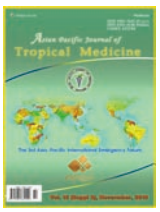

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Memantine inhibits 6-OHDA-induced apoptosis PC12 cells *via* the Nurr77 and caspase pathway

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ABSTRACT

Objective: To investigate the effect of memantine on Parkinson's disease cell models.

Methods: Parkinson's disease cell models were established using PC12 cells incubated with 6-hydroxydopamine (6-OHDA). Flow cytometry and microscopy were used to investigate the apoptotic process and the percentage of different apoptotic stages. PC12 cells were infected with lentiviral vectors to knockdown Nur77. Western blotting was used to detect the expression of Nur77 and caspase -3, -8, -9, -12 in PC12 cells withdifferent concentrations of 6-OHDA or 6-OHDA+memantine.

Results: 6-OHDA led to apoptosis PC12 cells, and increased the expression of Nur77 and caspases. Memantine significantly inhibited 6-OHDA-induced apoptosis of PC12 cells. Meanwhile, memantine could mitigate apoptosis of PC12 cells by regulating the Nur77 and caspase pathway.

Conclusions: Memantine has a protective effect on the PC12 cell model *via* regulating the Nur77 and caspases pathway.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra. The pathogenesis of PD remains unclear but may involve genetic diathesis, oxidative stress, mitochondrial dysfunction, neuroinflammation and excitototoxicity[1]. To explore the molecular pathways of neuronal death and to develop neuroprotective strategies, a number of *in vitro* and *in vivo* models have been characterized[2,3]. 6-hydroxydopamine (6-OHDA) has been used for PD models since the 1960s and it is regarded as the gold standard for studying mesencephalic dopamine (DA)-containing neurons[4]. It has been shown that caspases cascade were activated in 6-OHDA-induced apoptosis, and caspase 3 is the main effector. However, the crucial downstream targets of caspase 3 activation have not been clearly defined[2,5].

Nur77 (also known as nerve growth factor IB, NR4A1, or TR3), is a member of an orphan nuclear receptor superfamily and plays an important role in the regulation of cell survival and differentiation.

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Recent studies have indicated that Nur77 is involved in apoptosis in many cell lines[6]. Nur77 is known to be translocated from the nucleus to the cytoplasm, inducing cytochrome C release, and then initiating the apoptotic process related to mitochondrial impairment[7]. Nur77 was also reported to protect cells against oxidative stress and estrogen receptor stress-mediated apoptosis through regulation of caspase activities and C/EBP-homologous protein expression. However, the role of Nur77 in PD has not yet been elucidated. In this study, we focused on the potential role of Nur77 in apoptosis induced by 6-OHDA in PC12 cells[8,9].

Memantine (1-amino-3, 5-dimethyladamantane), a non-competitive N-methyl-*D*-aspartate receptor (NMDAR) antagonist, has been approved for the treatment of moderate to severe Alzheimer's disease. It has been reported that NMDARs are also found on dopaminergic neurons in the substantia nigra pars compacta. The activation of glutamatergic transmission through NMDARs could accelerate the degenerative process and induce apoptosis in dopaminergic neurons[10]. Our previous experiments have confirmed that the apoptosis of PC12 cells could be induced with the increase in Nur77 expression, but the effect could be blocked by the administration of memantine[11]. We speculated that memantine can protect PC12 cells from apoptosis through Nur77 regulation, however, the underlying mechanisms and signaling pathways are still unclear. In this research, we aimed to explore (1) activation of caspase cascade in 6-OHDA-induced apoptosis; (2) the role of Nur77 involved in 6-OHDA-lesion PC12 cells; (3) the protective effect of memantine in regulating apoptosis *via* Nur77 of PC12 cells.

2. Materials and methods

2.1. Reagents and cell line

PC12 cells (Cell bank of Sun Yat-Sen University), RPMI 1640 culture medium (GIBCO C11875500 BT), penicillin-streptomycin (GIBCO 15140-122), 0.25% trypsin-EDTA (1×) (GIBCO 25200-072), fetal bovine serum (FBS) (GIBCO 16000-044), HBS (GIBCO 26050-070), phosphate buffer pH7.4 basic (1×) (GIBCO C10010500BT), 6-OHDA (Sigma H116-5MG), vitamin C (Sangon Biotech A610021-100G), memantine (sigma), MTS (Promega G1112), CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (a) (CellTiter 96® AQueous (Promega G3580), PE annexin V Apoptosis Detection Kit Ⅰ (BD), APC annexin V (BD Biosciences550474), 7-AAD (BD Biosciences 559925), TritonX-100 (Amersham Biosciences), DeadEnd™ Fluorometric TUNEL System (Promega G3250), SSC, 20 (1:10) (Promega G329A), DAPI H1399 (1:500) (Invitrogen), mounting medium (Electron Microscopy Sciences), Nur77 (Santa sc5569, 1:200), caspase 3 (CST 9664, 1:1 000), caspase 8 (Santa Cruz, sc7890, 1:500), caspase 9 (Santa sc7885, 1:500), caspase 12 (Abcam ab62484, 1:1 000).

2.2. Cell culture and treatments

PC12 cells were cultured in 1640 medium (Roswell Park Memorial Medium Institute, USA) mixed with 10% FBS, 5% horse serum (HoS), 100 U/mL benzyl penicillin, and 100 mg/L streptomycin (Gibco Life Technologies). For all experiments, the cells were seeded in 96-well plates or 6-well plates at a density of 1×10^5 cells/mL for 24 h. For estimating the suitable concentration of 6-OHDA for a cellular damage model, PC12 cells were treated with either conditioned media alone or with 25, 50, or 100 μM 6-OHDA for 24 h; the cell viability was assayed by MTS. For the memantine toxicity measurement experiment, PC12 cells were treated with conditioned media alone, 6-OHDA, 6-OHDA mixed with memantine (1, 5, 10, and 20 μM, respectively) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. After the 24 h incubation, density and morphology of the cells were observed under optical mocroscopy, viability was assayed by MTS for examining the optimal concentration of memantine that provided neuroprotection to 6-OHDA-damaged PC12 cells. According to the results of the above-mentioned experiments, three experimental groups were established by treating with either media (DMEM), 50 μM 6-OHDA, or 50 μM 6-OHDA + 10 μM memantine. After 24 h, PC12 cell death was quantified *via* the MTS assay and flow cytometry.

2.3. Measurement of apoptotic cells by MTS assay and flow cytometry

PC12 cell viability was measured by the MTS assay (Promega Corporation), according to the manufacturer's instructions. In brief, 10 μL of the MTS kit reagent was added to the cells treated with 6-OHDA at 25, 50, or 100 μM in 96-well plates and incubated at 37℃ for 1 h. The cell viability was assessed at an absorbance of 490 nm with a microplate reader. Each treatment group was replicated in three wells. All results were normalized to optical density values measured from an identically conditioned well without cells. The results for the MTS assay were expressed as a percentage of the control group, which was set as 100%.

To investigate the apoptosis, PC12 cells and were seeded at a density of 1×10^5 cells/well in 24-well plates. After incubated with 6-OHDA and caspase inhibitors, PC12 cells were treated with the terminal deoxynucleotidyl transferase in dUTP Nick-End Labeling (TUNEL) kit and observed with a fluorescence microscope to investigate DNA damage. PC12 cells dyed with propidium iodide and annexin V were evaluated by flow cytometry (Bender MedSystems, Burlingame, CA, USA) for further investigation of the apoptotic process and the percentage of different apoptotic stages was calculated.

2.4. Effect of 6–OHDA and memantine on the expression of Nur77 and caspase 3

PC12 cells treated with 50 μM 6-OHDA or memantine (5, 10, 20 μM) or 6-OHDA+memantine (50 μM 6-OHDA, 1, 5, 10, 20 μM memantine) were washed twice in cold phosphate buffer (4℃) to remove serum proteins in the culture media. The cells were lysed by pouring 120 μL of RIPA buffer into each well of the culture plate. Cells were then harvested by using cell scrapers and placed in ice-cold Eppendorf tubes. Genomic DNA breakage was carried out by ultrasonic oscillation to reduce the viscosity of the cell lysate. After the protein was extracted and measured by means of Bradford measurement, the target protein was separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane. After it was sealed with 5% skim milk, the primary antibodies against Nur77 (Santa Cruz 1:200) and caspase 3 (CST, 9664, 1:1 000) were incubated with the PVDF membrane overnight at 4℃. After the immunoaffinity, the PVDF membrane was washed twice with tris-buffered saline tween-20 and incubated with the secondary antibody (conjugated with HRP, 1:5 000, Forevergen) at 25℃ for 1 to 2 h. Chemiluminescence (ECL, Forevergen) was carried out after the PVDF membrane was rinsed again with tris-buffered saline tween-20. Image J was used to analyze the optical density of the developed strip. GAPDH (HC301), was used at a concentration of 1:5 000, as the internal reference to compare the protein expression after different treatments.

2.5. Effect of 6–OHDA on apoptosis, Nur77 and caspase 3 expressions in cells incubated with caspase inhibitive reagents

PC12 cells were incubated with caspase inhibitive reagents, including Z-IETD-FMK for caspase 8 (20 μM), Z-LEHD-FMK for caspase 9 (20 μM), Z-ATAD-FMK for caspase 12 (30 μM) for 2 h, the control group was incubated with DMSO (the solvent of caspase inhibitive reagents) for 2 h. Subsequently, PC12 cells were incubated with 50 μM 6-OHDA for 6 h. TUNEL kit was used for detecting apoptosis through fluorescence microscopy and flow cytometry was used to determine the viability of cells. The viability rate of PC12 cells treated with different caspase inhibitive reagents was compared with statistical calculation. The effect of 6-OHDA on Nur77 and caspase 3 expressions in cells incubated with caspase inhibitive reagents was determined by Western blotting (WB) as mentioned in section 2.4.

2.6. Lentiviral vector construction/infection and short hairpin ribonucleic acid interference

Lentiviral vectors were used for the knockdown of Nur77 as previously described[11]. Briefly, 293T cells were cultured in DMEM containing 10% FBS, 5% HoS, penicillin (50 IU/mL), streptomycin (50 mg/mL), and *L*-glutamine (2 mM; Gibco) and transfected with pLV-gene, pGag/Pol, pRev, pVSV-G and Nur77 using the calcium phosphate method. Viral particles contained in the supernatant were harvested 1 and 2 d after transfection. After ultracentrifugation, viral titers were determined as previously described[11]. Briefly, serial dilutions of the concentrated virus were used for the transduction of 293T cells. Total genomic (DNA) was isolated 24 h after the transduction, using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. The number of inserted vector copies was determined by reverse transcription-polymerase chain reaction using the vector DNA for the standard curve: forward primer, 5'-AAC T CCAAGTTGGACTATTCCAA T TCAAGAGATTGGAATAGTCCAACTTGGTTTTTT C-3'; reverse primer, 5'-AACT TGGCCCAGAGTTCCCTGAA TTCAAGAGA TTCAGGGAACTCTGGGCCA TTTTTT C-3'.

2.7. Transduction of PC12 cells and the selection of transduced cells

PC12 cells were seeded on a 6-well plate at a density of 1×10^5 cells/mL and cultured for 24 h. For transduction, the medium was replaced with DMEM+10 μg/mL polybrene containing viral particles (multiplicity of infection 50 for knockdown). After overnight exposure to the virus, the medium containing viral particles was replaced with DMEM+10% FBS+5% HoS, and the cells were cultured for another 24 h. Screening with puromycin (2 μg/mL final concentration) was carried out after 3 d when transfection was completed. As a result of the screening, untransfected colonies were eliminated by puromycin selection and the stable colonies with puromycin resistance were amplified after 10-12 d. Stable colonies with the reporter gene, green fluorescent protein, were observed by fluorescence microscopy for preliminary verification of transfection. The transduction efficiency was evaluated by real-time quantitative (RT q)-PCR. The expression of Nur77 was compared using WB between the control group and the virus transfected group for further verification.

2.8. Effect of 6–OHDA on the expression of caspases in PC12 cells transfected with Nur77 lentiviral vector

PC12 cells were taransfered by lentiviral vectora as previously mentioned in section 2.5 and 2.6. Then the transduced cells were treated with 50 μM 6-OHDA. The expression of caspase-3, -8, -9, -12 were detected using WB among the control group, 6-OHDA group, the virus transfected group and virus transfected+6-OHDA group for further verification.

2.9. Effect of memantine on apoptosis of PC12 cells transfected with Nur77 lentiviral vector

PC12 cells were taransfered by lentiviral vectora as previously mentioned in section 2.6 and 2.7. Then the transduced cells were treated with 50 μM 6-OHDA or 50 μM 6-OHDA + 10 μM memantine for 24 h. TUNEL kit was used for detecting apoptosis through fluorescence microscopy and flow cytometry was used to determine the viability of cells. The viability rate of the cells was compared with statistical calculation.

2.10. Statistical analysis

The Statistical analyses were performed with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The data are expressed as the mean±SD. To compare the viability rate of cells and expressions of proteins, One-way analysis (ANOVA) was used for comparison among different groups. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Viability and apoptosis of 6-OHDA-induced PC12 cell

PC12 cells were treated with conditioned media alone or with 25, 50 and 100 μM 6-OHDA for 24 h, respectively. The cellular morphology was observed *via* microscopy (Figure 1A). It showed that PC12 cells incubated in 50 or 100 μM 6-OHDA were more damaged than the 25 μM groups and control group. In addition, the viability of PC12 cells incubated respectively in 25 μM, 50 μM, and 100 μM 6-OHDA was measured by MTS assay (Figure 1B). The viability of PC12 cells treated with 50 μM 6-OHDA sharply declined to (43.5±6.2)% compared with lower concentration groups. The viability of PC12 cells treated with 100 μM 6-OHDA was (12.7±5.8)%, which was too low to conduct experiments. Overall, 50 μM was selected to be the optimum concentration for the PC12 cells pathological model.

3.2. 6-OHDA induced PC12 cell apoptosis through activation of caspase pathways via Nurr77

Flow cytometry and TUNEL assay indicated that inhibiting the activation of caspases 3, 8, 9 and caspase 12 significantly mitigated apoptosis (Figure 2A, C). The caspase 9 inhibitor, LEHD, and the caspase 12, inhibitor ATAD significantly mitigated the apoptosis compared with the 6-OHDA group (Figure 2B).

The result showed that caspase 3 protein expression was increased in the cells incubated in 25 μM, 50 μM and 100 μM 6-OHDA, indicating that the apoptosis became more severe (Figure 3A).

Nur77 is one of the upstream signals of apoptosis. The expression of Nur77 protein was up-regulated with the increasing of 6-OHDA concentration (Figure 3B).

Three caspase inhibitors downregulated the expression of Nur77 and cleaved (cl) caspase 3 (Figure 3C, D, E). The expressions of several cl caspases in PC12 cells were detected after transfection

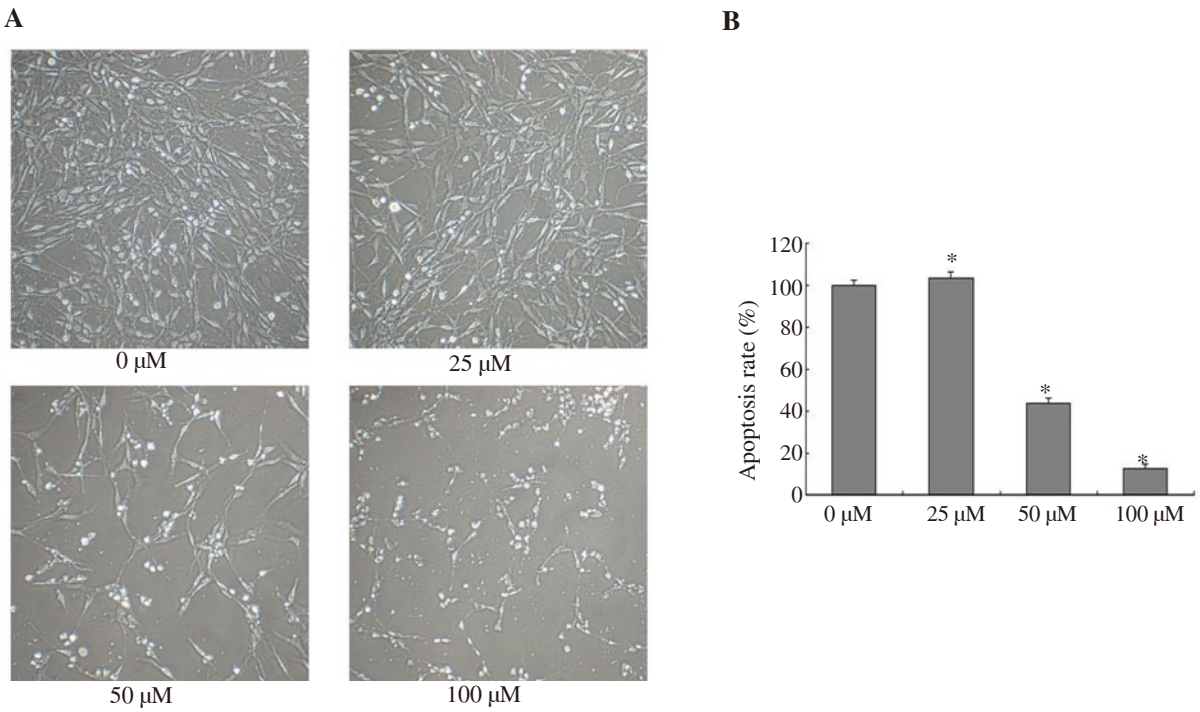

Figure 1. Cellular morphology of PC12 cells 6-hydroxydopamine (6-OHDA) (A) and the viability of PC12 cells 6-OHDA (B). The results are expressed as mean ± SD of 5 independent experiments. *P<0.05 *vs.* control.

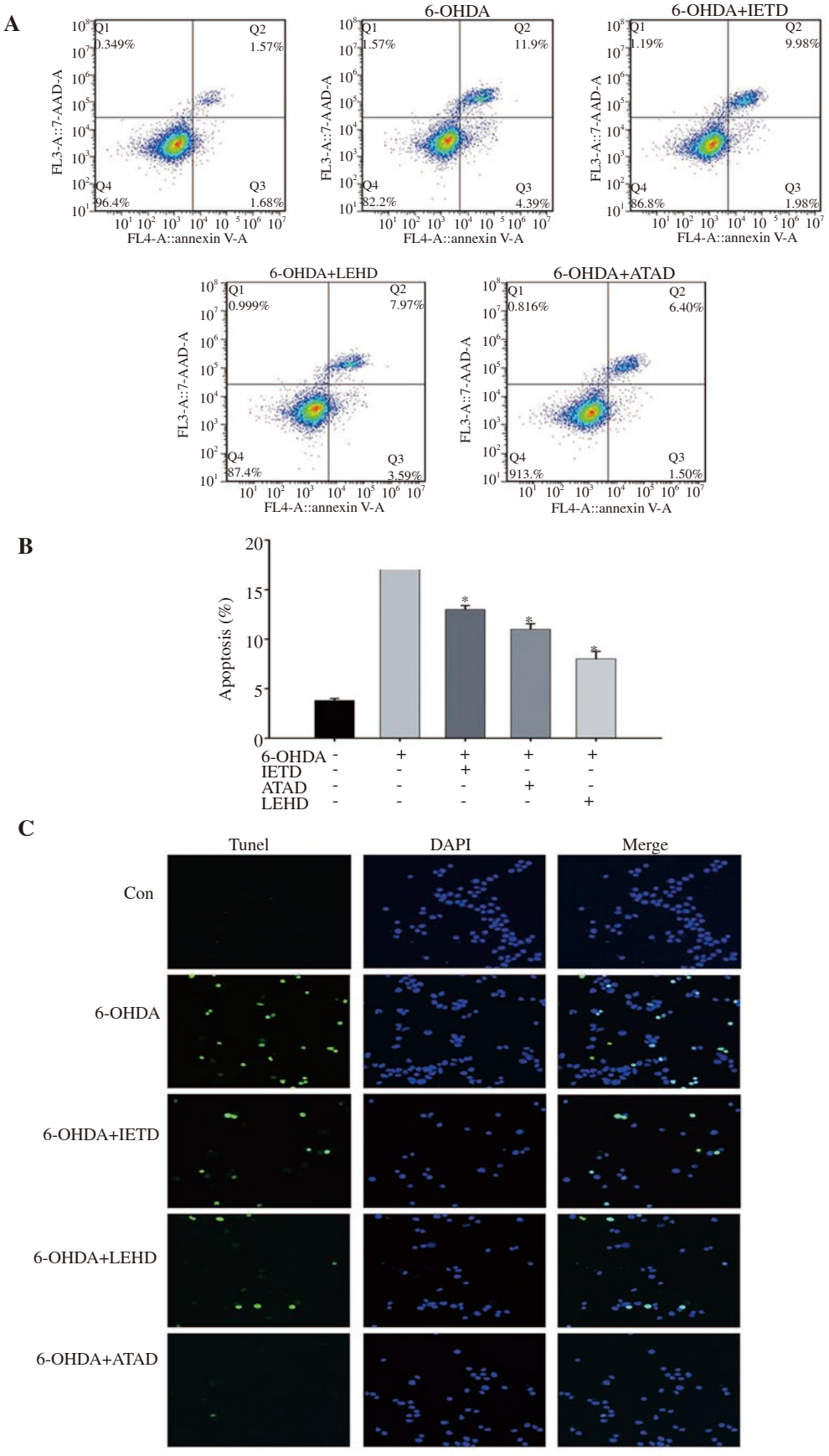

Figure 2. Apoptosis rate of 6-OHDA treated PC12 cells. A: flow cytometry, B: the apoptotic rate of PC12 cells treated with different concentrations of 6-OHDA; C: TUNEL assay. **P*<0.05 *vs.* control. 6-OHDA: 6-hydroxydopamine; Con: control; IETD: Z-IETD-FMK (20 μM); LEHD: Z-LEHD-FMK (20 μM); ATAD: Z-ATAD-FMK (30 μM).

with the Nur77 lentiviral vector. Compared with the normal saline group, the content of cl caspase 8, 9 and cl caspase 3 were significantly up-regulated in the 6-OHDA group. Compared with the 6-OHDA group, the shNur77+6-OHDA group showed lower content of cl caspase 8, 9 and cl caspase 3. The content of cl caspase 12 showed no difference according to the WB (Figure 4A&B).

3.3. Protective effects of different concentrations of memantine on PC12 cells PD models induced by 6-OHDA

The percentage of apoptosis decreased compared with the 6-OHDA group, as shown by flow cytometric data (Figure 5A, B). Content of cl caspases in the PC12 cells incubated with 50 μM 6-OHDA and continually diluted memantine was detected by WB (Figure 6A). The content of cl caspase 3, 9, 12 were all decreased in the memantine groups (Figure 6B, C, D, E).

3.4. Memantine regulates the levels of Nur77 and cl caspase 3 in 6-OHDA-treated PC12 cells

The apoptosis percentage in the PC12 cells was detected by flow cytometry after transfection with a lentiviral vector. Flow cytometry data showed that the apoptotic rate of the 6-OHDA group was significantly higher than all the other groups. Compared with the 6-OHDA group, the apoptosis rate of shNur77 cells was significantly decreased. Memantine showed a more obvious protective effect in the shNur77+6-OHDA+memantine group than in the 6-OHDA+memantine group (Figure 7A, B). Results of WB showed a significant decrease in the content of cl caspase 3 in the memantine group compared with the control group, along with down regulation of Nurr77. These results showed that the down-regulated expression of Nur77 could mitigate the damage inflicted by 6-OHDA and memantine protected damaged PC12 cells by down-regulation of Nur77 (Figure 8A, B, C).

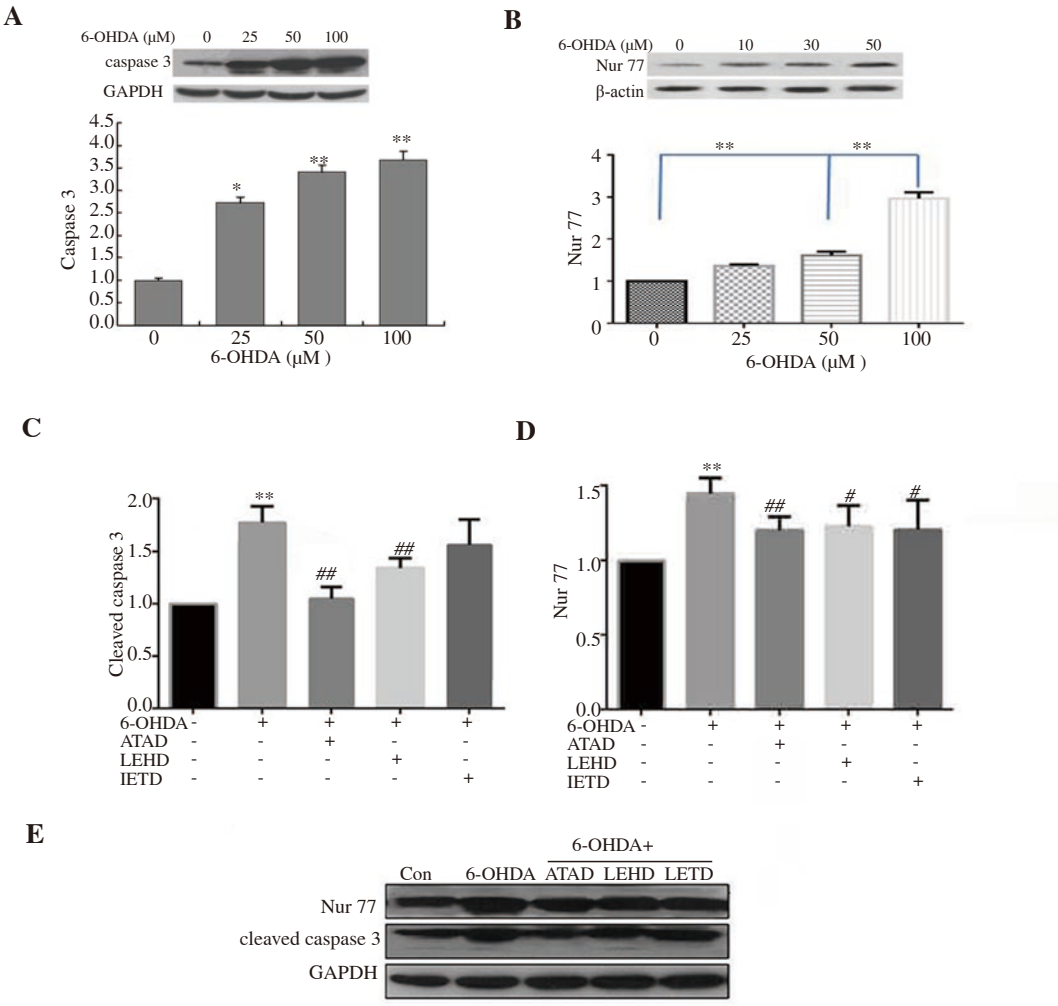

Figure 3. Expression of caspase 3 and Nur77 in PC12 cell after treatment with 6-hydroxydopamine (6-OHDA) and caspase inhibitors. A: caspase 3 expression in 6-OHDA PC12 cell; B: Nur77 expression in 6-OHDA PC12 cell; C: expression of cleaved caspase 3 after treatment with caspase inhibitors; D: expression of Nur77 after treatment with caspase inhibitors; E: Western blot results of treatment with caspase inhibitors. The results are expressed as mean ± SD of 5 independent experiments. **P*<0.05, *vs.* control; ***P*<0.01, *vs.* control. #*P*<0.05, *vs.* 6-OHDA; ##*P*<0.01, *vs.* 6-OHDA. Con: control; IETD: Z-IETD-FMK (20 μM); LEHD: Z-LEHD-FMK (20 μM); ATAD: Z-ATAD-FMK (30 μM).

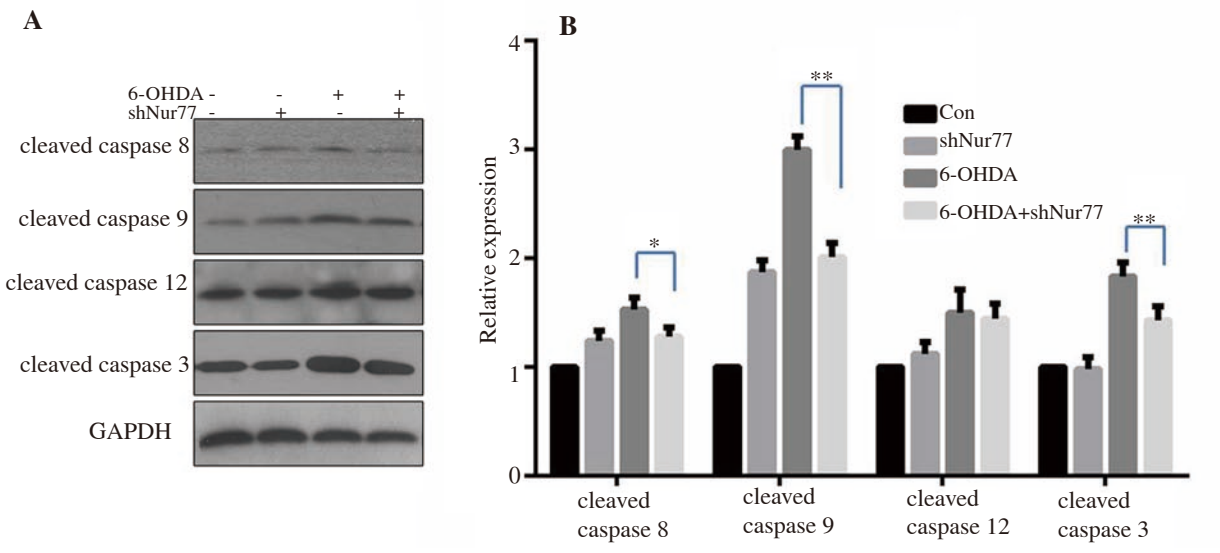

Figure 4. Expression of cleaved caspases after knockdown of Nur77. A: Representative immunoblotting images; B: Relative expression of cleaved caspases. **P*<0.05; ***P*<0.01. 6-OHDA: 6-hydroxydopamine; shNur77: knockdown of Nur77.

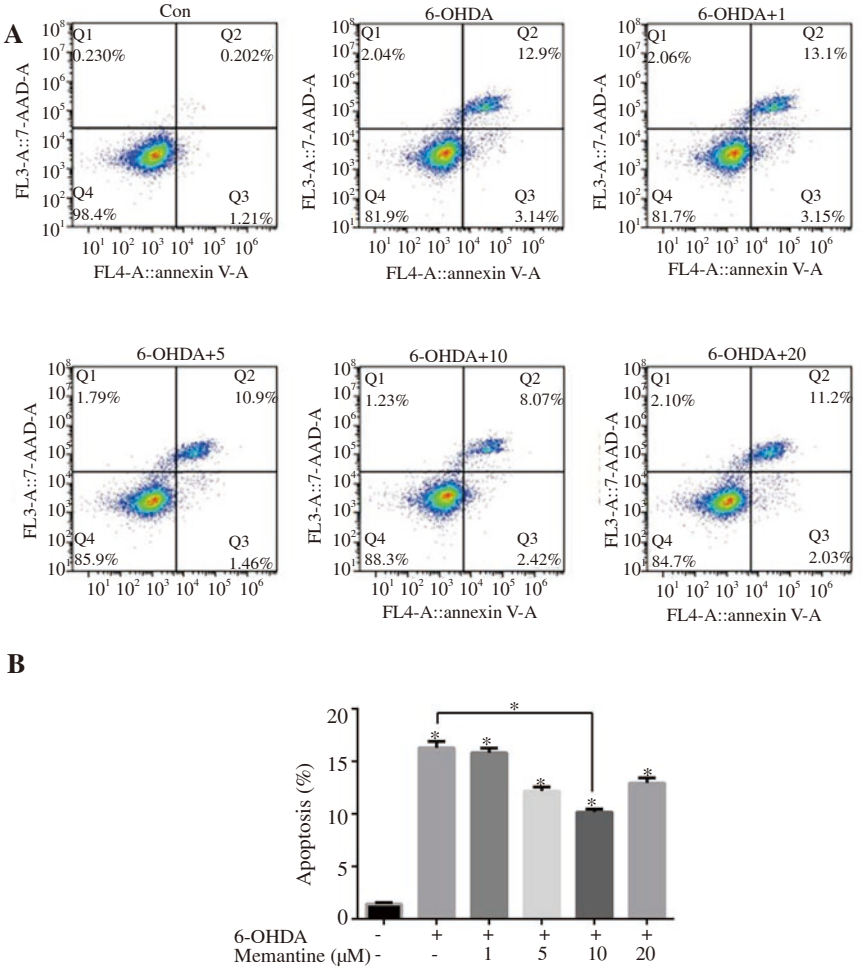

Figure 5. Apoptosis rate of PC12 cells treated with different concentrations of memantine. A: Flow cytometry; B: PC12 apoptosis rate at different concentrations of memantine, **P*<0.05. 6-OHDA: 6-hydroxydopamine; Con: control; 6-OHDA+1: 6-hydroxydopamine+memantine 1 μM; 6-OHDA+5: 6-hydroxydopamine+memantine 5 μM; 6-OHDA+10: 6-hydroxydopamine+memantine 10 μM; 6-OHDA+20: 6-hydroxydopamine+memantine 20 μM.

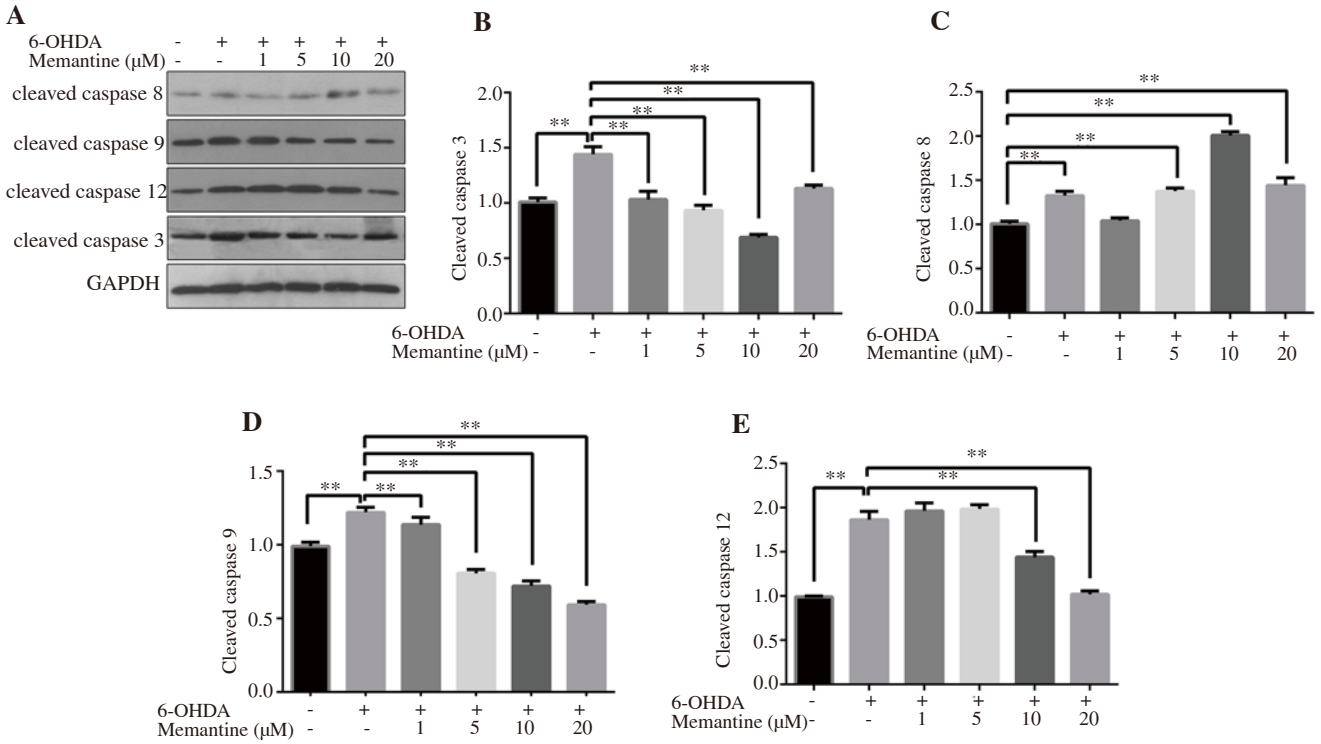

Figure 6. Expression of cleaved caspases in PC12 cells after treatment with memantine. A: Western blot result; B. Expression of cleaved caspase 3; C. Expression of cleaved caspase 8; D. Expression of cleaved caspase 9; E. Expression of cleaved caspase 12; ***P*<0.01. 6-OHDA: 6-hydroxydopamine; Con: control.

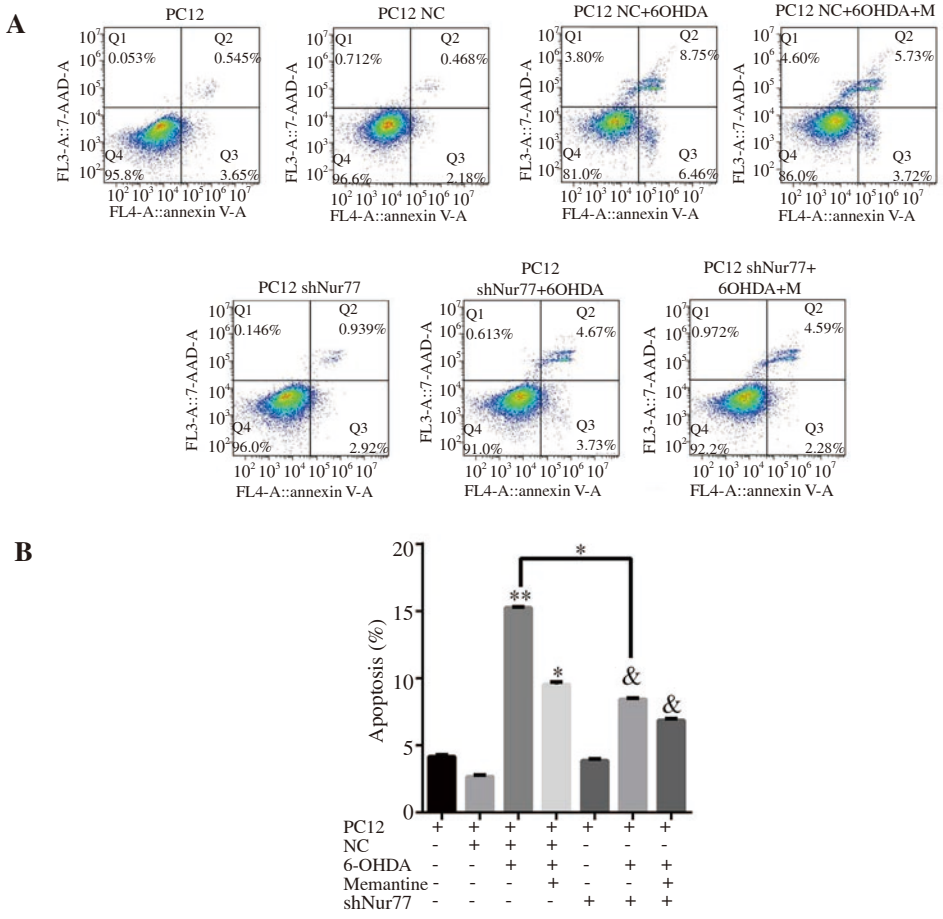

Figure 7. Apoptosis rate of PC12 cells and shNur77-/- PC12 cells. A: Flow cytometry result; B. Comparison of apoptosis rate of PC12 cells and shNur77-/- PC12 cells. **P*<0.05, ***P*<0.01 *vs*. PC12 NC; &*P*<0.05 *vs*. PC12 ShNur77. NC: normal control; 6-OHDA: 6-hydroxydopamine; M: Memantine; shNur77: knockdown of Nur77.

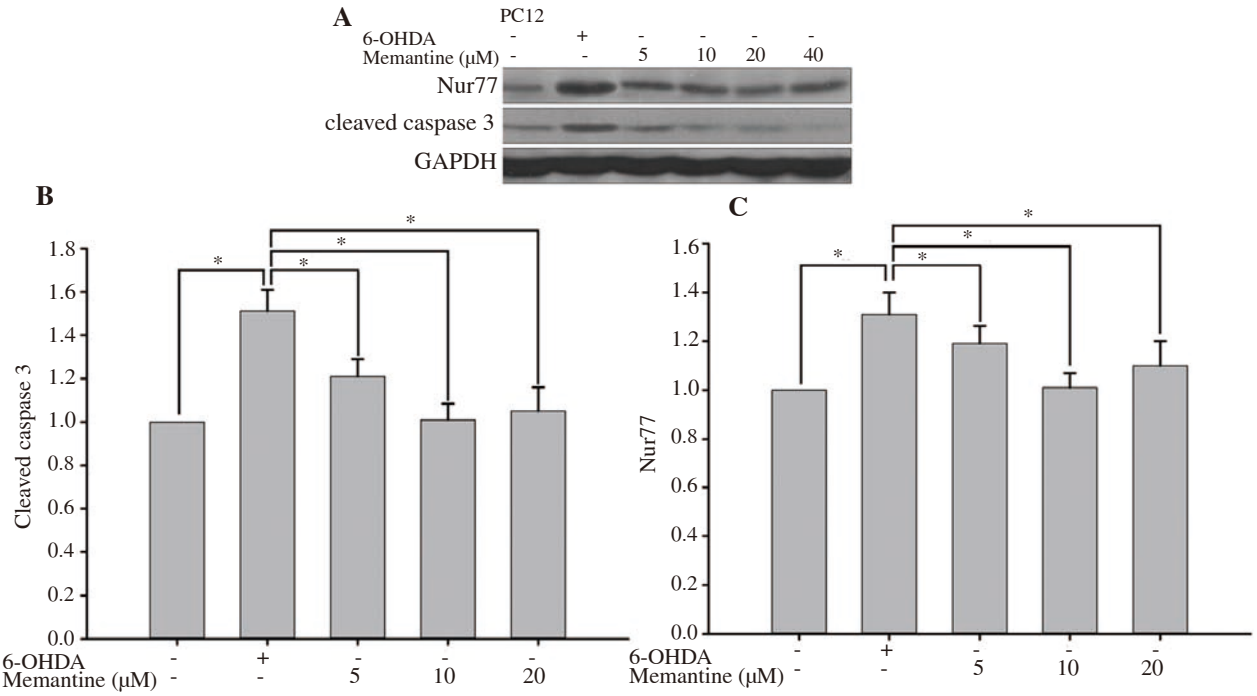

Figure 8. Expression of cleaved caspase 3 and Nur77 after treatment with memantine. A: Representative images; B: cleaved caspase 3 expression; C: Nur77 expression . The results are expressed as mean ± SD of 5 independent experiments. *P<0.05, *vs.* control.

4. Discussion

In this study, we investigated the variation of caspase cascade and Nur77 in PC12 cells incubated by 6-OHDA, furthermore, the protective effect of memantine in the PC12 cells induced by 6-OHDA was evaluated as well. Finally, we confirmed 3 novel findings: (1) 6-OHDA induced apoptosis *via* caspase cascade in PC12 cells; (2) In 6-OHDA incubated cells, there is mutual regulation between caspase cascades and Nur77; (3) Memantine can mitigate apoptosis induced by 6-OHDA *via* Nur77 and caspase cascades.

Currently, a number of histological studies have shown that dopaminergic neuronal apoptosis in the substantia nigra is the main cause of PD[12,13]. To further elucidate the pathogenesis of PD, researchers used a variety of neurotoxic compounds to construct PD cell models including 6-OHDA, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydro pyridine and rotenone. After careful consideration, 6-OHDA incubated PC12 cells was selected as the PD model. Our study confirmed that obviously apoptosis was induced in PC12 cells incubated with 6-OHDA, and the most appropriate concentration of 6-ODHA for PD cell model is 50 μM. Neuropathological studies showed that 6-OHDA can cause neuronal apoptosis in various ways. Two dominant ways are the oxidative stress response and excitatory amino acid toxicity[14]. The death effect domain of the compound can bind to caspase 8 or caspase 10 to trigger their activity. Activated caspase 8 and/or caspase 10 induced the downstream substrate caspase 3 and continued the process of apoptosis[15]. Our results also revealed that caspase 3 was increased after incubated by 6-OHDA and apoptosis was significantly mitigated after inhibiting the activation of caspase 8, 9 and caspase 12, which indicated that 6-OHDA induced apoptosis *via* caspase cascade. In summary, 6-OHDA could impair PC12 cells and induce apoptosis by caspase pathways mentioned above.

Nur77 has also been proven to be very important in apoptosis induced by 6-OHDA[16,17]. The results of this study revealed the caspase pathways which the orphan nuclear receptor Nur77 participates in. We found that Nur77 can be up-regulated with the increasing amount of 6-OHDA in PC12 cells. Furthermore, the content of Nur77 and caspase 3 were all decreased after inhibiting the activation of caspase 8, 9 and caspase 12, indicating that the apoptosis induced by 6-OHDA can be regulated by caspase cascade *via* Nur77. We also found that the content of caspase -8/9/12/3 were all down-regulated in the shNur77 group, which means caspase cascade can also be regulated by Nur77.

Memantine is a noncompetitive NMDAR antagonist. We further explored its protective effect on PC12 cell model and the mechanisms[11]. Our results revealed that memantine significantly mitigated the apoptosis induced by 6-OHDA in PC12 cells. The caspase cascades were also down-regulated, which means memantine can mitigate the apoptosis induced by 6-OHDA *via* the caspase pathway. Meanwhile, the content of Nur77 was also decreased after

incubated with memantine, indicating that memantine can mitigate the apoptosis induced by 6-OHDA *via* Nur77. Furthermore, we found that apoptosis can be more significantly mitigated after the administration of memantine and/or inhibition of Nur77.

We found that memantine has a protective effect on PC12 cell models. The levels of caspase 3 and 12 were decreased by more than a half. This mechanism may be related to the regulation of apoptosis. However, memantine could not inhibit al death receptor pathways of apoptosis. WB confirmed no decline in a key protein (caspase 8) in death receptor apoptosis after memantine treatment. Our study also found that Nur77 is involved in apoptosis in PC12 cell models. The mechanism of memantine inhibiting apoptosis may involve the down-regulation of Nur77. In PC12 cell models, the expression of Nur77 was up-regulated. Silencing of Nur77 significantly decreased apoptosis, meanwhile, significantly decreased the anti-apoptotic effect of memantine.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Authors' contribution

W.W.G. and W.W. contributed to the conception of the study. W.W., R.W. contributed significantly to analysis and manuscript preparation and wrote the manuscript; W.W., R.W., H.C.H., J.Z. and W.W.G. performed the data analyses; W.W., R.W. and W.W.G. helped perform the analysis with constructive discussions.

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