Research Article

MiR-25 protects PC-12 cells from H₂O₂ mediated oxidative damage via WNT/ β -catenin pathway

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Objective: Spinal cord injury (SCI) is associated with modulation of different microRNAs (miRs). This study aims to explore the role of miR-25 in PC-12 cells to reveal the potential of miR-25 in SCI treatment.

Methods: SCI model was established in C57BL/6 mice, then miR-expression in the injured spinal cords were detected by qRT-PCR. PC-12 cells were exposed to H₂O₂ conditions to establish an *in vitro* model of SCI. PC-12 cells were transfected with expressing vector or antisense oligonucleotides (ASO) of miR-25. The effects of miR-25 expression on H₂O₂-induced oxidative damage was evaluated by detection of cell viability, apoptosis, ROS activity, HIF- α and γ H2A expression, and the level of inflammatory mediators. The expression of Nrf2 in cells was silenced by transfection with Nrf2 siRNA, and the effects of Nrf2 silence on miR-25-mediated PC-12 cells were detected. Besides, the expression of main proteins in Wnt/ β -catenin and PI3 K/AKT/ERK signaling were assessed.

Results: miR-25 was low expressed in injured spinal cords. miR-25 protected PC-12 cells against H₂O₂-induced oxidative damage, as evidenced by significant suppression in cell apoptosis, increase in cell viability, decrease in the level of ROS, HIF- α and γ H2A, and decrease in inflammatory mediators (IL-1 β , TNF- α , IL-6, and MCP-1). However, Nrf2 silence abolished the protective functions of miR-25 on H₂O₂-induced damage. Furthermore, we found that Wnt/ β -catenin and PI3 K/AKT/ERK signaling were activated by miR-25.

Conclusions: miR-25 protects PC-12 cells against H₂O₂-induced oxidative damage though regulation of Nrf2 and activation of Wnt/ β -catenin and PI3 K/AKT/ERK signaling.

Keywords: miR-25, Spinal cord injury (SCI), H₂O₂, PC-12 cells, Nrf2, Wnt/ β -catenin signaling, PI3 K/AKT/ERK signaling

Introduction

The role of different microRNAs (miRs) in the biological system is well established; miRs are responsible for suppression of gene expression through modification at the post-transcriptional stage.¹⁻³ It has been established that miRs control the expression of about 20–30% of total human genes.¹ miRs are endogenous non-coding RNAs consisting of approximately 18–22 nucleotides with genetic sequence only partially complementary to their respective target mRNAs.⁴ Currently thousands of miRs have been identified which participate in a number of biological processes like cellular growth, proliferation, development, and metabolism.⁴

miR-25, a member of miR-106b-25 cluster, has been reported as an oncogene in many cancers, such as gastric carcinoma,⁵ glioblastoma multiforme,⁶ and

ovarian cancer.⁷ It exerts pro-growth, anti-apoptotic, and cell cycle-promoting activities in tumor cells via regulation of its target genes, including Bim and LATS2.^{7,8} In addition, miR-25 has been reported to be a major miRs marker in neural cells, and is highly expressed in ischemic brain tissues.⁹ Knocking down of miR-25 resulted in a decrease in neural stem/progenitor cells proliferation,¹⁰ and miR-25 was implicated in the regulation of neuronal migration and differentiation.¹¹ Although previous studies have revealed the importance of miR-25 on neural cells, little was focused on the potential of miR-25 on oxidative damaged neurons.

Spinal cord injury (SCI) is a dreaded injury with an incidence rate of 23 new cases per million as estimated in 2007.¹² Since ancient times SCI was known as a catastrophic injury to the spinal cord.¹³ Recovery (even partial) was considered to be impossible, until David and Aguayo in 1981 demonstrated in adult rat models that following focal central nervous system

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injury axonal regeneration is possible to some extent under specific circumstances with the use of peripheral nerve segments as “bridges”.¹⁴ The pathogenesis of SCI is a rather complex one involving the neurons, vessels and the immune system.¹³ The underlying pathogenesis takes time develop; usually months after the initial trauma.^{15,16} The pathogenesis can be discussed under two phases; the primary mechanism involves the initial mechanical injury followed by the secondary phase mediated by alteration vascular abnormality, change in biochemical processes and ultimately inflammatory damage and apoptosis of the neurons.¹⁶

Hydrogen peroxide (H₂O₂) is a diffusible and relatively stable oxygen species (ROS) that is present in low micromolar concentrations under physiological conditions.¹⁷ H₂O₂ levels are significantly increased in mitochondrial fractions after SCI,¹⁸ and it plays an important role in the pathogenesis of SCI.^{19,20} Although the mechanisms of neuronal damage induced by H₂O₂ are not entirely understood, H₂O₂ exposure has been commonly used to mimic an *in vitro* model of SCI.²⁰ Pheochromocytoma (PC-12) cells have a similar morphology and function to sympathetic neurons in the presence of nerve growth factor (NGF), and subsequently have been widely utilized as a model for the study of neuron responses to stress and injury.^{21,22} In the current study, PC-12 cells were used and exposed to H₂O₂ conditions to establish an *in vitro* model of oxidative damage of neural-like cells. Thereafter, the effects of miR-25 on H₂O₂-damaged cells were detected, to reveal the potentials of miR-25 on SCI treatment.

Materials and methods

In vivo SCI model

All the experimental research protocols were reviewed and approved by the Animal Ethical Committee of Jining No. 1 People's Hospital. Specific pathogen-free grade of male C57BL/6 mice (8 weeks old) were purchased from Vital River Laboratories (Beijing, China). Mice (n = 6 in each group) were anaesthetized by intraperitoneal injection with 125 mg/kg 2,2,2-tribromoethanol anesthesia (Sigma-Aldrich, St Louis, MO, USA). After laminectomy at the Th9 spinal vertebra, SCI model was established in mice as previously described.²³ Control mice received only a laminectomy without any direct manipulation of the spinal cord. Spinal cords were removed at 6 hours, 12 hours, 1 day, 3 day and 7 day after injury from SCI mice, and 12 hours after laminectomy from control mice. Spinal cords (the injured site T11–12 and 5 mm on either

side) were then harvested for RNA isolation as described below.

Cell culture and treatment

PC-12 cells were purchased from Kunming Institute of Zoology (Kunming, China) and used throughout the study. The cells were seeded onto flasks at a density of 1×10^4 cells/ml in DMEM with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. Culture medium was changed every other day.

For the H₂O₂ treatment, cells were planted in cell culture multi-well plates (Thermo Scientific, Nunc™, Naerum, Denmark) at a density of 5×10^4 cells/ml for 24 hours. The cells were treated with fresh medium with H₂O₂ (final concentration of 0–500 μ M) for 4 hours to construct the SCI injury model. The control group was treated with the same medium without H₂O₂.

For inhibition of Wnt pathway, 10 μ M ICG001 purchased from Selleck Chemicals (Houston, TX, USA) were used for treating cells for 12 hours.

Establishment cell line with stable expression of miR-25

Lentiviral vectors which overexpress miR-25 were purchased from GeneChem (Shanghai, China). A lentiviral vector expressing scrambled RNA was used as the control and the sequence was 5'-TTCTCCGAACGTGTCACGT-3'. PC-12 cells were infected with lentiviral vector, and polyclonal cells with green fluorescent protein signals were selected for further experiments using fluorescence-activated cell sorting flow cytometry. The antisense oligonucleotides (ASO) of miR-25 and ASO-NC (no silencing) were synthesized by GenePharma Co (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Total RNA from these cell clones was isolated, and levels of miR-25 were quantified using quantitative RT-PCR (qRT-PCR).

qRT-PCR

Total RNA was extracted from PC-12 cells by Trizol Reagent (Invitrogen). For miR-25 detection, reverse-transcribed complementary DNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and qRT-PCR was performed with SYBR Premix ExTaq (TaKaRa) with the Stratagene Mx3000P real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). Expression levels were normalized against the endogenous snRNA U6 control. For mRNA analyses, cDNA was synthesized using Moloney murine

leukaemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed with SYBR Premix ExTaq with the Stratagene Mx3000P real-time PCR system. GAPDH was used as internal controls for mRNA quantification. The relative expression ratio of miRNA and mRNA was calculated by the $2^{-\Delta\Delta CT}$ method. PCR reactions for each gene were repeated three times. Independent experiments were done in triplicate.

Cell viability assay

The miR-transfected PC-12 cells were seeded in 96-well plate with 5000 cells/well, and then treated by 300 μ M H₂O₂ for 4 hours. Cells were then cultured in fresh medium for 1–4 days at 37°C, and 20 μ l Cell Counting Kit-8 solution (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD) was added to the culture medium. The cultures were incubated for 1 hour at 37°C in humidified 95% air and 5% CO₂. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Flow cytometry

Apoptosis assay was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). PC-12 cells (100,000 cells/well) were seeded in 6 well-plate. After miR-transfection and 4 hours of treatment with 300 μ M H₂O₂, cells were washed twice with cold PBS and resuspended in Annexin-binding buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter Life Sciences, Indianapolis, IN, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

ROS assay

ROS was measured by flow cytometry using 2, 7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, Nanjing, China). The cells were seeded into a 6-well cells were washed twice with PBS and co-incubated with serum-free culture medium containing 10 μ M DCFH-DA (20 minutes, 37°C, in dark). Subsequently, the cells were washed with PBS, a trypsin digestion method was used for sample collection. All samples were centrifuged and the supernatants were removed. The cells were resuspended to 500 μ l PBS and the fluorescent intensities were measured using a flow cytometer (488 nm excitation, 521 nm emission).

Western blot

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 hour at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μ l Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean $\pm$ SD. Statistical analyses were performed using Graphpad statistical software (Graphpad Inc, La Jolla, CA, USA). The P-values were calculated using one-way analysis of variance (ANOVA) or two-way ANOVA. P-value of < 0.05 was considered to indicate a statistically significant result.

Results

miR-25 was low expressed in SCI model

At 6 hours, 12 hours, 1 day, 3 day and 7 day after SCI injury in mice, the expression level of miR-25 in spinal cords detected by qRT-PCR analysis. We found that, the expression of miR-25 in the injured spinal cords was much lower than in the control group (spinal cords without SCI injury at 12 hours post-laminectomy) ($P < 0.05$, $P < 0.01$, or $P < 0.001$, Fig. 1). These data indicated miR-25 was low expressed following SCI injury, which suggesting miR-25 may play a significant role in SCI injury.

H₂O₂ induced PC-12 cells injury

Then we detected the functional effects of miR-25 on the *in vitro* model of SCI. Firstly, various concentrations of H₂O₂ (0, 100, 200, 300, 400, and 500 μ M) was used to treat PC-12 cells for 4 hours. We found that, exposure

Figure 1 miR-25 was low expressed in SCI model. SCI model was established in mice, and then the expression of miR-25 in spinal cords was measured by qRT-PCR at 6 h, 12 h, 1 day, 3 day and 7 day post-surgery. The expression of miR-25 in spinal cords without SCI injury after 12 h of laminectomy was used as control. $n = 6$. mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control group.

to 100 μ M concentration of H₂O₂ did not significantly decrease viability of the PC-12 cells in comparison to control group of cells (PC-12 cells with no exposure to H₂O₂). However, subsequent exposure of PC-12 cells to increasing concentrations of H₂O₂ revealed significant decrease in cell viability ($P < 0.01$ at 200 μ M and 300 μ M H₂O₂, and $P < 0.001$ at 400 μ M and 500 μ M H₂O₂) (Fig. 2). The concentration of 300 μ M H₂O₂ was used in the following experiments.

Overexpression of miR-25 improved PC-12 cells viability and reduced apoptosis

The expression of miR-25 in PC-12 cells was altered by transfection with miR-25 expressing vectors and ASO of miR-25. qRT-PCR analysis results showed that, the expression of miR-25 was significantly overexpressed and suppressed following transfection (both $P < 0.01$, Figs. 3A and 3B).

Figure 2 H₂O₂ induced PC-12 cells injury. PC-12 cells were exposed to different concentrations of H₂O₂ (0–500 μ M) for 4 hours, thereafter cell viability was measured for evaluation cell oxidative damage. $n = 3$. mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$ vs. cells without H₂O₂ treatment.

The effects of miR-25 expression on H₂O₂-diminished cell viability were detected in PC-12 cells. We found that viability of PC-12 cells was significantly decreased after H₂O₂ exposure ($P < 0.001$). The viability of H₂O₂ + miR-25 group of cells was much higher than the H₂O₂ + scramble group of cells ($P < 0.01$, Fig. 3C), while cell viability of H₂O₂ + ASO-miR-25 group was lower than the H₂O₂ + ASO-NC group ($P < 0.05$, Fig. 3D).

The functional impacts of miR-25 on H₂O₂-induced apoptosis were then evaluated by detection of apoptotic cells rate and the expression changes of apoptosis-associated proteins following transfection. As shown in Figs. 3E and 3F, the percentage of apoptotic cells was significantly decreased following H₂O₂ exposure ($P < 0.001$). Apoptotic cells rate was decreased in H₂O₂ + miR-25 group in comparison to scramble cells treated with H₂O₂ (H₂O₂ + scramble) ($P < 0.01$, Fig. 3E). Of contrast, apoptotic cells rate was significantly increased in H₂O₂ + ASO-miR-25 group when compared to H₂O₂ + ASO-NC ($P < 0.01$, Fig. 3F). Furthermore, western blot revealed that in H₂O₂ + miR-25 group of cells the levels of p53, cytochrome c, and cleaved caspase-3 were lower and the level of Bcl-2 was higher in comparison to the H₂O₂ + scramble cells (Fig. 3G). As expected, p53, cytochrome c, and cleaved caspase-3 were up-regulated, while Bcl-2 was down-regulated in H₂O₂ + ASO-miR-25 group of cells compared to H₂O₂ + ASO-NC group (Fig. 3H). These data suggested that miR-25 overexpression protects PC-12 cells against H₂O₂-induced apoptosis.

Overexpression of miR-25 decreased the level of ROS, HIF- α and γ H2A

Given that studies have reported that apoptosis is modulated not only by ROS, but also by HIF- α (24, 25), we detected the ROS activity and HIF- α level after miR-25 transfection and H₂O₂ exposure. As results given in Figs. 4A and 4B, both ROS activity and HIF- α level was significantly increased after H₂O₂ exposure (both $P < 0.001$). More importantly, both of them are reduced almost to the normal level (without treatment) after the miR-25 expression was overexpressed in presence of H₂O₂ in comparison to H₂O₂ + scramble group (both $P < 0.01$). However, ROS activity and HIF- α level were significantly higher in H₂O₂ + ASO-miR-25 group in comparison to the H₂O₂ + ASO-NC group (both $P < 0.05$). Furthermore, western blot analysis was performed to assess the expression changes in the DNA damage marker γ H2A. As results given in Fig. 4C, the expression of γ H2A was

Figure 3 Overexpression of miR-25 improved cell viability and reduced apoptosis. (A) and (B) The expressions of miR-25 in PC-12 cells were overexpressed or suppressed by transfection, and then transfection efficiency was verified using qRT-PCR. $n = 3$. mean $\pm$ SD. ** $P < 0.01$ vs. Scramble group or ASO-NC group. After miR-transfection, PC-12 cells were exposed to 300 μ M H₂O₂ for 4 h. (C) and (D) Following another incubation in fresh medium for 1–4 days, cell viability was measured by CCK-8. $n = 3$. mean $\pm$ SD. ### $P < 0.001$ vs. control group; * $P < 0.05$, and ** $P < 0.01$ vs. H₂O₂ + ASO-NC group and H₂O₂ + Scramble. (E) and (F) apoptotic cells rate were measured by flow cytometry detection. $n = 3$. mean $\pm$ SD. ** $P < 0.01$, and *** $P < 0.001$. (G) and (H) The expression levels of apoptosis-associated proteins were detected by western blot analysis.

remarkably induced by H₂O₂, implying DNA was damaged after H₂O₂ exposure in PC-12 cells. Lower amount of γ H2A was found in H₂O₂ + miR-25 group

in comparison to the H₂O₂ + scramble; higher level was found in H₂O₂ + ASO-miR-25 group when compared to the H₂O₂ + ASO-NC group.

Figure 4 Overexpression of miR-25 decreased the level of ROS, HIF- α and γ H2A. After miR-transfection, PC-12 cells were exposed to 300 μ M H₂O₂ for 4 h. Thereafter, (A) and (B) the mean fluorescence intensity of ROS, (C) and the mRNA level of HIF- α were respectively measured flow cytometry and qRT-PCR. $n = 3$. mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (C) The protein expression of γ H2A was detected by western blot analysis.

Overexpression of miR-25 decreased inflammatory response in PC-12 cells

Next, we asked whether miR-25 expression could modulate H₂O₂-induced inflammatory response in PC-12 cells. Western blot analysis results showed that, the expression levels of IL-1 β , TNF- α , IL-6, and MCP-1 were all up-regulated after H₂O₂ exposure, indicating inflammatory response was induced by H₂O₂. Significant decreases of these factors were found in H₂O₂ + miR-25 group of cells in comparison to the H₂O₂ + scramble cells. Meanwhile, cells in H₂O₂ + ASO-miR-25 group were found to express the maximum amount of these inflammatory cytokines in comparison to other group of cells (Fig. 5).

Figure 5 Overexpression of miR-25 decreased inflammatory response in PC-12 cells. After miR-transfection, PC-12 cells were exposed to 300 μ M H₂O₂ for 4 hours. The expressions of inflammatory mediators, including IL-1 β , TNF- α , IL-6, and MCP-1 in PC cells were detected by western blot analysis.

miR-25 protected PC-12 cells from oxidative damage via up-regulating Nrf2

It is well-established that, Nrf2 is a critical transcription factor in protecting cell from oxidative damage (26). Based on this reason, we detected the regulation between miR-25 and Nrf2, in order to further reveal the underlying mechanism(s) via which miR-25 protected PC-12 cells from H₂O₂-induced damage. As shown in Figs. 6A-6C, both the mRNA and protein levels of Nrf2 was up-regulated by miR-25 overexpression, while was down-regulated by miR-25 knockdown ($P < 0.05$), indicating a positive correlation between the expression of miR-25 and Nrf2. Then, we silenced Nrf2 in PC-12 cells to evaluate the impacts of Nrf2 silencing on the protective functions of miR-25. qRT-PCR and western blot analyses results showed that, the expressions of Nrf2 were significantly reduced after transfection with Nrf2 siRNA ($P < 0.01$, Fig. 6D and 6E). The percentage of apoptotic cells was significantly higher in H₂O₂ + miR-25 + si-Nrf2 group when compared to the group of H₂O₂ + miR-25 ($P < 0.01$, Fig. 6F), indicating the protective role of miR-25 in H₂O₂-induced damage was abolished by Nrf2 silence. These above mentioned findings were further supported by the fact that the expressions of p53, cytochrome c and cleaved caspase-3 were increased, while Bcl-2 was decreased in H₂O₂ + miR-25 + si-Nrf2 group when compared to the group of H₂O₂ + miR-25 (Fig. 6G).

Figure 6 miR-25 protected PC-12 cells from oxidative damage via up-regulating Nrf2. (A)–(C) PC-12 cells were transfected with either miR-25 expressing vector or ASO-miR-25, and then the mRNA and protein levels of Nrf2 were monitored by qRT-PCR and western blot analysis. (D) and (E) PC-12 cells were transfected with Nrf2 targeted siRNA, and then the mRNA and protein levels of Nrf2 were measured by qRT-PCR and western blot analysis. PC-12 cells were co-transfected with miR-25 expressing vector and Nrf2 siRNA, and exposed to 300 μ M H₂O₂ for 4 hours. (F) Apoptotic cells rate was assessed by flow cytometry. $n = 3$. mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (G) The protein expressions of apoptosis-associated factors were detected by western blot analysis.

miR-25 regulated Nrf2 expression via Wnt/ β -catenin and PI3 K/AKT/ERK signaling

To further detect the molecular mechanism(s) in which miR-25 modulated PC-12 cells, we focused on Wnt/ β -catenin and PI3 K/AKT/ERK pathway signaling. As shown in Fig. 7A, Wnt3a, Wnt5a, β -catenin, Nrf2 were all up-regulated in PC-12 cells with overexpression of miR-25, while were down-regulated with miR-25 suppression. Following addition Wnt pathway inhibitor ICG001 to the miR-25 overexpressing-cells, the expression of these four proteins were dramatically reduced, suggesting

miR-25 activated Wnt/ β -catenin pathway was blocked by ICG001 (Fig. 7B). In addition, activation of PI3 K, AKT, and ERK1/2 were found in miR-25 overexpressing-cells, and deactivation of these proteins were observed in miR-25 suppressing-cells (Fig. 7C). Altogether, these findings suggested miR-25 regulated Nrf2 expression possibly via Wnt/ β -catenin and PI3 K/AKT/ERK signaling.

Discussion

In the present study, we demonstrated that miR-25 was low expressed following SCI injury in mice. miR-25

Figure 7 miR-25 regulated Nrf2 expression via Wnt/ β -catenin and PI3 K/AKT/ERK signaling. (A) PC-12 cells were transfected with miR-25 expressing vector or ASO-miR-25, the expression of Nrf2, Wnt3a, Wnt5a, and β -catenin were detected by western blotting. (B) PC-12 cells were transfected with miR-25 expressing vector and were treated with 10 μ M ICG001 for 12 hours, and then Nrf2 and main factors in Wnt pathway were detected again. (C) PC-12 cells were transfected with miR-25 expressing vector or ASO-miR-25, the expression of p-PI3 K, PI3 K, p-AKT, AKT, p-ERK1/2, and ERK1/2 were detected by western blot analysis.

overexpression protected PC-12 cells against H₂O₂-induced oxidative damage, as evidenced by significant decrease in the percentage of apoptotic cells and increase in cell viability. Overexpression of miR-25 protected PC-12 cells against inflammatory and oxidative damage, as evidenced by significant down-regulations of IL-1, TNF- α , IL-6, and MCP-1 expression, and decreases in ROS, HIF- α , and γ H2A level. The expression of Nrf2 was positively regulated by miR-25, and Nrf2 silence could abolish the protective functions of miR-25 on H₂O₂-induced oxidative damage. Furthermore, we found that Wnt/ β -catenin and PI3 K/AKT/ERK signaling were activated by miR-25 overexpression.

Following exposure of PC-12 cells to increasing concentrations of H₂O₂, the viability of these cells significantly decreased at a rate directly proportional to the concentration of H₂O₂. The damaging effects of H₂O₂ on PC-12 cells were also established in other studies.²⁷ To date, several miRs have been implicated in H₂O₂ damaged neural and neural-like cells. For instance, anti-sense-miR-146a reversed the decrease of both the SOD2 (endogenous antioxidant enzyme) level and cell viability in H₂O₂ treated PC-12 cells.²⁸ Down-regulation of miR-100 effectively protected H₂O₂-induced apoptosis in RGC-5 cells via regulation of IGF1R.²⁹ However, our

study provided the first evidence that miR-25 was also implicated in H₂O₂-induced neural-like cells damage. Overexpression of miR-25 improved PC-12 cells viability and reduced apoptotic cells rate following H₂O₂ exposure. Together with our findings that miR-25 was low expressed following SCI injury in mice, we inferred that miR-25 may have protective potentials during SCI.

Previous study has described the protective role of miR-25 against oxidative damage in cardiomyocytes.³⁰ Similarly, we found out that overexpression of miR-25 protected PC-12 cells against oxidative damage by fall in ROS, and TNF- α , which indicating miR-25 suppressed apoptosis by modulating both ROS and HIF- α . Additionally, DNA damage was alleviated after miR-25 was overexpressed, as evidenced by decrease in the level of γ H2A, a biomarker for DNA damage.

In the current study, we also found out that overexpression of miR-25 in PC cells decreased the expression of inflammatory mediators, including IL-1 β , TNF- α , IL-6, and MCP-1. Kuhn *et al.* explored the role of miR-25 in human airway smooth muscles; similar to our finding, they also concluded that overexpression of miR-25 suppresses expression inflammatory mediators like TNF- α .³¹ Together with this previous study, our findings suggested miR-25 could alter the expressions

of inflammatory factors, which might be involved in the protective role of miR-25 in H₂O₂-induced damage in PC-12 cells.

The role of Nrf2 is well-established in human genome, and it acts as an important regulator in the reactions of biotransformation, maintenance of redox potential, and metabolism.^{32–34} The protective role of Nrf2 against inflammation in various experimental models has already been established. Mao *et al.* have demonstrated that protective role of Nrf2 against inflammatory damage after SCI in mice model and thus have suggested the potential of Nrf2 in management of SCI.³⁵ In our study we found that Nrf2 in PC-12 cells was highly expressed after miR-25 was overexpressed, suggesting the stimulatory role of miR-25 on expression of Nrf2. This fact provides us with a new perspective that the protective role of miR-25 in H₂O₂-induced damage possibly via regulation of Nrf2. To confirm this hypothesis, we knocked down the expression of Nrf2 in PC-12 cells by transfection with its specific siRNA, and we found that Nrf2 silencing significantly abolished miR-25 overexpressing-induced decrease in cell apoptosis. Also knockdown of Nrf2 expression increased expression of apoptotic factors and decreased expression of anti-apoptotic factors. These findings confirmed the anti-apoptotic role of Nrf2, and provided the evidence that miR-25 suppresses apoptosis via increased expression of Nrf2.

The role of Wnt/ β -catenin signaling pathway in development of neurons, relief of neuropathic pain and survival of neurons, has already been established in various studies.^{36,37} Strand *et al.* had established that Wnt signaling pathway stimulates regeneration of spinal cord following SCI in zebrafish.³⁸ Again Kai Gao *et al.*, have established that rapamycin acts as a neuroprotective agent in SCI through activation of Wnt pathway.³⁹ In our study we have also established that expressions of different factors associated with Wnt/ β -catenin pathway were increased in miR-25 overexpressing-cells; however addition of Wnt/ β -catenin pathway inhibitor ICG001 led to decreased expression of those factors. In addition to Wnt/ β -catenin, we also focused on PI3 K/AKT/ERK signaling, in order to further explore the molecular mechanisms via which miR-25 protects PC-12 cells against H₂O₂-induced oxidative damage. Previous studies have reported that PI3 K/AKT/ERK signaling is in central sensitization induced by various stimuli,⁴⁰ and it is implicated in neuroprotective functions against SCI.⁴¹ In the current study, PI3 K, AKT and ERK1/2 were all phosphorylated by miR-25 overexpression in PC-12 cells. Altogether, our findings suggested the protective

effects of miR-25 on PC-12 cells possibly via regulation of Nrf2, and thus activation of Wnt/ β -catenin and PI3 K/AKT/ERK signaling.

In conclusion, we demonstrated that miR-25 plays a protective role in H₂O₂-induced oxidative damage in PC-12 cells possibly via regulation of Nrf2 and activation of Wnt/ β -catenin and PI3 K/AKT/ERK signaling. These findings will provide evidence that modulation of miR-25 may be a viable option for SCI treatment.

Declaration of interest

The authors report no declarations of interest.

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