of Dex was not clear. Hence, we collected PC12 cells to validate how the expression of miR-199a induced by Dex played a role in neurodegenerative diseases. Further results manifested that Dex-caused the decline of cell viability and the increase of apoptotic percentage through increasing the level of miR-199a. We additionally found that the increase of miR-199a resulted in ROS generation. The above all results illustrated that Dex could suppress oxidative damage driven by H2O2 via down-regulating miR-199a expression. Our study uncovered that Dex decreased the expression of miR-199a to play its neuroprotective role. There was a study released that the silence of long non-coding RNAs (lncRNA MALAT1) improved the protective effect of Dex on hypoxic-ischemic brain damage by sponging miR-429 [38]. Another article also showed lncRNA SNHG12 could target miR-199a to ameliorate brain microvascular endothelial cell injury [39]. Thus, we speculated that Dex possibly reduced miR-199a via the regulation of certain lncRNA (such as lncRNA SNHG12). Hence, we need further experiments to verify this.

The PI3K/AKT/mTOR and Wnt/β-catenin pathways took part in neurodegenerative diseases. For instance, simulation of the PI3K/AKT/mTOR pathway could inhibit cell apoptosis to mediate the neuroprotective effect in a cellular model of PD [40]. Research also explained that the blockage of Wnt/β-catenin pathway was partially activated to protect nerve cells in PD and AD [41,42]. The report also explicated that miRNAs protected cell from damage via regulating the above two kinds of pathways in neurodegenerative diseases [43,44]. For instance, up-regulating miR-100 expression evoked the simulation of PI3K/AKT/mTOR pathway to decrease the incidence of PD [43]. Down-regulating miR-214 expression induced the simulation of Wnt/β-catenin pathway to decline the rate of HD [44]. Although a few studies tested the miRNAs expression regulated the above pathways in neurodegenerative diseases, related research is still insufficient. The study has exhibited that PI3K/AKT/mTOR signalling pathway is activated by miR-199a-5p inhibitor [45]. This was similar to

Figure 6. Dex activated PI3K/AKT/mTOR and Wnt/β-catenin pathways by down-regulating miR-199a. After transfection by NC mimic or miR-199a mimic, PC12 cells were incubated by 50 μM H2O2 in combination with Dex (1 nM). (A, B) The phosphorylation levels of PI3K, AKT, and mTOR were measured by Western blot. (C, D) The levels of β-catenin and Wnt3a expression were also tested by adopting Western blot. *p < .05 and **p < .01 vs. control (Ctrl) group. #p < .05 and ##p < .01 vs. H2O2 group. *p < .05 and **p < .01 vs. H2O2 + Dex + NC mimic group.
our experimental results. Our study released miR-199a overexpression could repress the mobilization of PI3K/AKT/mTOR and Wnt/β-catenin signalling pathways in PC12 cells. The focus of our study is on how Dex regulates those two pathways in PC12 cells. The data displayed H₂O₂ prevented the activation of the above two signalling pathways. Moreover, Dex induced the activation of those two pathways in PC12 cells via declining miR-199a expression.

In brief, this article depicted the effect of Dex on oxidative damage in PC12 cells. This study indicated that Dex could protect PC12 cells from oxidative damage. In addition, Dex attenuated H₂O₂-induced oxidative damage and induced the simulation of PI3K/AKT/mTOR and Wnt/β-catenin signalling pathways in PC12 cells through declining miR-199a expression. Although Dex has been used in clinical studies, the mechanism of Dex’s action is not fully known to this day. Our research put forward an idea that Dex exerted its neuroprotective function by reducing miR-199. This not only lays a better foundation for further study but also provides a theoretical basis for clinical application of Dex. In a clinical study, Dex acts as a neuroprotective drug from oxidative damage.

Disclosure statement
No potential conflict of interest was reported by the authors.

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