

Lenalidomide improves H₂O₂-induced PC12 cell injury by blocking the Notch signaling pathway

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Abstract. Lenalidomide (LEN) has been reported to exert antitumor, anti-inflammatory, anti-angiogenic and immunomodulatory activities. However, the effects of LEN in spinal cord injury (SCI) are yet to be fully elucidated. The present study was conducted to identify whether LEN has a healing effect on SCI and to determine the underlying mechanism of action. The viability of H₂O₂-stimulated PC12 cells was detected using a Cell Counting Kit-8 assay. The viability of PC12 cells treated with LEN was examined with an MTT assay. The level of lactate dehydrogenase (LDH) was measured using an LDH assay kit, while the levels of the oxidative stress-related factors malondialdehyde, superoxide dismutase, glutathione peroxidase and catalase in PC12 cells were determined using commercial kits. Oxidative stress-related proteins were examined via western blotting. PC12 cell apoptosis was observed using a TUNEL assay, while apoptosis markers and Notch signaling-related proteins were examined via western blotting. The results of the present study demonstrated that H₂O₂ decreased PC12 cell viability in a dose-dependent manner. However, treatment with LEN significantly improved the viability of H₂O₂-stimulated PC12 cells and alleviated H₂O₂-induced cytotoxic injury. Additionally, LEN treatment inhibited the oxidative stress and apoptosis induced by H₂O₂ in PC12 cells. Notch signaling-related protein expression was downregulated after LEN treatment in H₂O₂-stimulated PC12 cells. In addition, a Notch agonist reversed the effects of LEN on H₂O₂-induced oxidative stress and PC12 cell apoptosis. In summary, it was demonstrated that LEN alleviated the H₂O₂-induced injury of PC12 cells by blocking the Notch signaling pathway, suggesting the value of applying LEN to the treatment of SCI.

Introduction

Spinal cord injury (SCI) is a common and destructive injury caused by external forces. SCI refers to fracture and/or severe complications following dislocation of the spinal cord (1). SCI is known to be mostly caused by road traffic accidents, falls and sports-related accidents, and could further lead to sensory disturbance, motor dysfunction and lower extremity paralysis (2-4). As a result of the unremitting efforts made by global researchers and the successful translation into clinical practice, recovery from SCI through rehabilitation therapies and orthopedic appliances is now possible for mild cases, whereas patients with severe cases continue to risk disability and even death (5,6). Surgical techniques, biological therapies such as stem cell and precursor cell transplantation, and pharmacological therapies such as methylprednisolone (MP) and gangliosides have been approved for the treatment of SCI; however, clinically significant effects have not been reported (7,8). Thus, there is an extremely urgent need to find new therapies for SCI and secondary injury following SCI.

Lenalidomide (LEN), chemically known as (RS)-3-(4-amino-1-oxoisindolin-2-yl)piperidine-2,6-dione (molecular formula, C₁₃H₁₃N₃O₃), exerts antitumor, anti-inflammatory, anti-angiogenic and immunomodulatory effects (9,10). LEN also improves the motor behavior defect caused by the striatum and ameliorates dopaminergic fiber loss. This protective effect is accompanied by a decrease in microgliosis in the striatum and hippocampus and reduction in NF-κB activity in Parkinson's disease (11). LEN has also shown other protective potential, such as exerting anti-inflammatory and neuroprotective effects in a G93A mutant superoxide dismutase (SOD)-1 mouse model of amyotrophic lateral sclerosis (12). The anti-inflammatory effect of LEN is exerted via the regulation of cytokine production by human myeloid-derived primary dendritic cells, which allows for beneficial immunoregulation and is therefore facilitative for treating inflammation-related diseases, including multiple myeloma (MM) (13). LEN inhibits the downstream genes of Notch2 signaling transduction, including recombination signal binding protein for immunoglobulin κ J region (also called CSL or CBF1) and hes family bHLH transcription factor 1 (HES1). Under LEN treatment, Notch2 signaling combines with the expression of multiple drug-resistance proteins to modulate the inhibitory effect on cancer cell proliferation (14).

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A previous study reported a significant decline in the expression levels of Notch signaling molecules, including the receptors, the ligands and the downstream cytokines, after treatment with LEN in mesenchymal stem cells (MSCs) from patients with MM (MM-MSCs). It was concluded that treatment with LEN inactivated Notch signaling to restore the osteogenic differentiation of MM-MSCs (15). These previous studies have collectively confirmed the interaction between LEN and Notch signaling, which may play a role in the clinical treatment for SCI.

The present study aimed to verify this hypothesis and the results may contribute to the exploration of the therapeutic potentials of LEN other than for treating MM.

Materials and methods

Cell culture and treatment. The rat adrenal pheochromocytoma cell line, PC12, acquired from the American Type Culture Collection (ATCC), was cultured in ATCC-formulated RPMI-1640 medium (cat. no. 30-2001) supplemented with 10% heat-inactivated horse serum and 5% FBS (all Gibco; Thermo Fisher Scientific, Inc.) in an environment of 95% air and 5% CO₂ at 37°C. The cells were pretreated for 24 h with LEN (cat. no. EY0006; AMQUAR Corporation) dissolved in DMSO (Shanghai Aladdin Biochemical Technology Co., Ltd.) at a dose of 1.25, 2.5, 5 and 10 μM. H₂O₂ at a dose of 100, 200, 300 and 400 μM was selected to treat PC12 cells for 1 h after LEN treatment. Cells in the JAG + LEN + H₂O₂ group were pre-treated with 50 μg/ml Notch agonist Jagged-1 (JAG; cat. no. AS-61298; AnaSpec) peptide before treatment with LEN and H₂O₂. Untreated cells were regarded as the control group.

Cell Counting Kit-8 (CCK-8) assay. The viability of H₂O₂-stimulated PC12 cells was detected using a CCK-8 Cell Proliferation and Cytotoxicity assay (Beijing Solarbio Science & Technology Co., Ltd.). In total, 100 μl cell suspension was added to a 96-well plate at a density of 5x10³ cells/well and precultured in an incubator at 37°C in 5% CO₂. Subsequently, 10 μl CCK-8 solution was added to each well and incubated with the cells for 4 h. A microplate reader (RT-3001; Thermo Fisher Scientific, Inc.) was used to measure the absorbance at a wavelength of 450 nm.

MTT assay. PC12 cells in the logarithmic growth phase were collected, and 100 μl cell suspension was added to a 96-well plate at a density of 5x10³ cells/well. The cells were treated with a concentration gradient of LEN after the formation of the cell monolayer on the bottom of the well. Following 48 h of incubation with LEN in 5% CO₂ at 37°C, 20 μl MTT solution (Procell Life Science & Technology Co., Ltd.) was added to each well and the incubation was continued for 4 h. The incubation was then terminated, and the culture medium was discarded before the addition of 150 μl DMSO to each well. The plate was vibrated using a shaking bed for 10 min at low speed to fully dissolve the crystalized substances. The optical density was measured at a wavelength of 490 nm using a universal microplate spectrophotometer.

Lactate dehydrogenase (LDH) cytotoxicity assay. Cell cytotoxicity was measured by an LDH assay using the LDH

kit (cat. no. A020-1-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols. Briefly, the H₂O₂-stimulated cells were seeded into a 96-well plate at a density of 1x10⁴ cells/well and received the appropriate treatments of incubation with LEN at doses of 1.25, 2.5, 5 and 10 μM for 24 h at 37°C. Next, the cell culture media was collected and the LDH activity was measured at 530 nm using a microplate reader (Benchmark; Bio-Rad Laboratories, Inc.).

Determination of oxidative stress level. Oxidative stress in PC12 cells was reflected by the levels of malondialdehyde (MDA), SOD, glutathione peroxidase (GSH-Px) and catalase (CAT), as detected using a Lipid Peroxidation MDA Assay kit (cat. no. S0131S; Beyotime Institute of Biotechnology), a SOD Activity Assay kit (cat. no. ab65354; Abcam), GSH-Px Assay kit (cat. no. EKC39116; BioVision, Inc.) and a CAT Assay kit Without Hydrogen Peroxide (cat. no. 700910; Cayman Chemical Company), respectively. All operational procedures followed the manufacturer's protocols.

Western blot analysis. Total protein was extracted from PC12 cells using RIPA lysis buffer (Absin Bioscience, Inc.). The protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology). Complete denaturation of the samples was performed by incubating the protein in boiling water for 5 min. After electrophoresis using 10% SDS-PAGE (Beijing Solarbio Science & Technology Co., Ltd.), the protein samples (30 μg per lane) were transferred to a PVDF membrane (Corning, Inc.). Subsequently, 5% skimmed milk (Absin Bioscience, Inc.) was used to block the membrane for 2 h at 25°C, followed by incubation with primary antibodies targeting NADPH oxidase (Nox)2 (1:5,000; cat. no. ab129068; Abcam), Nox4 (1:1,000; cat. no. ab133303; Abcam), Bcl-2 (1:1,000; cat. no. ab196495; Abcam), Bax (1:1,000; cat. no. 2772; Cell Signaling Technology, Inc.), caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), cleaved caspase-3 (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), caspase-9 (1:1,000; cat. no. ab184786; Abcam), cleaved caspase-9 (1:1,000; cat. no. ab2324; Abcam), Notch2 (1:1,000; cat. no. 5732; Cell Signaling Technology, Inc.), HES-related family bHLH transcription factor with YRPW motif 1 (Hey1; 1:1,000; cat. no. ab154077; Abcam), HES1 (1:1,000; cat. no. #11988; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. ab8245; Abcam) at 4°C overnight. After the membrane was washed with 0.5% TBST, it was incubated with horseradish peroxidase-labeled secondary antibodies [goat anti-rabbit IgG (1:2,000; cat. no. 7074) or horse anti-mouse IgG (1:2,000; cat. no. 7076); both Cell Signaling Technology, Inc.] at room temperature for 2 h. An ECL kit (Beyotime Institute of Biotechnology) was used to visualize the protein bands and ImageJ software (v6; National Institutes of Health) was used to analyze the protein bands.

TUNEL assay. A colorimetric TUNEL Apoptosis Assay kit (cat. no. C1088; Beyotime Institute of Biotechnology) was utilized to observe PC12 cell apoptosis. The cells (5x10⁵/well) seeded into a 24-well plate were fixed with 4% paraformaldehyde (Shanghai Macklin Biochemical Co., Ltd.) at room temperature for 30 min, and incubated with Enhanced Immunostaining Permeabilization Buffer (Beyotime Institute

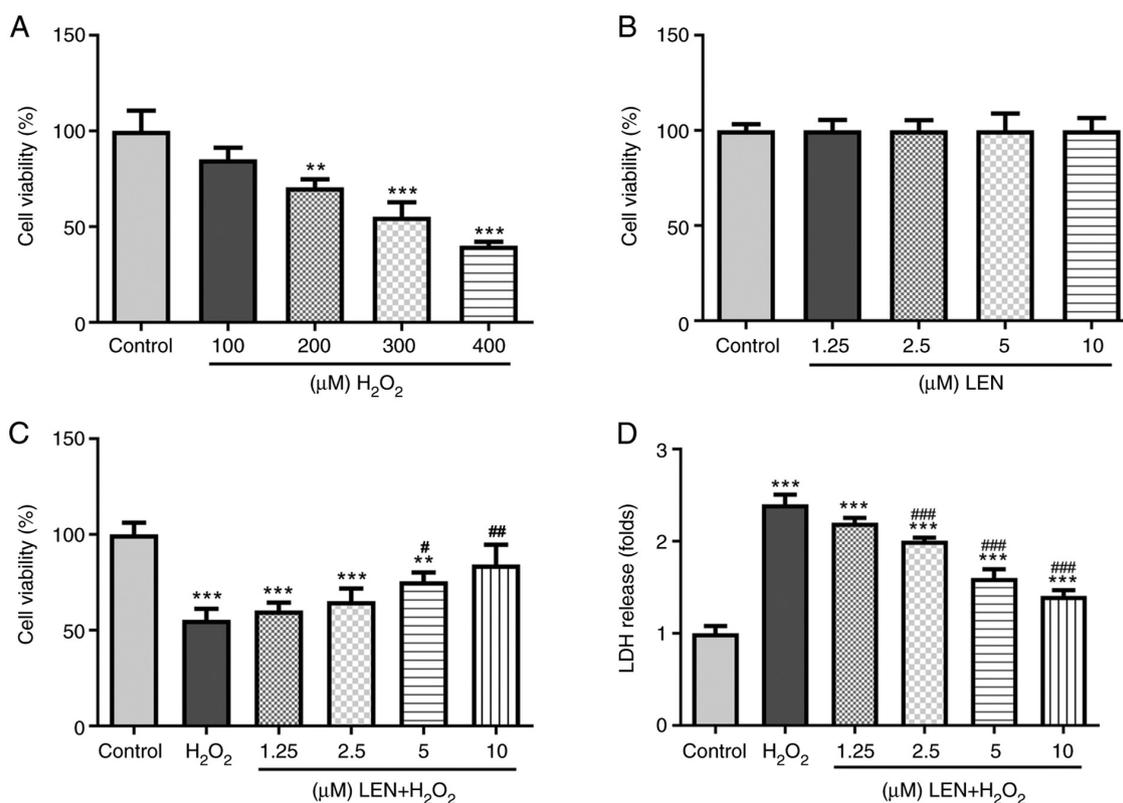


Figure 1. Effects of LEN on the viability of H₂O₂-stimulated PC12 cells. (A) The viability of PC12 cells after treatment with H₂O₂, detected by Cell Counting Kit-8. (B) The viability of PC12 cells after treatment with LEN, detected by MTT. (C) The viability of H₂O₂-stimulated PC12 cells after treatment with LEN at different doses, detected by MTT. (D) The LDH level in H₂O₂-stimulated PC12 cells after treatment with LEN in different doses, detected by LDH assay. **P<0.01 and ***P<0.001 vs. control; *P<0.05, #P<0.01 and ###P<0.001 vs. H₂O₂. LEN, lenalidomide; LDH, lactate dehydrogenase.

of Biotechnology) at room temperature for 5 min. After incubation with 0.3% H₂O₂ in PBS (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min, 50 μl biotin-dUTP was added to label the samples for 1 h at 37°C in the dark. To develop the colors, the samples were then incubated with 50 μl streptavidin-HRP for 30 min at 37°C, followed by incubation in 0.5 ml DAB solution for another 30 min at 37°C and the anti-fluorescence quencher was added dropwise for mounting. The coloration was observed microscopically from three random fields of view using a fluorescence microscope (magnification, x200; Olympus Corporation).

Statistical analysis. The data were analyzed and graphs were generated using GraphPad Prism 6 software (GraphPad Software, Inc.). Data are presented as the mean ± SD. Differences among multiple groups were analyzed using one-way ANOVA with a post hoc Bonferroni multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

LEN increases the viability of H₂O₂-stimulated PC12 cells. The SCI model was established by treating PC12 cells with H₂O₂. The viability of PC12 cells showed a declining trend as the dose of H₂O₂ was increased from 100 to 400 μM (Fig. 1A). According to a previous experiment (16), 300 μM was selected as the final dose of H₂O₂ for the SCI model in view of the

appropriate degree of cell death under this dose. Additionally, the viability of PC12 cells treated with different doses of LEN revealed no significant difference compared with the control group (Fig. 1B), indicating the non-cytotoxicity of LEN. After pre-treatment with increasing doses of LEN, the viability of H₂O₂-stimulated PC12 cells was noticeably improved (Fig. 1C). In addition, the level of LDH in H₂O₂-stimulated PC12 cells was notably decreased by LEN in a dose-dependent manner (Fig. 1D). Thus, these results indicated that treatment with LEN could revitalize H₂O₂-stimulated PC12 cells.

LEN inhibits the oxidative stress level in H₂O₂-stimulated PC12 cells. By examining the production of MDA, SOD, GSH-Px and CAT, the present study investigated the effect of LEN on H₂O₂-induced oxidative stress in PC12 cells. It was identified that, while H₂O₂ induced an increased production of MDA, this was notably decreased after pre-treatment with LEN in PC12 cells (Fig. 2A). Conversely, H₂O₂-induced suppression of SOD, GSH-Px and CAT production was attenuated in a dose-dependent manner by LEN (Fig. 2B-D). Furthermore, the oxidative stress-related proteins, Nox2 and Nox4, were examined and both were revealed to be upregulated under H₂O₂ stimulation, but downregulated by LEN treatment (Fig. 2E). These results suggested an inhibitory effect of LEN on H₂O₂-induced oxidative stress in PC12 cells.

LEN extenuates the apoptosis of H₂O₂-stimulated PC12 cells. H₂O₂-induced PC12 cell apoptosis was detected in the absence

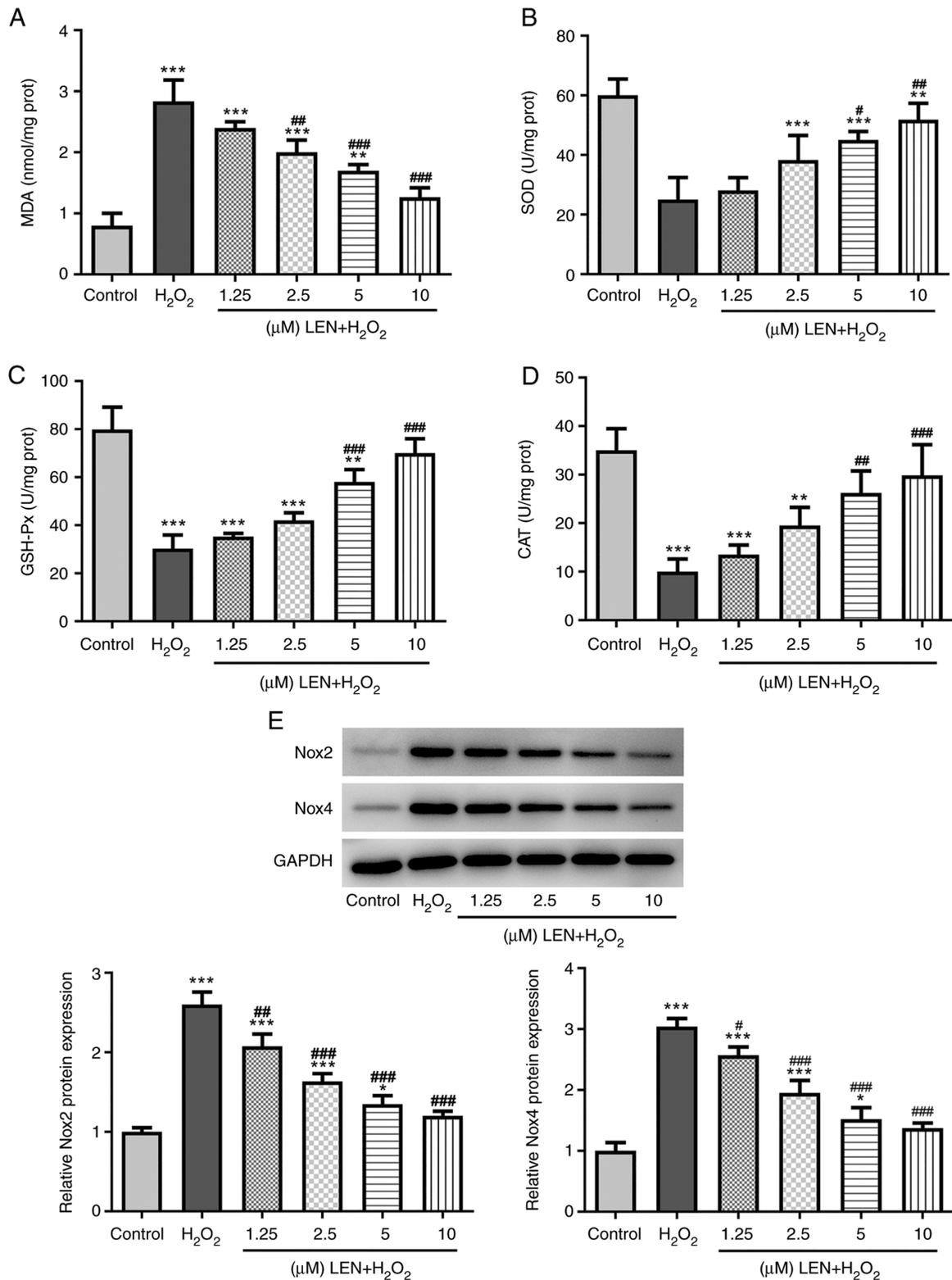


Figure 2. Effects of LEN on oxidative stress level in H₂O₂-stimulated PC12 cells. (A-D) The production of (A) MDA, (B) SOD, (C) GSH-Px and (D) CAT in H₂O₂-stimulated PC12 cells in the presence and absence of LEN at different doses, detected by corresponding commercial kits. (E) Relative protein expression of oxidative stress-related Nox2 and Nox4 in H₂O₂-stimulated PC12 cells in the presence and absence of LEN at different doses, detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. H₂O₂. LEN, lenalidomide; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; Nox, NADPH oxidase.

and presence of pre-treatment with LEN. The results of the TUNEL assay demonstrated a notable increase in the number of apoptotic cells (green fluorescence) in the H₂O₂ group, but

this was significantly decreased by pre-treatment with LEN (Fig. 3A and B). The expression levels of apoptosis-related proteins in PC12 cells were also detected (Fig. 3C), among

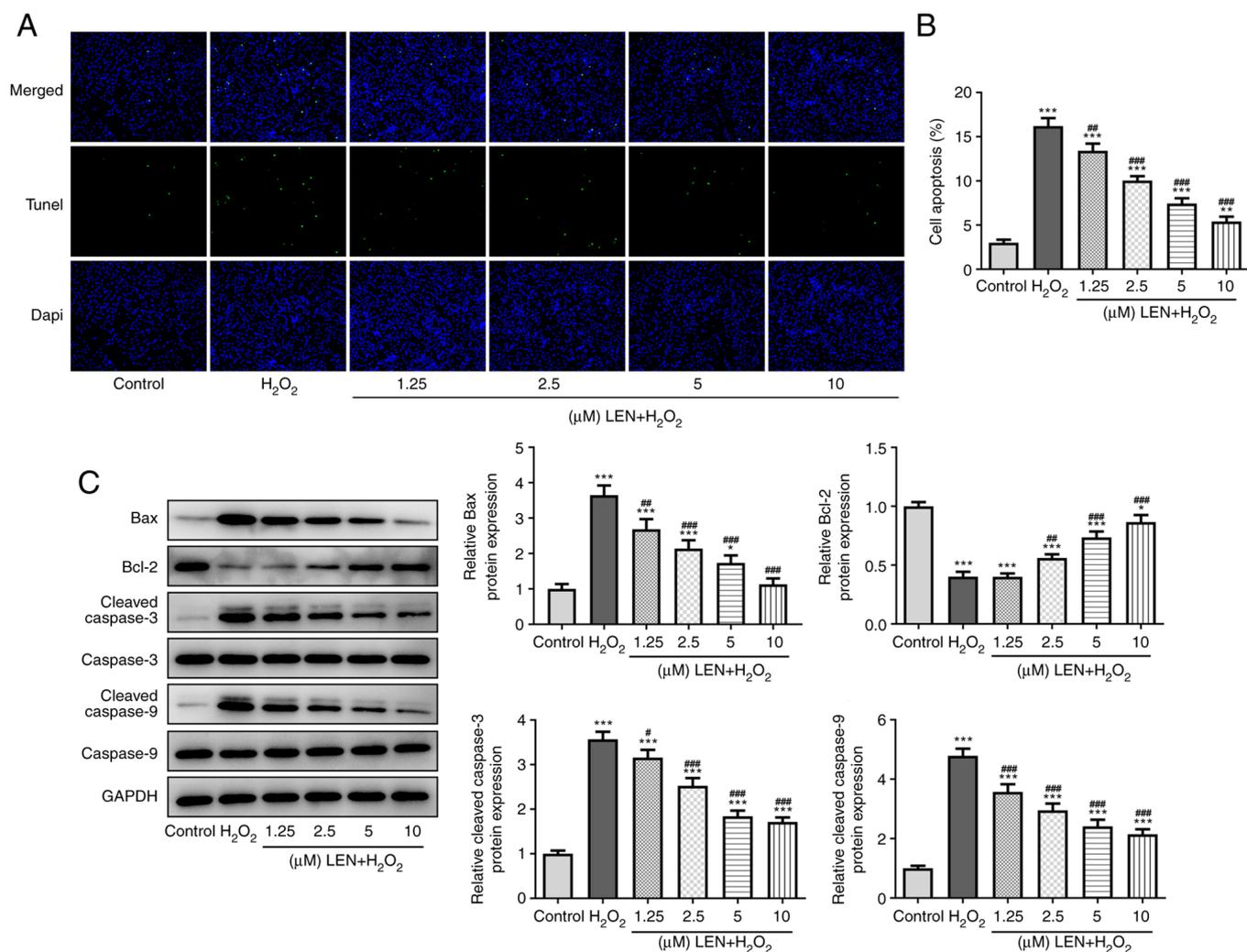


Figure 3. Effects of LEN on the apoptosis of H₂O₂-stimulated PC12 cells. (A and B) The apoptosis of H₂O₂-stimulated PC12 cells in the presence and absence of LEN at different doses, detected by TUNEL (magnification, x200). (C) Relative protein expression of apoptosis-related Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9 in H₂O₂-stimulated PC12 cells in the presence and absence of LEN at different doses, detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. H₂O₂. LEN, lenalidomide.

which the expression level of anti-apoptotic protein Bcl-2 was decreased by H₂O₂ and dose-dependently increased after LEN treatment, while Bax, cleaved caspase-3 and cleaved caspase-9 expression exhibited the opposite trend. These results indicated a suppressive effect of LEN on H₂O₂-induced PC12 cell apoptosis.

LEN blocks the Notch signaling pathway in H₂O₂-stimulated PC12 cells. Whether LEN interacts with the Notch signaling pathway was preliminarily examined by detecting related protein expression levels in H₂O₂-stimulated PC12 cells. It was identified that the expression levels of Notch2, Hey1 and HES1 were all significantly increased in H₂O₂-stimulated PC12 cells, but were gradually decreased along with the increasing doses of LEN (Fig. 4). These findings suggested that treatment with LEN may block the expression of the Notch signaling pathway in H₂O₂-stimulated PC12 cells.

LEN inhibits H₂O₂-induced oxidative stress and apoptosis of PC12 cells by blocking the Notch signaling pathway. To verify the involvement of Notch in the action mechanism

of LEN, rescue experiments were conducted by pre-treating the cells with the Notch agonist Jagged-1 (JAG) peptide at a concentration of 50 μg/ml, as previously described (17). In addition, 10 μM LEN was chosen for these subsequent procedures. As revealed in Fig. 5A, the level of MDA formerly downregulated by LEN in H₂O₂-stimulated PC12 cells was significantly upregulated by treatment with JAG. In addition, the levels of SOD, GSH-Px and CAT in LEN-treated H₂O₂-stimulated PC12 cells were significantly decreased by JAG (Fig. 5B-D). The expression levels of oxidative stress-related proteins in LEN-treated H₂O₂-stimulated PC12 cells were also rescued after pre-treatment with JAG, as both Nox2 and Nox4 exhibited re-upregulation (Fig. 5E). Similarly, reduced apoptosis of H₂O₂-stimulated PC12 cells by LEN was promoted after treatment with JAG (Fig. 5F and G), evidenced by the downregulation of Bcl-2 expression and the upregulation of Bax, cleaved caspase-3 and cleaved caspase-9 expression (Fig. 5H and I). Collectively, these results demonstrated that the Notch agonist could reverse the effects of LEN on the H₂O₂-induced oxidative stress and apoptosis of PC12 cells.

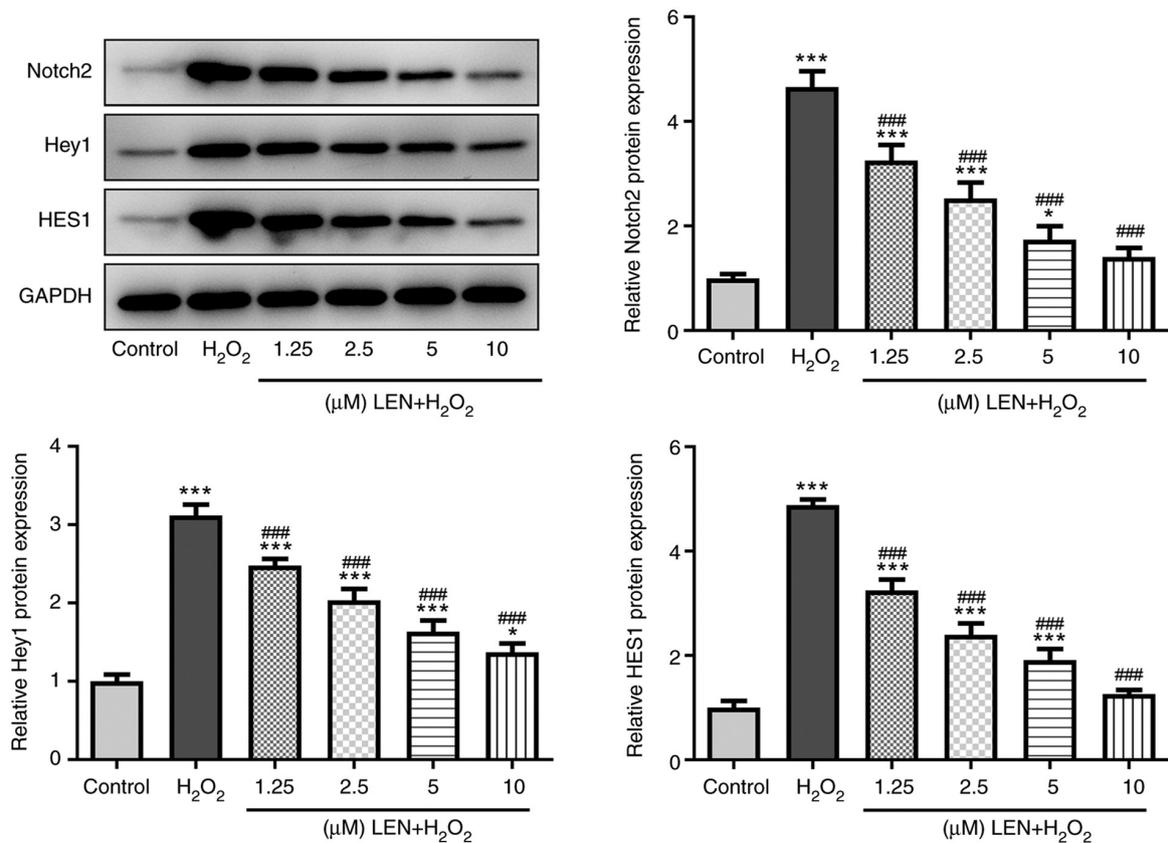


Figure 4. Effects of LEN on the Notch signaling pathway in H₂O₂-stimulated PC12 cells. Relative protein expression of Notch signaling pathway-related Notch2, Hey1 and HES1 in H₂O₂-stimulated PC12 cells in the presence and absence of LEN at different doses, detected by western blot analysis. *P<0.05 and ***P<0.001 vs. control; ###P<0.001 vs. H₂O₂. LEN, lenalidomide; Hey1, HES-related family BHLH transcription factor with YRPW motif 1; HES1, hes family BHLH transcription factor 1.

Discussion

The incidence of SCI has been continuously increasing with the world's economic development, which will not only bring serious physical and psychological harm to the patients themselves, but also result in significant financial burden to society (4,18). Hence, the prevention and treatment of SCI have become major issues in the medical field. In the present study, H₂O₂ was chosen for the modeling of SCI, considering its wide use in SCI models in previous studies (19,20), and the significantly decreased viability in H₂O₂-treated PC12 cells was confirmed.

LEN is a Food and Drug Administration-approved drug that has immunomodulatory, antitumor and anti-angiogenic activities, and is commonly used for the treatment of MM and myelodysplastic syndrome (21,22). However, little is known regarding the impact of LEN on other diseases, including SCI. PC12 cells pre-treated with LEN in the present study showed no indication of cytotoxicity, and their viability was significantly improved following stimulation with H₂O₂, compared with that in cells without LEN pre-treatment. The LDH level, reflecting cytotoxic injury, in H₂O₂-stimulated PC12 cells was also reduced by LEN. It was therefore suggested that LEN treatment benefits the survival of PC12 cells under the stimulation of H₂O₂.

Previous studies have reported that an elevated oxidative stress level characterizes the occurrence of SCI and is

considered to be a treatment target, with the overproduction of free radicals and lipid peroxidation found in damaged spinal neurons (23,24). LEN has been revealed to have a regulatory effect on inflammatory cytokines as well as certain stress signals (25,26). In addition, it has been reported that combined treatment with LEN and nanoceria produces a suppressive effect *in vivo* on central nervous system autoimmunity-induced inflammation and oxidative stress (27). In the present study, increased MDA and decreased SOD, GSH-Px and CAT production were observed in H₂O₂-stimulated PC12 cells. Pre-treatment with LEN effectively inhibited the production of MDA, while promoting that of the anti-oxidants SOD, GSH-Px and CAT. Consistently, the gene expression levels of the reactive oxygen species, Nox2 and Nox4, were comparatively higher in H₂O₂-stimulated PC12 cells in the absence of LEN and were significantly downregulated in the presence of LEN. These results suggested an anti-oxidative stress role of LEN in the model of SCI.

Inflammation and apoptosis occur in the secondary injury phase that follows the primary mechanical injury in SCI, further resulting in the dysfunction or damage of the central nervous system (28,29). Qu *et al* (30) revealed that LEN could restrict the formation of osteoclasts and protect osteocytes from IL-1 β -induced apoptosis in a mouse model of osteoarthritis. The present study observed a significantly increased number of apoptotic PC12 cells after treatment with H₂O₂, in addition to the downregulation of anti-apoptotic Bcl-2 protein

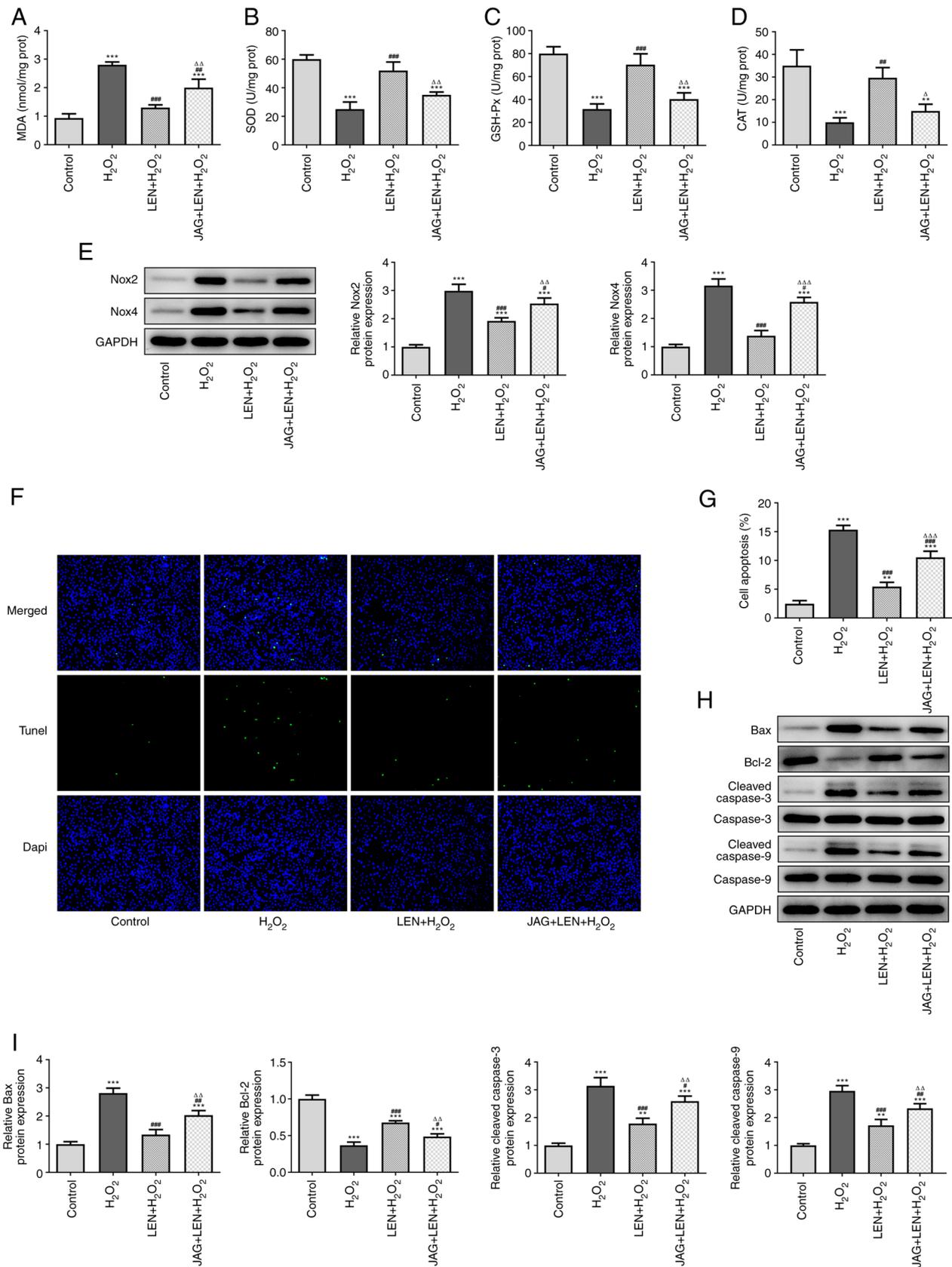


Figure 5. LEN suppresses H₂O₂-induced oxidative stress and apoptosis of PC12 cells by blocking the Notch signaling pathway. The production of (A) MDA, (B) SOD, (C) GSH-Px and (D) CAT in H₂O₂-stimulated PC12 cells treated with LEN in the presence or absence of Notch agonist JAG, detected by corresponding commercial kits. (E) Relative protein expression of Nox2 and Nox4 in H₂O₂-stimulated PC12 cells treated with LEN in the presence or absence of Notch agonist JAG, detected by western blotting. (F and G) The apoptosis of H₂O₂-stimulated PC12 cells treated with LEN in the presence or absence of Notch agonist JAG, detected by TUNEL (magnification, x200). (H and I) Relative protein expression of Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9 in H₂O₂-stimulated PC12 cells treated with LEN in the presence or absence of Notch agonist JAG, detected by western blotting. ^{***}P<0.01 and ^{***}P<0.001 vs. control; [#]P<0.05, ^{##}P<0.01 and ^{###}P<0.001 vs. H₂O₂; ^ΔP<0.05, ^{ΔΔ}P<0.01 vs. LEN + H₂O₂ and ^{ΔΔΔ}P<0.001 vs. LEN + H₂O₂. LEN, lenalidomide; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; Nox, NADPH oxidase; JAG, Jagged-1.

expression and upregulation of the pro-apoptotic proteins Bax, cleaved caspase-3 and cleaved caspase-9. However, pre-treatment with LEN decreased the number of apoptotic cells and the expression levels of pro-apoptotic proteins, while increasing the expression of Bcl-2. An anti-apoptotic effect of LEN on H₂O₂-stimulated PC12 cells was thus demonstrated in the present study.

The present study also investigated the effects of LEN by examining the underlying mechanism. Previous studies determined the role of the interaction between LEN and Notch signaling in inhibiting human gastric cancer cell proliferation and promoting osteogenic differentiation in MM (14,15). Additionally, Cai *et al* (31) identified that Notch inhibition *in vivo* by microRNA-139-5p upregulation could potentially attenuate the oxidative stress-induced liver injury in a diabetic model. Notch is also known as an essential regulatory signaling pathway in the process of cellular apoptosis in cerebrovascular diseases (32). More importantly, Notch expression is likely to be inhibited by resveratrol to facilitate the recovery from SCI, according to a recent review (33). In addition, a previous study revealed that circular RNA_0005075 knockdown could alleviate neuropathic pain by inactivating the Notch2 signaling pathway (34). It was observed in the present study that the expression levels of Notch-related proteins, Notch2, Hey1 and HES1, were notably increased in H₂O₂-stimulated PC12 cells, but were significantly decreased by LEN treatment in a dose-dependent manner. This result preliminarily validated the interaction of LEN and the Notch signaling pathway. The present study further investigated the role of Notch in the effects of LEN by introducing the Notch agonist JAG to H₂O₂-stimulated PC12 cells pre-treated with LEN. It was revealed that the formerly inhibited oxidative stress level and mitigated apoptosis caused by LEN treatment were significantly promoted again by Notch activation. This suggested that LEN inhibits the H₂O₂-induced oxidative stress and apoptosis of PC12 cells by blocking the Notch signaling pathway.

In conclusion, the present study demonstrated that LEN has the ability to alleviate PC12 cell injury induced by H₂O₂ stimulation, likely by blocking the Notch signaling pathway, revealing the value of LEN in restoring the viability of spinal cord neurons following SCI.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZL and KW designed the study, and drafted and revised the manuscript. SY and ZC analyzed the data and searched the literature. All authors performed the experiments. All authors

read and approved the final manuscript. ZL and SY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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