Puerarin attenuates remifentanil-induced postoperative hyperalgesia via targeting PAX6 to regulate the transcription of TRPV1

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Abstract. Remifentanil-induced hyperalgesia (RIH) is characterized by the emergence of stimulation-induced pain, including phenomena such as allodynia and thermal hyperalgesia following remifentanil infusion. As a sequence-specific DNA binding transcription factor, PAX6 positively and negatively regulates transcription and is expressed in multiple cell types in the developing and adult central nervous system. It was hypothesized that puerarin could relieve RIH via targeting PAX6 to regulate transcription of transient receptor potential cation channel subfamily V Member 1 (TRPV1). A total of 32 rats were randomly divided into five groups, namely control group, RI group, RI + 10 mg/kg puerarin group (RI + puerarin10), RI + 20 mg/kg puerarin group (RI + puerarin20), and RI + 40 mg/kg puerarin group (RI + puerarin40). Mechanical and thermal hyperalgesia were tested at -24, 2, 6, 24 and 48 h after remifentanil infusion. Following the sacrifice of rats after the last behavioral test, western blot was used to detect the expression levels of TRPV1 in the tissues; Immunofluorescence staining and western blotting were used to detect the expression of PAX6 in the spinal cord. PharmMapper and JASPAR were used to predict the binding sites of puerarin/PAX6/TRPV1. Chromatin immunoprecipitation-PCR and dual luciferase reporter assay were used to verify the targeting relationship between PAX6 and TRPV1. Immunofluorescence was used to detect the expression levels of TRPV1 and p-NR2B. The results revealed that puerarin (10, 20, 40 mg/kg) dose-dependently reduced thermal and mechanical hyperalgesia from 2 to 48 h after remifentanil infusion. Remifentanil infusion remarkably stimulated the expression of phosphorylated (p-)NR2B. Nevertheless, the increased amount of p-NR2B by RIH was dose-dependently suppressed by puerarin in rats. In conclusion, puerarin was revealed to attenuate postoperative RIH via targeting PAX6 to regulate the transcription of TRPV1.

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Introduction

With the increasing number of cancer patients every year, the side effects caused by chemotherapy drugs have become the focus of medical workers. Chemotherapy-induced neuropathic pain seriously affects the quality of life of millions of individuals, while bringing a great economic burden worldwide (1,2). At present, opioids are the most widely used painkillers in clinical practice, but their side effects cannot be ignored (3). Remifentanil has some intraoperatively and postoperatively clinical value, but its unique pharmacokinetics are associated with the development of opioid-induced hyperalgesia (OIH). Hyperalgesia could hinder patient's recovery process and reduce their quality of life after surgery (4). Therefore, to relieve pain, it is urgent to find new targets for the treatment of neuropathic pain and explore potential therapeutic drugs.

According to previous studies (5,6), the members of the Transient Receptor Potential family are key regulatory factors in the induction of hyperalgesia. As a non-selective cationic channel, transient receptor potential cation channel subfamily V Member 1 (TRPV1) could be activated by protons, cold stimulation, and capsaicin to transmit neuropathic pain signals, and it is highly expressed in dorsal root ganglion neurons (7). TRPV1 antagonists could effectively inhibit neuropathic pain mediated by various neurotransmitters and activates the CaMKII-PKC signaling pathway in DRG neurons, thus exacerbating the persistence of postoperative remifentanil-induced hyperalgesia (RIH) (8). Meanwhile, TRPV1 is a potentially meaningful target molecule for the treatment of opiate-induced hyperalgesia.

Chinese herbal extracts are known to be effective in treating various diseases including cancer, diabetes, kidney disease and chronic pain. As a kind of traditional Chinese medicine monomer extracted from plants of genus *Pueraria*, puerarin has been widely reported for its anti-inflammatory and antioxidant effects. Liu *et al* (9) found that puerarin could regulate the NF-κB signaling pathway, downregulate inflammatory factors,

and relieve diabetic neuropathic pain. In addition, puerarin could reduce the stress transmission mediated by the P2X3 receptor and relieve pain hypersensitivity after burn (10). It was hypothesized that puerarin could alleviate postoperative RIH, but the mechanism remains unclear. In the present study, it was intended to explore the effects of puerarin on OIH and the molecular mechanism of TRPV1 regulation in rat pain models.

Materials and methods

Animals and groups. The adult male Sprague-Dawley rats (80 rats; age, 8-week-old) used in the study, weighing 220-250 g, were purchased from a company Beijing Vital River Laboratory Animal Technology Co. Ltd. Animals were raised in an environment of 23±2˚C in a 12/12-h day-night cycle and they had access to a standard diet and water. The surgical treatment of experimental animals was performed in accordance with the guidelines of the General Hospital of the Western Theater Command of the PLA on the use of experimental animals. The animal experiments in the present study were approved (approval no. 20210329015) by the Experimental Animal Ethics Committee of the General Hospital of the Western Theater Command of the PLA (Chengdu, China).

Drugs and groups. Remifentanil hydrochloride (Renfu Pharmaceutical Co., Yichang, China) was administered 1.0 μg/kg/min for 60 min via the tail vein. Moreover, intravenous saline (0.1 ml/min) for 60 min was used as the control group. Puerarin was dissolved in 10% DMSO and diluted with normal saline.

Puerarin was injected intraperitoneally every 24 h for 21 days before remifentanil-induced pain. In the animal experiment I, the rats were divided into five groups: Control, RI (remifentanil-induced group), RI + 10 mg/kg puerarin (RI + 10 mg/kg puerarin), RI + 20 mg/kg puerarin group (RI + 20 mg/kg puerarin) and RI + 40 mg/kg puerarin group (RI + 40 mg/kg puerarin). In experiment II, the rats were divided into five groups: Control, RI, RI + puerarin (40 mg/kg), RI + MK-801 (0.3 mg/kg), and RI + puerarin + MK-801. There were eight rats in each group, for a total of 80 rats. Feed and water were supplemented at 10 a.m. every day, and the rats were monitored. After 21 days of experimentation, 80 rats were euthanized by intraperitoneal injection of 120 mg/kg pentobarbital sodium anesthesia. To confirm death, rats were monitored for several signs such as no response when the toes were pressed with tweezers, no rising and falling of the chest and no palpable heartbeat. The tissues were harvested within 24 h after the end of the last behavioral experiment for subsequent detection.

Behavioral test. As previously reported (11), to assess the sensitivity to mechanical and thermal pain, the mechanical paw withdrawal threshold (PWT) and the paw withdrawal latency (PWL) were recorded using electronic Von Frey filaments and a hot plate (Thermo Fisher Scientific, Inc.) at 24 h (baseline) before remifentanil intervention and at 2, 6, 24 and 48 h after remifentanil intervention. Behavioral tests were performed in different groups, and the observers of the behavioral tests were blind to the animal randomization and treatment conditions throughout the experiment.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from spinal cord tissue using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA was synthesized from total RNA using Prime Script Reagent kit following the manufacturer's instructions. RT-qPCR analysis was performed by ABI 7500 PCR system using SYBRSYBRTM Green Master Mix (Applied Biosystems). The following thermocycling conditions were used for qPCR: 95˚C for 30 s, 40 cycles of 95˚C for 10 s and 60˚C for 30 s, followed by 95˚C for 10 s, 65˚C for 60 s and 97˚C for 1 s. The relative mRNA expression of TRPV1 was calculated and analyzed by the $2^{-\Delta\Delta Cq}$ method (12), and GAPDH was used as the internal control. Primer sequences were as follows: TRPV1 forward, 5'-TGCACAATGGGCAGAATGAC-3' and reverse, 5'-GTCATGTTCCGCCGTTCAAT-3'; and GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCACCCTGTTGCTGTA-3'.

Bioinformatics analysis. PharmMapper database (lilab.ecust.edu.cn/pharmmapper/) was used to search potential targets of puerarin, and then JASPAR database (jaspar2020.genereg.net/) was used to predict the binding sites of TRPV1 and PAX6.

Dual luciferase reporter assay. HEK293 cells were transiently transfected with the wild-type construct of pGL3-TRPV1-promoter (WT), mutant construct of pGL3-TRPV1-promoter (MUT), empty plasmid (Vector), or PAX6 expression plasmid (PAX6) using lipofectamine 3000. After 48 h, A dual luciferase reporting assay system (cat. no. E1910, Promega Corporation) was used to detect luciferase activity of firefly and Renilla.

Western blotting. RIPA lysate (Solarbio, Beijing) was used to extract the protein from spinal cord tissue and protein concentration was determined by BCA kit (cat. no. ab287853; Abcam). As previously reported (13), western blot assay was performed in accordance with standardized procedures. Western blot was carried out by separating 40 μg of protein by 10% SDS-PAGE and electro-transferred onto a PVDF) membrane. The membranes were blocked with 5% non-fat milk and washed with TBST buffer and then incubated with primary antibody overnight in 4˚C. Next, the membranes were washed 3 times with TBST and incubated with secondary antibody (ZB2306, 1:10,000, ZSGB-Bio) at a 1:5,000 dilutions for 1 h at room temperature. Finally, the expression of the proteins was evaluated using enhanced chemiluminescence kit (cat. no. PE0010; Beijing Solarbio, Beijing). The following primary antibodies were used: anti-PAX6 (cat. no. ab195045; Abcam, 1:1,000), anti-t-NR1 (cat. no. 5704s; Cell Signaling Technology, Inc., 1:1,000), anti-m-NR1 (cat. no. ab14596; Abcam, 1:1,000), anti-NR2B (cat. no. 4207s; Cell Signaling Technology, Inc., 1:1,000), anti-p-NR2B (cat. no. 4208s; Cell Signaling Technology, Inc., 1:1,000) and anti-GAPDH (cat. no. 97166s; Cell Signaling Technology, Inc., 1:1,000). GAPDH was used as the internal reference. Finally, Image J software (National Institutes of Health) was used to measure the gray value of protein bands and calculate the relative expression of target proteins.

Immunofluorescent staining. The expression levels of PAX6, p-NR2B and TRPV1 were determined by immunofluorescence

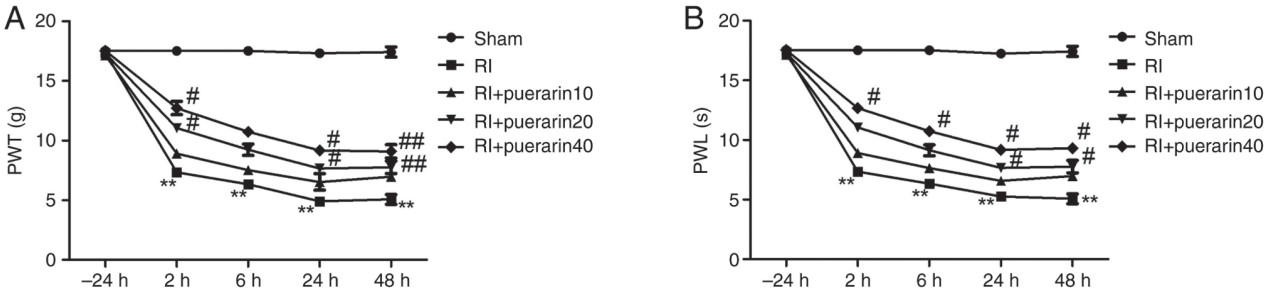

Figure 1. Effects of puerarin on remifentanil-induced hyperalgesia. When compared with sham group, the PWT (A) and PWL (B) in RI group were significantly decreased. Besides, pretreatment with puerarin (20 and 40 mg/kg) before remifentanil infusion prevented mechanical tenderness and hyperalgesia in a dose-dependent manner. The data were presented as the mean ± SD (n=8). **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the RI group. PWT, mechanical paw withdrawal threshold; PWL, paw withdrawal latency; RI, Remifentanil.

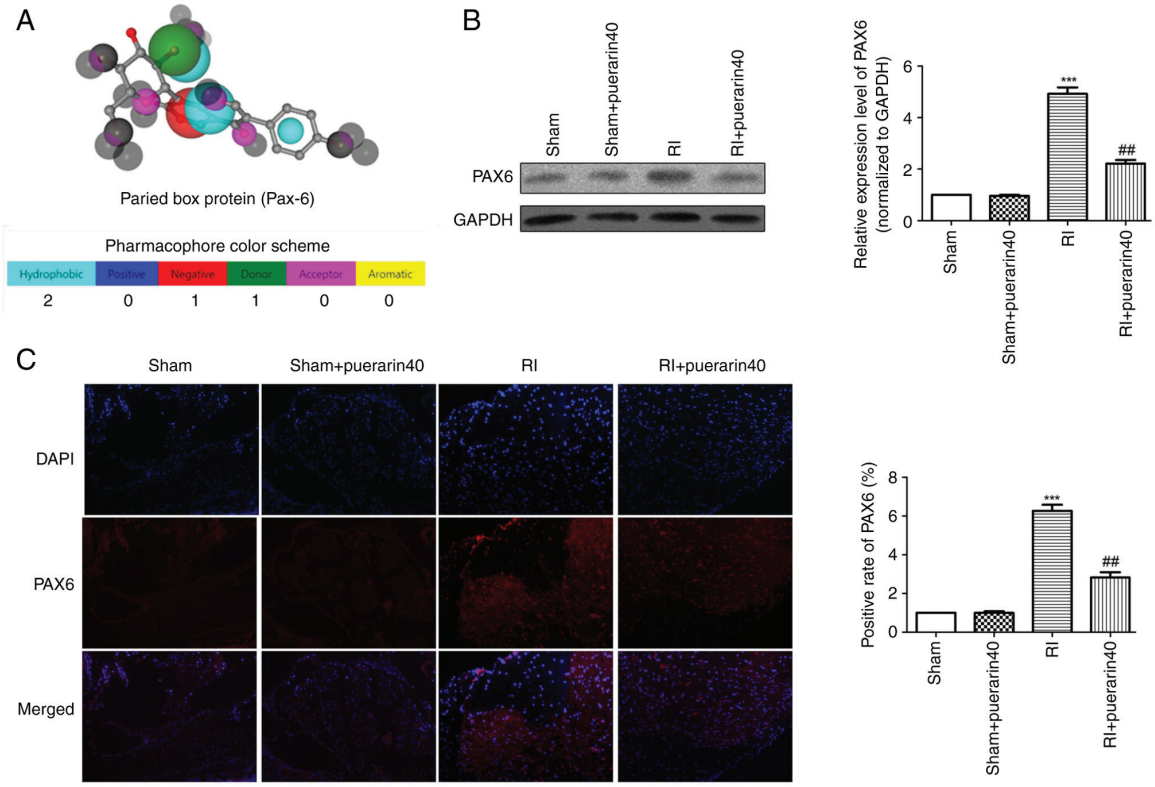

Figure 2. Puerarin alleviates the expression of PAX6 in remifentanil-induced spinal cord. (A) Predicted binding sites of puerarin to molecular PAX6 from Pharmmapper. (B) Western blot was used to detect the expression of PAX6 in different groups. (C) Immunofluorescence staining was used to detect the expression of PAX6 in the spinal cord. The data were presented as the mean ± SD (n=3). ***P<0.001 vs. the control group; $^{\#\#}$P<0.01 vs. the RI group. RI, Remifentanil.

staining according to the previously reported methods (14). Finally, the stained images were viewed under a fluorescence microscope and images were captured. Image J software (National Institutes of Health, Version 7.2) was used to analyze the fluorescence intensity and evaluate the positive signal.

Chromatin immunoprecipitation (Chip)-PCR. Chip-PCR analysis was performed as previously reported (15). Finally, PCR was used to quantify immunoprecipitated DNA, and all values were normalized to the input.

Statistical analysis. The data in the study were expressed as the mean ± SD. SPSS 22.0 software (IBM Corp.) package was used for statistical analysis. Comparison between the two groups was performed by unpaired t-test. One-way analysis of variance and minimum significant difference (LSD) tests were used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference. In animal experiments, there were six rats in each group.

Results

Effects of puerarin on RIH. As shown in Fig. 1, there were no significant differences between baseline PWLs to thermal stimulation and baseline PWTs to von Frey filaments in the treatment groups before the surgery (-24 h) (P<0.05, Fig. 1A and B). After surgery, PWT and PWL in the incision and + remifentanil group decreased at 2, 6, 24 and 48 h, and

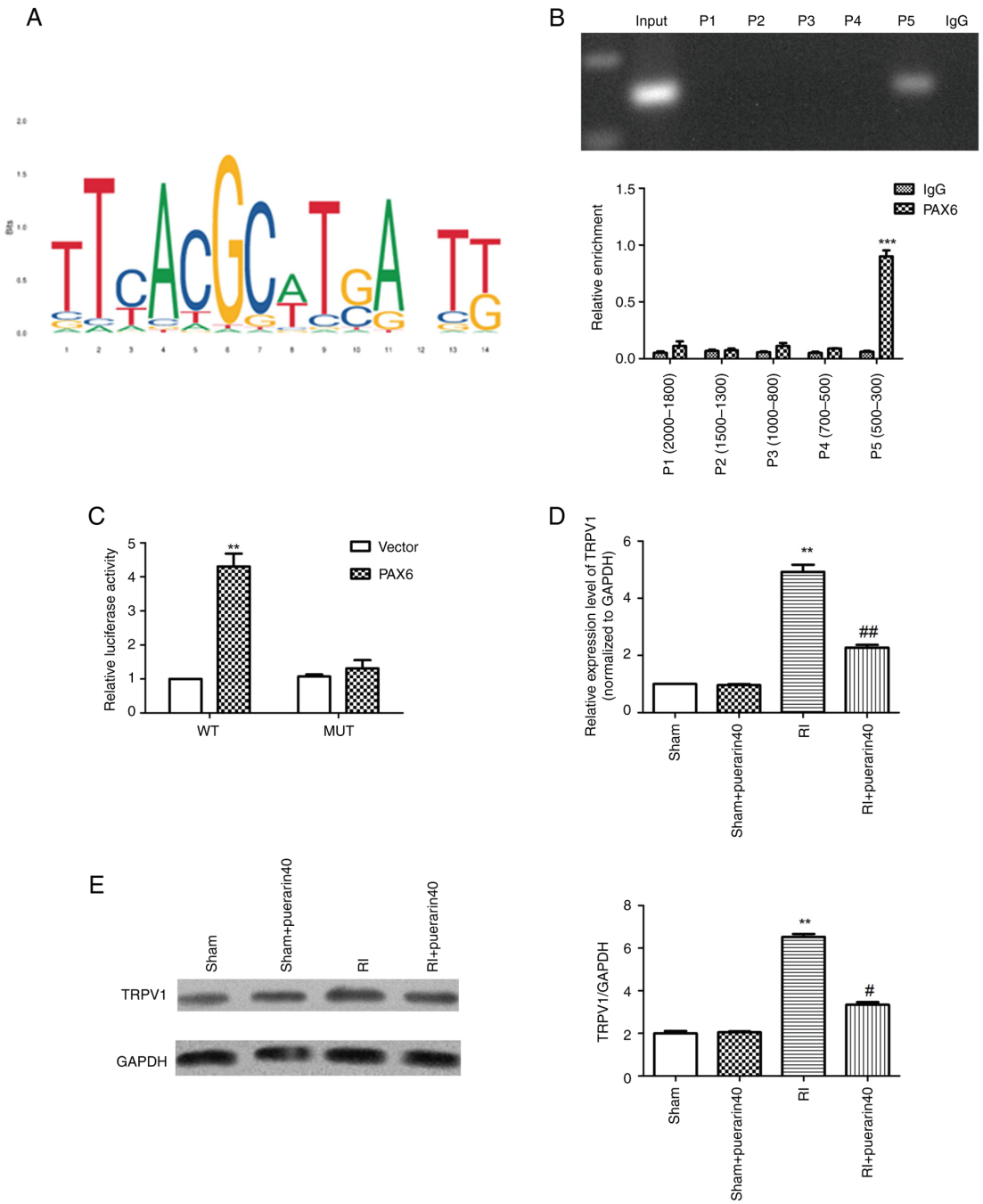

Figure 3. Puerarin regulates the transcription of TRPV1 via targeting PAX6. (A) Schematic diagram of canonical PAX6 binding motif (JASPAR database). (B) Chromatin immunoprecipitation-PCR assay was used to detect the relative enrichment. (C) Dual luciferase reporter assay was performed to detect the binding relationship between PAX6 and TRPV1. (D) Reverse transcription-quantitative PCR analysis was used to detect the mRNA expression levels of TRPV1 in different groups. (E) Western blot was used to determine the protein expression levels of TRPV1 in the spinal cord. The data were presented as the mean ± SD. **P<0.01 vs. the sham group; ***P<0.001, vs. IgG group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the RI group. TRPV1, transient receptor potential cation channel subfamily V Member 1; RI, Remifentanil; WT, wild-type; Mut, mutant.

postoperative RIH was noticeable and maintained from 2 to 48 h. Moreover, it reached a peak at 24 h and remifentanil appeared to facilitate hyperalgesia induced by incision. On the contrary, PWT and PWL did not demonstrate significant changes in saline rats (P<0.05, Fig. 1A and B). The results reflected that the postoperative RIH rat model was successfully established.

Puerarin alleviates the expression of PAX6 in remifentanil-induced spinal cord. Combined with Pharmmapper prediction (Fig. 2A), the sites of targeted binding of puerarin to PAX6 were analyzed and identified. Subsequently, western blot results (Fig. 2B) indicated that compared with the sham group, the expression levels of PAX6 were increased significantly in the RI group. While the expression of PAX6 in RI + puerarin40 group was reduced significantly compared with the RI group. Consistent with western blot results, immunofluorescence staining proved the same trend (Fig. 2C). The present findings suggested that Puerarin alleviates the expression of PAX6 in remifentanil-induced spinal cord.

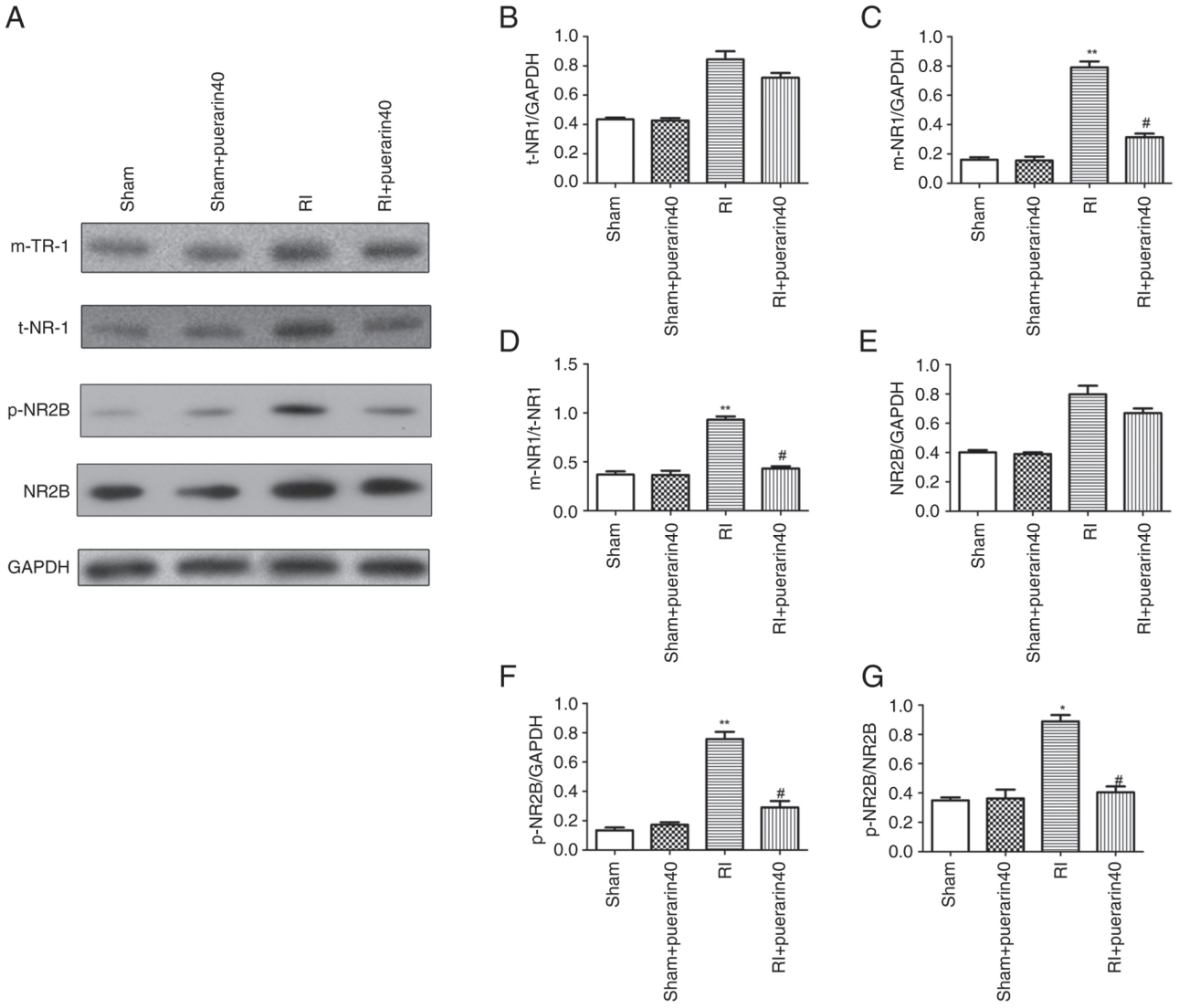

Figure 4. Effects of puerarin on NR1 trafficking in remifentanil-induced postoperative hyperalgesia. (A-G) The expression levels of t-NR1, m-NR1, p-NR2B and NR2B were detected by western blotting assay. (A) Bands of western blot analysis for protein expression levels. GAPDH was used as a loading control. Quantification of (B-D) t-NR1 and m-NR1, and (E-G) p-NR2B/NR2B in different groups. The data were presented as the mean ± SD. *P<0.05 and **P<0.01 vs. the sham group; $^{\#}$P<0.05 vs. the RI group. RI, Remifentanil.

Puerarin regulates the transcription of TRPV1 via targeting PAX6. Next, JASPAR was used to predict the base binding sequence between TRPV1 and PAX6 (Fig. 3A). Chip-PCR results (Fig. 3B) illustrated ~8-fold enrichment of PAX6-bound TRPV1 compared with the IgG group. Furthermore, dual luciferase reporter assay results (Fig. 3C) indicated that compared with the vector group, the luciferase activity was significantly increased. RT-qPCR results (Fig. 3D) demonstrated that the mRNA expression levels of TRPV1 in the tissues were increased significantly in the RI group compared with the sham group, while the expression of TRPV1 was reduced significantly in RI + puerarin group. Western blot results (Fig. 3E) revealed the same trend as RT-qPCR results. Overall, it was found that puerarin could regulate the transcription of TRPV1 via targeting PAX6.

The effects of puerarin on NR1 trafficking in postoperative RIH. In order to confirm the effect of puerarin on NR1 transport after remifentanil hyperalgesia, the NR1 protein expression level was detected by western blot assay. As demonstrated in Fig. 4A-D, compared with the sham group, the expression of NR1 protein and membrane protein in the spinal cord were significantly upregulated and downregulated after treatment. Moreover, western blot results revealed that the expression of p-NR2B was reduced significantly compared with the RI group (Fig. 4E-G). The aforementioned results suggested that puerarin may play a role in influencing the NR1 trafficking and NR2B phosphorylation of the spinal cord.

Overexpression of TRPV1 reverses the effects of puerarin on postoperative RIH. Immunofluorescence staining results (Fig. 5A) identified that the positive rate of TRPV1 in the tissue was significantly increased in the RI group compared with sham group. Compared with RI group, the positive rate of TRPV1 was reduced significantly in the RI + puerarin + AAV-con group, while the positive rate of TRPV1 in the RI + puerarin + AAV-TRPV1 group was reversed. Immunofluorescence staining results (Fig. 5B) proved that the

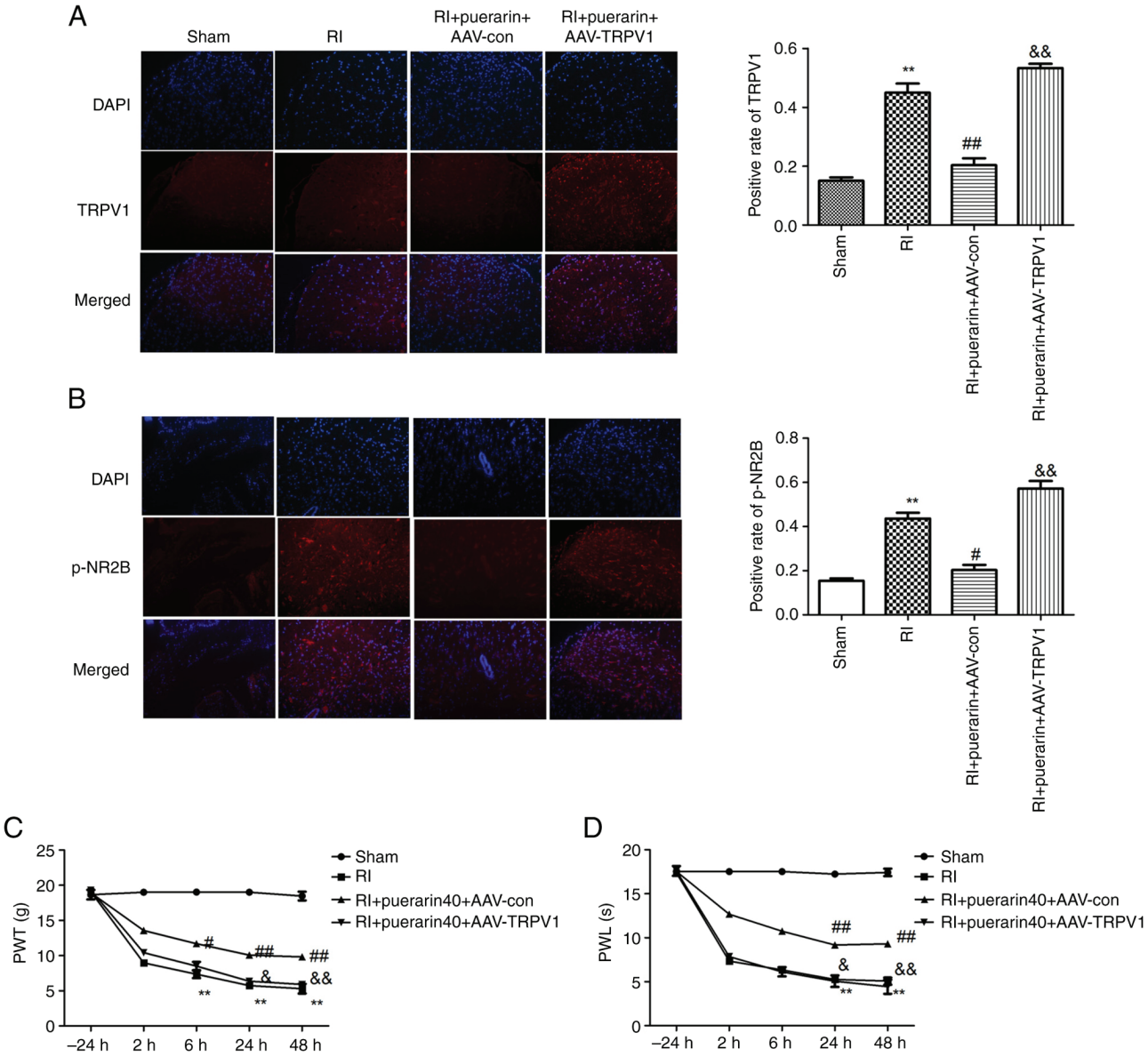

Figure 5. Effects of N-methyl-D-aspartate receptor antagonist MK-801 and puerarin in postoperative RIH. (A and B) Immunofluorescence staining was used to detect the expression levels of (A) TRPV1 and (B) p-NR2B in the tissues. The (C) PWT and (D) PWL in the RI group were detected. The data were presented as the mean ± SD (n=8). **P<0.01 vs. the sham group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the RI group; $^{\&}$P<0.05 and $^{\&\&}$P<0.01 vs. RI+puerarin40+AAV-con. TRPV1, transient receptor potential cation channel subfamily V Member 1; PWT, mechanical paw withdrawal threshold; PWL, paw withdrawal latency; RI, Remifentanil.

expression level of p-NR2B had the same trend. After surgery, PWT and PWL in the RI group decreased at 2, 6, 24 and 48 h, and postoperative RIH was noticeable and maintained from 2 to 48 h (Fig. 5C and D). In addition, both PWT and PWL were significantly increased in the RI + puerarin + AAV-con compared with the RI group. While the PWT and PWL showed a downtrend compared with the RI + puerarin + AAV-TRPV1 group. These results reflected that the overexpression of TRPV1 reversed the effects of puerarin on postoperative RIH.

Discussion

Although opioids relieve the pain caused by cancer, long-term exposure to opioids can lead to hyperalgesia (16,17). The present study mainly investigated the effect of puerarin on postoperative pain and allergy induced by remifentanil. It was discovered that puerarin regulates NR1 transmembrane transport and NR2B phosphorylation, and alleviates postoperative pain and hypersensitivity induced by remifentanil. The protective mechanism of puerarin is related to its targeted regulation of PAX6 and its effects on TRPV1 transcription.

As a traditional Chinese medicine, the pharmacological effects of puerarin have been widely reported, including clinical applications (18-20). It has been reported that puerarin can alleviate osteoporosis in OVX-induced mice by inhibiting osteoclast generation by inhibiting the TRAF6/ROS-dependent MAPK/NF-κB signaling pathway (21). In addition, puerarin could inhibit apoptosis by regulating SIRT3/SOD2 signaling pathway and alleviating nerve function defects in mice with subarachnoid hemorrhage (22). Fu *et al* (23) found that puerarin could downregulate the PPARγ signaling pathway and has therapeutic effect on atherosclerosis. Zhang *et al* (24) identified

that puerarin may preferentially block the β1 subunit of Nav1.8 in sensory neurons and participate in the anti-paclitaxel-induced neuropathic pain. Regarding opiate-induced postoperative hyperalgesia, it was found that puerarin could inhibit NR1 transport and NR2B phosphorylation and relieve OIH.

As a member of the paired box family, PAX6 plays a key role in brain development (25). As a conserved transcription factor, PAX6 has 2 distinct DNA-binding domains and also mediates embryonic and adult neurogenesis (26). DNMT3b-mediated hypomethylation of PAX6 gene may be involved in mechanical allodynia after drug therapy (27). It was hypothesized that PAX6 may also play an important role in opiate-induced postoperative hyperalgesia. Most importantly, further molecular mechanism studies (28,29) suggested that overexpression of TRPV1 could reverse the inhibitory effect of puerarin on OIH. In addition, it was demonstrated that puerarin could target and regulate the expression of PAX6 and affect the transcription of TRPV1.

Puerarin could relieve postoperative hyperalgesia caused by remifentanil that may be associated with N-methyl-D-aspartate (NMDA) activation through intracellular pathways, thereby upregulating the function of NMDA activity (30). The distribution of NMDA receptor NR2B in the superficial dorsal horn is almost limited. The expression of NR2B subunit in the superficial dorsal horn was significantly increased due to the sensitivity signal transmission of the spinal cord. The findings of the present study suggested that puerarin could inhibit the transmembrane transport of NR1 and the phosphorylation of NR2B.

The present results confirmed that puerarin could alleviate remifentanil-induced postoperative pain by targeting PAX6 to regulate TRPV1 transcriptional expression. On the other hand, the current findings explained the molecular mechanism through which puerarin alleviates OIH, which may provide theoretical support for the further expansion of its range of clinical applications.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LY wrote the manuscript, conceptualized the study, developed methodology and performed software analysis. YL collected the data, wrote a draft of the manuscript, performed the data presentation and produced the figures. YS visualized data and conducted investigation. LR supervised the study and developed methodology. XG performed software analysis, validated and curated data. LC developed methodology and performed software analysis. GZ visualized data and performed software analysis. XS validated data. QH performed software analysis. XC developed methodology. GG designed experiments and reviewed and edited the manuscript. YS and GG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The animal experiments in the present study were approved (approval no. 20210329015) by the Experimental Animal Ethics Committee of the General Hospital of the Western Theater Command of the PLA (Chengdu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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