Article

Discovery Medicine 2026; 38(207): 1219–1229
https://doi.org/10.24976/Discov.Med.202638207.97

Driven by Regulation of the NOX4/ROS/NF-κB Signaling Cascade, Angiopoietin-1 Emerges as a Potent Modulator of Post-Surgical Pain

Feifei Ma[1], Bing Han[2], Xiufang Wang[1], Lulu Liu[1], Mengmeng Li[1], Junhua Hu[3], Xinnan Song[4,*]

[1]Anesthesiology Department, Jinan Third People's Hospital, 250132 Jinan, Shandong, China
[2]The Department of Respiratory Medicine, Jinan Third People's Hospital, 250132 Jinan, Shandong, China
[3]Neurosurgery Department, The 990th Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army, 464000 Zhumadian, Henan, China
[4]The Fifth Clinical Medical College, Guilin Medical University, 541199 Guilin, Guangxi Zhuang Autonomous Region, China
*Correspondence: songxinnan_Sn@163.com (Xinnan Song)
Submitted: 12 December 2025 Revised: 11 February 2026 Accepted: 19 March 2026 Published: 20 April 2026

Background: Post-surgical pain is a common clinical complication following surgical procedures, significantly impacting patient comfort, functional recovery, and long-term prognosis. Its pathological mechanisms, particularly those involving vascular and inflammatory pathways, remain incompletely understood, which limits therapy options and necessitates the exploration of novel therapeutic targets. Angiopoietin-1 (Ang-1), a crucial growth factor, is essential for vascular homeostasis and inflammatory regulation, processes implicated in pain pathogenesis, while playing an unknown role in post-surgical pain. Herein, we aimed to elucidate the role of Ang-1 in post-surgical pain and its potential molecular mechanisms for pain relief.
Methods: Post-surgical pain rat models were established using skin/muscle incision and retraction (SMIR), followed by assessment of pain hypersensitivity (paw withdrawal threshold (PWT) and paw withdrawal latency (PWL)). After Ang-1 treatment, examinations were performed on oxidative stress and inflammation in dorsal root ganglion (DRG) neurons, as well as whether NADPH oxidase 4 (NOX4)–reactive oxygen species (ROS)–nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway was implicated.
Results: SMIR induced pronounced pain hypersensitivity, reflected by downregulated PWT and PWL, accompanied by reduced Ang-1 expression in DRG neurons ($p < 0.001$). Elevation of Ang-1 alleviated post-surgical pain behaviors and attenuated the upregulation of ROS, NOX4, Tumor Necrosis Factor-alpha (TNF-α), and Interleukin-6 (IL-6) in SMIR-exposed DRG neurons ($p < 0.001$). Moreover, NF-κB level was enhanced by SMIR, yet later suppressed by Bay ($p < 0.001$). Ang-1 also reduced DRG neuronal apoptosis and inflammation, suggesting the inhibitory role of NF-κB ($p < 0.001$).
Conclusion: Ang-1 alleviates post-surgical pain by modulating oxidative stress and inflammatory responses via NOX4/ROS/NF-κB signaling pathway in DRG neurons. These findings identify Ang-1 as a promising candidate for effective post-surgical pain management and the development of novel analgesic strategies.

Keywords: angiopoietin-1; post-surgical pain; reactive oxygen species; inflammation; NADPH oxidase 4

Introduction

Post-surgical pain is defined as persistent or intermittent pain following surgical procedures, commonly categorized by acute pain (typically lasting less than 3 months) and persistent (or chronic) post-surgical pain (lasting beyond 3 months) [1]. This pain is primarily associated with surgical trauma, inflammation, and subsequent tissue healing [2]. While acute pain is an expected and often unavoidable physiological response, persistent post-surgical pain represents a significant clinical challenge that requires further intervention. Beyond causing immediate discomfort, especially when persistent, post-surgical pain can trigger a cascade of adverse physiological and psychological effects, ultimately hindering post-surgical recovery [3]. For example, inadequately controlled post-surgical pain frequently delays mobilization, impairs functional outcomes, and compromises rehabilitation efficiency [4]. Moreover, persistent or severe pain can aggravate psychological disturbances, including insomnia, anxiety, and depression [5]. Accumulating evidence indicates that post-surgical pain results from complex and multifactorial mechanisms, including peripheral nerve injury, altered spinal neuronal excitability, and central sensitization, all of which contribute to the onset and persistence of pain [6]. Although current treatment strategies (non-steroidal anti-inflammatory drugs, opioids,

Copyright: © 2026 The Author(s). Published by Discovery Medicine. This is an open access article under the CC BY 4.0 license.
Note: *Discovery Medicine* stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

DM DISCOVERY MEDICINE 2001

regional anesthesia, and physical therapy) are widely used, their effectiveness remains suboptimal and is often accompanied by adverse effects [7]. Additionally, neuroimmune interactions within the dorsal root ganglion (DRG) and spinal cord play a key role in the pathogenesis of post-surgical pain. Surgical injury can activate DRG neurons and trigger spinal glial responses, including microglia and astrocytes, leading to the release of pro-inflammatory mediators and sustained nociceptive signaling [8,9]. This neuroinflammatory signaling cascade is a key driver of central sensitization and chronic pain persistence. Therefore, identifying novel therapeutic targets is essential for improving pain management and accelerating post-surgical recovery.

Angiopoietin-1 (Ang-1) from the angiopoietin family is predominantly expressed in endothelial cells and can impact vascular maturation and stabilization [10]. Through binding to its receptor Tie2, Ang-1 maintains vascular integrity by regulating angiogenesis and endothelial cell homeostasis [11]. Notably, to date, direct evidence supporting the role of Ang-1 in brain endothelial cells or neurological diseases remains scarce. Growing evidence has demonstrated that Ang-1 plays a significant role in the progression of various diseases. For instance, in diabetic retinopathy, Ang-1 exerts protective effects by stabilizing the retinal vasculature [12]. Additionally, aberrant angiogenesis is implicated in the development and persistence of pain, suggesting a potential mechanistic link between vascular regulation and nociception [13]. Inflammation, a major driver of post-surgical pain, is also modulated by Ang-1, which exhibits anti-inflammatory properties and influences nuclear factor-κB (NF-κB)-mediated signaling pathways [14,15]. These findings imply that post-surgical alterations in Ang-1 expression may affect vascular repair and inflammation responses, thereby influencing both the intensity and duration of pain.

Herein, Ang-1 expression was quantitatively evaluated in rat models of post-surgical pain, followed by assessment of its impact on mechanical hypersensitivity. Previous studies have shown that Ang-1 confers neuroprotection by counteracting apoptotic stress [16]. Building on this, we further investigated the role of Ang-1 in modulating inflammation and apoptosis in L4/L5 dorsal root ganglion (DRG) neurons, with a focus on the NADPH oxidase 4 (NOX4)/reactive oxygen species (ROS)/NF-κB signaling pathway. Collectively, we elucidate the mechanistic involvement of Ang-1 in post-surgical pain and provide a theoretical basis for developing innovative therapeutic strategies.

Materials and Methods

Model Construction

Eighteen male Sprague–Dawley rats (10 weeks old, 200–220 g, Hangzhou Medical College, China) were housed (22–24 °C, 45–65% humidity, 12 h light/dark cycle) with food and water available *ad libitum*. Experiments were approved by the Zhejiang Experimental Animal Centre (No. ZJCLA-IACUC-20040181). Four groups of rats (n = 3 per group) were established: Control, skin/muscle incision and retraction (SMIR), SMIR alone (serving as control for SMIR+Ang-1), and SMIR+Ang-1. SMIR modeling [17] was performed under anesthesia (1% pentobarbital sodium; 50 mg/kg, P3761, Sigma-Aldrich, St. Louis, MO, USA) by making a 1.5–2 cm incision along the medial thigh to expose the muscle, followed by a 7–10 mm incision of the superficial adductor muscle layer. The muscle was separated to allow placement of a microdissection retractor (Biomedical Research Instruments Inc., USA), with all four prongs placed beneath the superficial muscle layer. The skin and superficial muscle were retracted by 2 cm for 1 h. Additional anesthesia was administered as needed using 4% isoflurane (26675-46-7, Jizhi Biotech, Shanghai, China). After 1 h, the incision was closed using 3.0 and 4.0 Vicryl sutures (Hanfei Medical, Shanghai, China). Control rats underwent skin and muscle incision without retraction. For Ang-1 treatment, angiopoietin-1 peptide (1075214-55-9; Cote Biosciences, Shanghai, China) was dissolved in distilled water to prepare an 800 µg/mL solution. Rats in the SMIR+Ang-1 group received 0.5 mL Ang-1 solution (400 µg) via tail vein once daily for one week, starting post-SMIR [18]. After behavioral testing, rats were euthanized with pentobarbital sodium (50 mg/kg) followed by cervical dislocation.

Behavioral Testing

Before baseline (BL) testing, rats were acclimated to the behavioral apparatus for at least three days. Mechanical hypersensitivity was assessed using Von Frey filaments (NC12775, Yuyan Instruments, Shanghai, China), and paw withdrawal threshold (PWT) was measured using the up-and-down method [19]. Thermal hyperalgesia was evaluated by paw withdrawal latency (PWL) using a thermal stimulator (37550, Yuyan Instruments, Shanghai, China) [20], with radiant heat applied to the plantar surface of each hind paw through a glass floor. At each time point (BL, days 1, 4, 7, 14, 21), three measurements per paw were recorded and averaged, with at least 5 min intervals between tests. Left and right paws were tested alternately.

Culture of DRG Neurons

Two groups (Con and SMIR; n = 3 per group) of rats received treatment (14 days), anesthesia with pentobarbital sodium (50 mg/kg), and euthanasia by cervical dislocation. DRG (L4–L5) extraction was performed according to established protocols [21]. Ganglia were dissected in ice-cold oxygenated buffer and incubated in a solution containing collagenase D (1 mg/mL, COLLD-RO, Sigma-Aldrich, St. Louis, MO, USA) and trypsin (40101ES25, YEASEN, Shanghai, China) for 1 h. Dissociated DRG cells were cultured in Neurobasal-A medium (10888022,

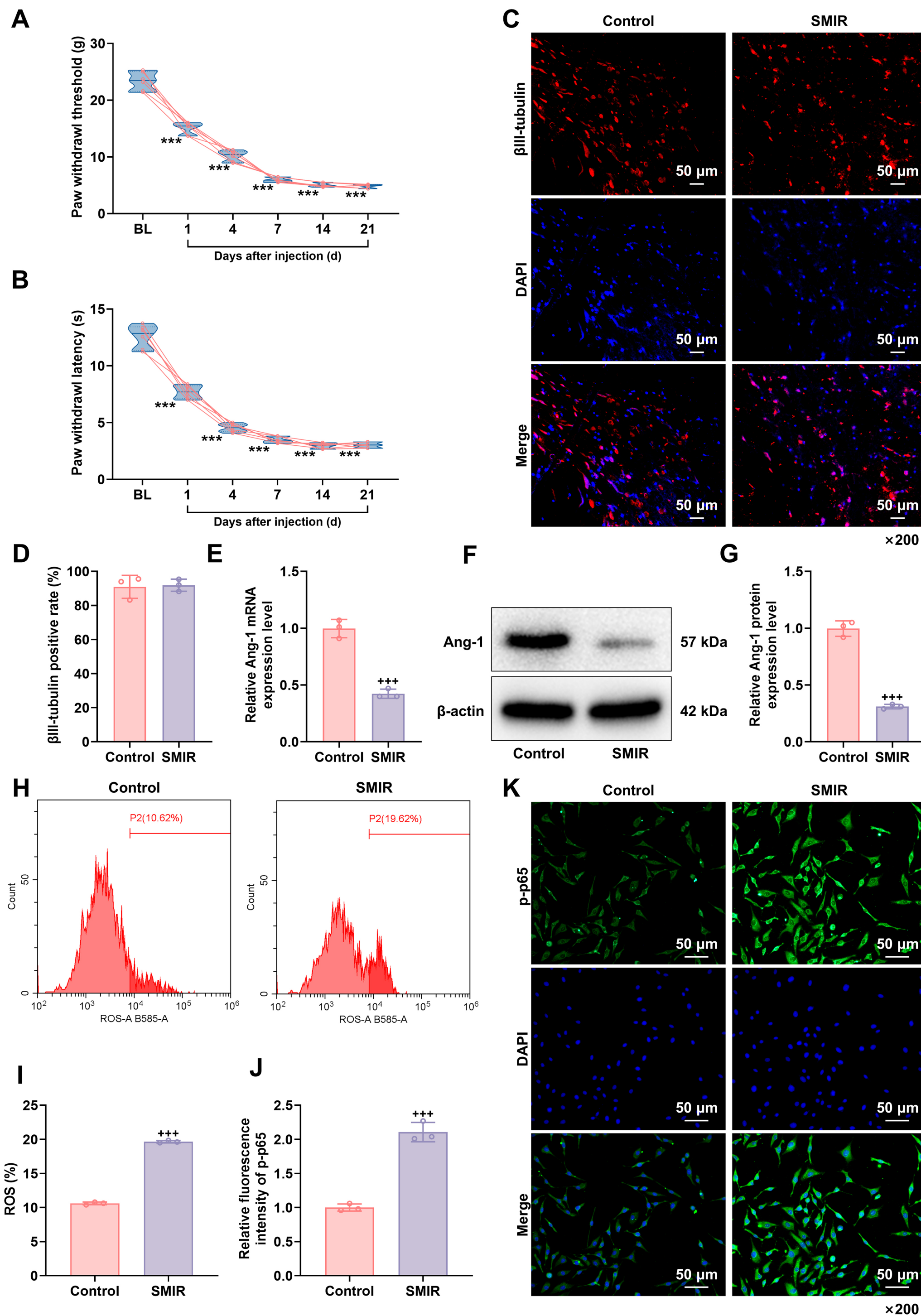

Fig. 1. Changes in Pain Sensitivity, Angiopoietin-1 Expression, Reactive Oxygen Species (ROS), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) Activation in Dorsal Root Ganglion (DRG) neurons after post-skin/muscle incision and retraction (SMIR) surgery. (A,B) Paw Withdrawal Threshold (PWT) and Paw Withdrawal Latency (PWL) at baseline (BL) and on days 1, 4, 7, 14, and 21 post-SMIR surgery. (C) Immunofluorescence staining was used to detect the purity of DRG neurons. Magnification 200×, scale bar 50 µm. (D) Quantitative analysis of DRG neuron purity based on immunofluorescence staining results. (E) *Ang-1* mRNA levels in DRG neurons (Quantitative real-time polymerase chain reaction (qRT-PCR), *β-actin* as an internal control). (F,G) Ang-1 protein levels in DRG neurons (Western blotting (WB), β-actin as an internal control). (H,I) ROS levels in DRG neurons (Flow cytometry). (J,K) Phosphorylated p65 (p-p65) in the nuclei of DRG neurons (Immunofluorescence). Magnification 200×, scale bar 50 µm. Each experiment was performed independently three times. $^{***}p < 0.001$ compared to BL. $^{+++}p < 0.001$ compared to Control group. Ang-1, Angiopoietin-1.

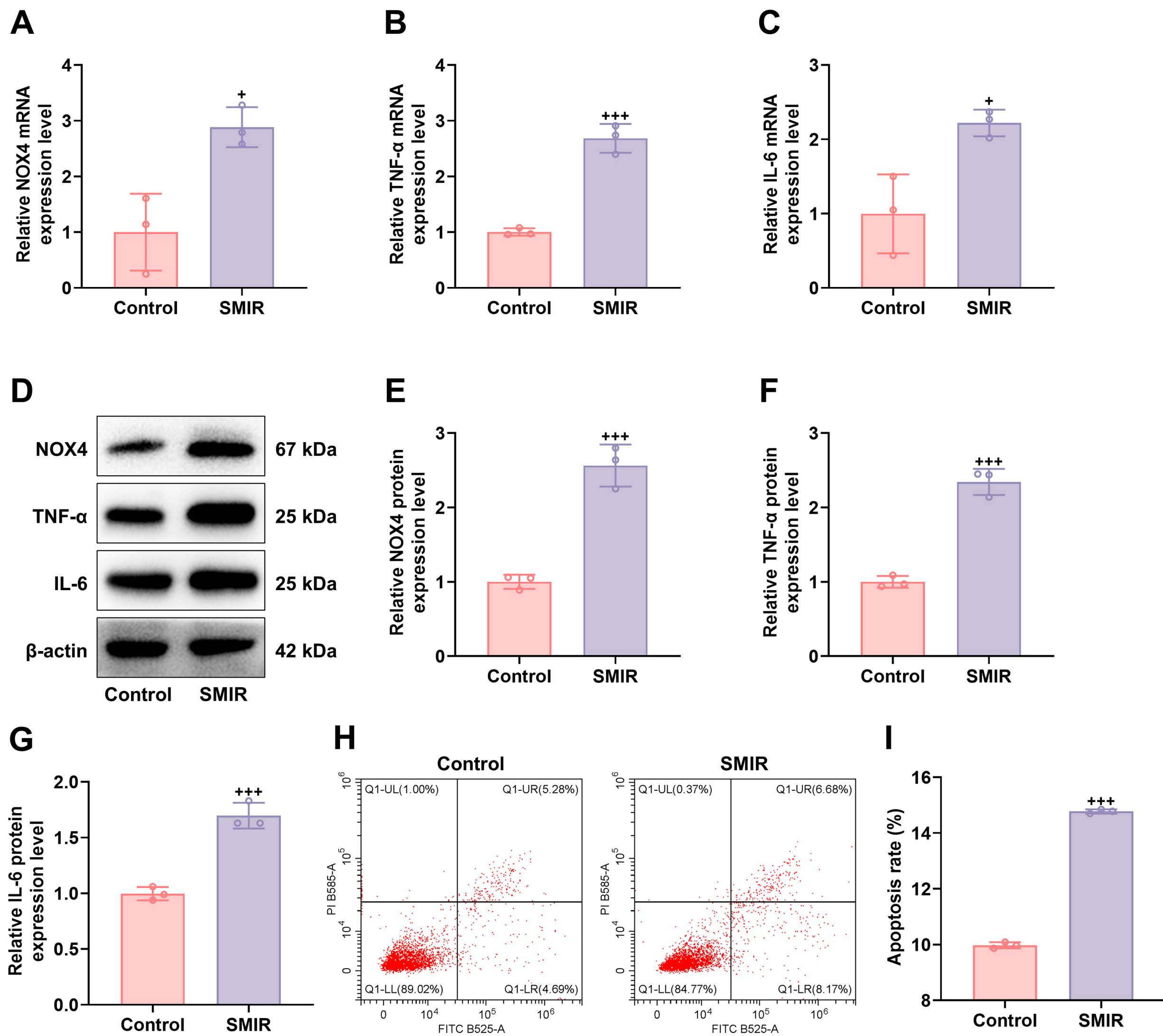

Fig. 2. mRNA and protein expression of NADPH oxidase 4 (NOX4), Tumor Necrosis Factor-alpha (TNF-α), Interleukin-6 (IL-6) and apoptosis levels in DRG neurons after SMIR. (A–C) *NOX4* (A), *TNF-α* (B), and *IL-6* (C) mRNA levels in DRG neurons (qRT-PCR, *β-actin* as an internal control). (D–G) Representative Western blot bands (D) and quantitative analysis of NOX4 (E), TNF-α (F), and IL-6 (G) protein levels in DRG neurons (Western blotting, β-actin as an internal control). (H,I) Apoptosis in DRG neurons (Flow cytometry). Each experiment was performed independently three times. $^{+}p < 0.05$, $^{+++}p < 0.001$ compared to Control group.

Thermo Fisher, Waltham, MA, USA) supplemented with 2% B27 (12587010), 2 mM GlutaMAX (35050061), and penicillin–streptomycin (15140122) at 5% CO_2 and 80% humidity. To remove non-neuronal cells, cultures were treated with uridine (ST1735, Beyotime, Shanghai, China) and 20 mM 5-fluoro-2′-deoxyuridine (50-91-9, MERCK, Darmstadt, Germany) for three days. To verify the neuronal purity of the cultures, cells were fixed and immunostained with an antibody against the neuron-specific marker β-III-tubulin (T2200, 10-20 µg/mL, Sigma-Aldrich, CA, USA). Nuclei were counterstained with DAPI. Cultures were imaged using a fluorescence microscope. Only cultures with neuronal purity exceeding 90% were used for subsequent experiments.

Cell Treatment

Angiopoietin-1 overexpression vector (oe-Ang-1; pRP [Exp]-EGFP-CMV>rAngpt1 [NM_053546.2]) and negative control (NC) were purchased from Yunzhou Biotech (Guangzhou, China). Six groups were established: Control (Con), Con+Bay, SMIR, SMIR+NC, SMIR+oe-Ang-1, and SMIR+Bay. DRG neurons from sham-operated rats served as the Con group, while DRG from SMIR rats served as the SMIR group.

For transfection, 2 µg of either NC or oe-Ang-1 plasmid was introduced into DRG neurons using Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA), and cells were harvested 48 h later. Transfection efficiency was examined using quantitative real-time polymerase chain reac-

tion (qRT-PCR). For NF-κB inhibition, DRG neurons were pretreated for 2 h with Bay 11-7082 (2 µM; S44144, Yuanye Biotech, Shanghai, China) in the Con+Bay and SMIR+Bay groups [22].

qRT-PCR

Total RNA was extracted using TRIzol reagent (R0016, Beyotime, Shanghai, China), and its purity was assessed with SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). cDNA was then synthesized using the Transcriptor First Strand cDNA Synthesis Kit (11483188001, Roche, Mannheim, Germany). qRT-PCR was conducted on an ABI 7500 system with SYBR Green PCR Master Mix (4344463, Thermo Fisher,Waltham, MA, USA). Relative mRNA levels were quantified using the $2^{-\Delta\Delta CT}$ method and normalized to *β-actin* [23]. The primer sequences (Sangon, Shanghai, China) are listed in Table 1.

Western Blotting (WB)

DRG neurons were lysed using RIPA buffer (P0013, Beyotime, Shanghai, China), and protein concentrations were determined with BCA kit (P0009, Beyotime). Equal amounts of protein (60 µg) were separated via SDS-PAGE (P0690, Beyotime) and transferred onto PVDF membranes (FFP24, Beyotime). After 5% skim milk blockage, membranes were incubated overnight at 4 °C with primary antibodies (Abcam, Cambridge, UK unless otherwise specified), Ang-1 (ab8451, rabbit polyclonal, 1:1000), p-p65 (ab76302, rabbit monoclonal, 1:1000), p65 (10745-1-AP, rabbit polyclonal, 1:2000, Proteintech, Wuhan, China), NOX4 (14347-1-AP, rabbit polyclonal, 1:2000), IL-6 (ab259341, rabbit monoclonal, 1:1000), TNF-α (ab183218, rabbit monoclonal, 1:1000), and β-actin (ab8227, rabbit polyclonal, 1:1000), followed by incubation with HRP-conjugated secondary antibodies (ab6721, goat anti-rabbit IgG, 1:5000) for 1 h at room temperature (RT). Signals were visualized using an enhanced chemiluminescence kit (KGC4902, Keygene, Jiangsu, China) and imaged with a gel documentation system (Bio-Rad, Hercules, CA, USA). Protein band intensities were quantified using ImageJ 1.54p (NIH, Bethesda, MD, USA).

ROS Analysis

Intracellular ROS levels were quantified using flow cytometry in combination with the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; 50101ES01, YEASEN, Shanghai, China). The probe was diluted 1:1000 in Neuro Basal-A medium and added to the cultured DRG neurons. Cells were incubated for 30 min at 37 °C in the dark, followed by three washes with fresh Neuro Basal-A medium to remove excess dye. ROS fluorescence intensity was measured using a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Immunofluorescence

DRG neurons were fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) for 30 min at RT and washed with PBS (C0221A, Beyotime, Shanghai, China). After blocking with blocking buffer (P0096, Beyotime, Shanghai, China) for 1 h, cells were incubated overnight at 4 °C with NF-κB p-p65 antibody (SAB5700333, rabbit polyclonal, 1:50, Sigma-Aldrich, St. Louis, MO, USA) diluted in the same blocking buffer. Following washing with PBS containing 0.1% Tween-20 (ST828, Beyotime, Shanghai, China), cells were cultivated with goat anti-rabbit H&L (Alexa Fluor® 488) (ab150077, 1:1000, Abcam, Cambridge, UK) for 2 h at RT in the dark. Nuclei were counterstained with DAPI (C1005, Beyotime, Shanghai, China) for 5–10 min in the dark. Confocal images were captured using a confocal microscope (200×, Olympus, Tokyo, Japan) equipped with an Andor camera (Manchester, CT, USA). Green fluorescence intensity representing p-p65 expression was analyzed using Imaris software 11.0 (Bitplane AG, Andor Technology, Belfast, UK).

Cell Apoptosis

DRG neurons were digested and resuspended in binding buffer to prepare a single-cell suspension. Apoptotic cells were labeled using the Annexin V-FITC Apoptosis Detection Reagent (C1062, Beyotime, Shanghai, China), with staining performed for 30 min at RT in the dark. Apoptosis was assessed using flow cytometry.

Table 1. Primer sequences used in quantitative real-time polymerase chain reaction (qRT-PCR).

Gene	Forward (5′-3′)	Reverse (5′-3′)
Ang-1	TGCCATTACCAGTCAGAGG	CAAGCATCAAACCACCATC
NOX4	ACAGTCCTGGCTTACCTTCG	TTCTGGGATCCTCATTCTGG
TNF-α	GACCCTCACACTCAGATCATCTTC	TGCTACGACGTGGGCTACG
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
β-actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

Ang-1, Angiopoietin-1; NOX4, NADPH oxidase 4; TNF-α, Tumor Necrosis Factor-alpha; IL-6, Interleukin-6.

DM DISCOVERY MEDICINE 2001

Statistical Analysis

All data are presented as mean ± standard deviation (SD) and analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Comparisons between the two groups were performed using independent-samples *t*-tests. For intergroup comparisons, one-way repeated-measures ANOVA followed by Dunnett's post hoc test was employed, while multiple comparisons were analyzed using two-way repeated-measures ANOVA with Sidak's post hoc test. For other datasets involving more than two groups (n > 2), one-way ANOVA followed by Tukey's post hoc test was conducted. $p < 0.05$ denoted statistical significance.

Results

SMIR Treatment Suppresses Ang-1 Expression and Activates the NOX4/ROS/NF-κB Pathway in DRG Neurons

Behavioral assessments showed PWT and PWL were significantly decreased in the SMIR group from days 1 to 21, reaching their lowest value on day 14 ($p < 0.001$, Fig. 1A,B). DRG neurons collected on day 14 post-SMIR were used for subsequent experiments. The cells in our cultures were positively stained for β-III-tubulin, confirming a high purity of neurons (Fig. 1C,D). The qRT-PCR and WB analyses revealed significantly decreased Ang-1 mRNA and protein levels in DRG neurons following SMIR ($p < 0.001$, Fig. 1E–G) and elevated ROS levels ($p < 0.001$, Fig. 1H,I). Immunofluorescence staining demonstrated that nuclear p-p65 expression was markedly increased in DRG neurons following SMIR ($p < 0.001$, Fig. 1J,K). WB and qRT-PCR results further showed that SMIR upregulated NOX4, TNF-α, and IL-6 proteins and mRNA in DRG neurons ($p < 0.05$, Fig. 2A–G), as well as enhanced DRG neuronal apoptosis ($p < 0.001$, Fig. 2H,I).

Ang-1 Alleviates Post-Surgical Pain by Inhibiting the NOX4/ROS/NF-κB Pathway

SMIR-induced Ang-1 downregulation in DRG neurons was significantly reversed by Ang-1 overexpression ($p < 0.001$, Fig. 3A). The elevated ROS production and nuclear p-p65 levels in the SMIR group were markedly reduced following Ang-1 overexpression ($p < 0.01$, Fig. 3B–E). Ang-1 overexpression effectively reversed SMIR-induced increases in NOX4, TNF-α, and IL-6 levels ($p < 0.001$, Fig. 4A–D). SMIR group exhibited upregulation of NOX4, p-p65/p65, TNF-α, and IL-6 levels ($p < 0.001$, Fig. 4E–I). After SMIR, NF-κB inhibition decreased p-p65/p65, TNF-α, and IL-6 levels ($p < 0.01$), whereas NOX4 levels remained unchanged (Fig. 4E–I). Ang-1 overexpression also reversed the increase of apoptosis in SMIR-triggered DRG neurons ($p < 0.001$, Fig. 5A,C). Consistently, apoptosis was higher in the SMIR group and lower in the control+BAY group compared with their respective controls ($p < 0.001$, Fig. 5B,D). Furthermore, apoptosis was markedly reduced in the SMIR+BAY group ($p < 0.001$, Fig. 5B,D). *In vivo*, both PWT and PWL were decreased progressively from day 1 to day 21 in the SMIR group ($p < 0.01$, Fig. 5E,F), which was offset by Ang-1 overexpression ($p < 0.01$, Fig. 5E,F). Notably, in the SMIR+Ang-1 group, PWT remained below BL on days 1 and 4 but exceeded BL on days 7, 14 and 21 ($p < 0.05$, Fig. 5E). Similarly, PWL exceeded BL on days 4, 7, and 21 but remained below BL on days 1 and 14 ($p < 0.05$, Fig. 5F).

Discussion

Herein, we demonstrated that SMIR-treated animals exhibited reduced PWT and PWL, which was reversed by exogenous Ang-1, revealing the alleviating role of Ang-1 in post-surgical pain. Notably, SMIR treatment diminished Ang-1 expression and augmented ROS levels in DRG neurons. ROS, as critical mediators of oxidative stress, are closely associated with cellular damage and dysfunction [24]. Previous studies have emphasized the critical role of oxidative stress in post-surgical pain, as tissue injury triggers ROS accumulation, pain-related signaling pathway activation, and amplification of pain via pro-inflammatory mediator release [25–27]. Antioxidant interventions relieve post-surgical pain, underscoring oxidative stress as a contributor to pain [28]. Herein, Ang-1 overexpression effectively suppressed the SMIR-induced increase in ROS, highlighting its antioxidative role. Inflammation, a protective response to tissue injury or infection, releases pro-inflammatory factors such as TNF-α and IL-6 to coordinate immunity [29]. TNF-α exerts pro-inflammatory effects by recruitment and activation of inflammatory cells, while IL-6 modulates immune responses by driving acute-phase protein synthesis and regulating immune cell activity [30,31]. Post-surgical local inflammation sensitizes nociceptors and exacerbates pain, with TNF-α and IL-6 as vital factors [32]. In this study, Ang-1 abrogated SMIR-induced increases in TNF-α and IL-6 in DRG neurons, signifying that Ang-1 attenuates post-surgical pain through dual suppression of oxidative stress and inflammation. While TNF-α, NF-κB, IL-6 and Ang-1 are often linked to non-neuronal cells, this study focused on their roles within DRG neurons for two reasons. First, activation of inflammatory pathways in sensory neurons represents a direct mechanism driving nociceptive sensitization [33]. Second, we propose a paracrine regulatory axis, where mediators like Ang-1 may modulate neuronal signaling pathways, such as NOX4/ROS/NF-κB [34], highlighting a direct cross-cellular interaction in pain pathology.

NOX4 from the NADPH oxidase family resides in multiple cell types (endothelial and smooth muscle cells) [35]. It produces ROS through redox reactions and regulates proliferation, apoptosis, inflammation, and fibrosis [36,37]. NOX4 drives ROS generation and

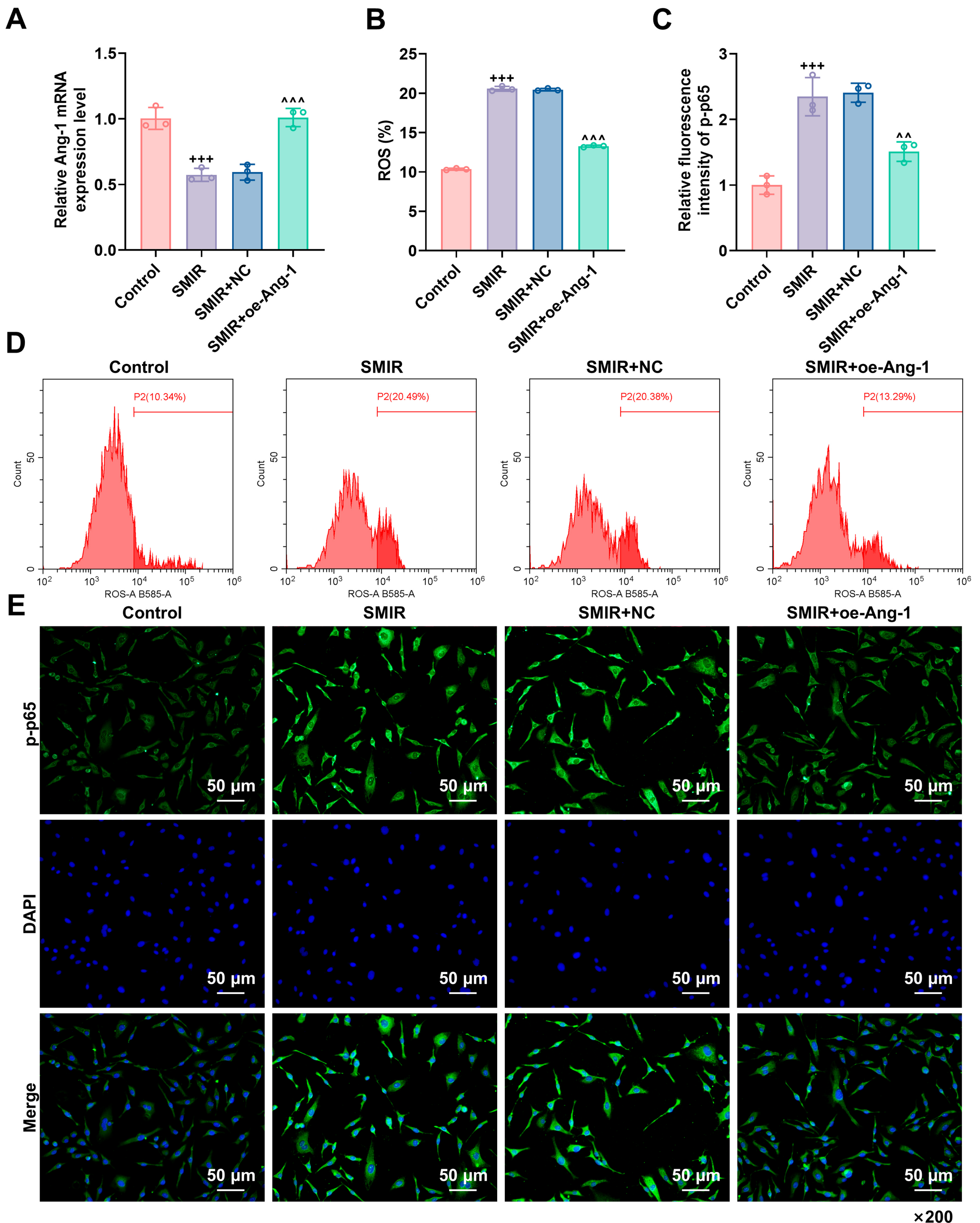

Fig. 3. Effects of Ang-1 overexpression on ROS levels, p-p65 expression, and Angiopoietin-1 mRNA levels in DRG neurons following SMIR surgery. (A) *Ang-1* mRNA levels in DRG neurons (qRT-PCR, *β-actin* as an internal control). (B,D) ROS levels in DRG neurons (Flow cytometry). (C,E) p-p65 in the nuclei of DRG neurons (Immunofluorescence). Magnification 200×, scale bar 50 µm. Each experiment was performed independently three times. $^{+++}p < 0.001$ compared to Control group. $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ compared to SMIR+NC group.

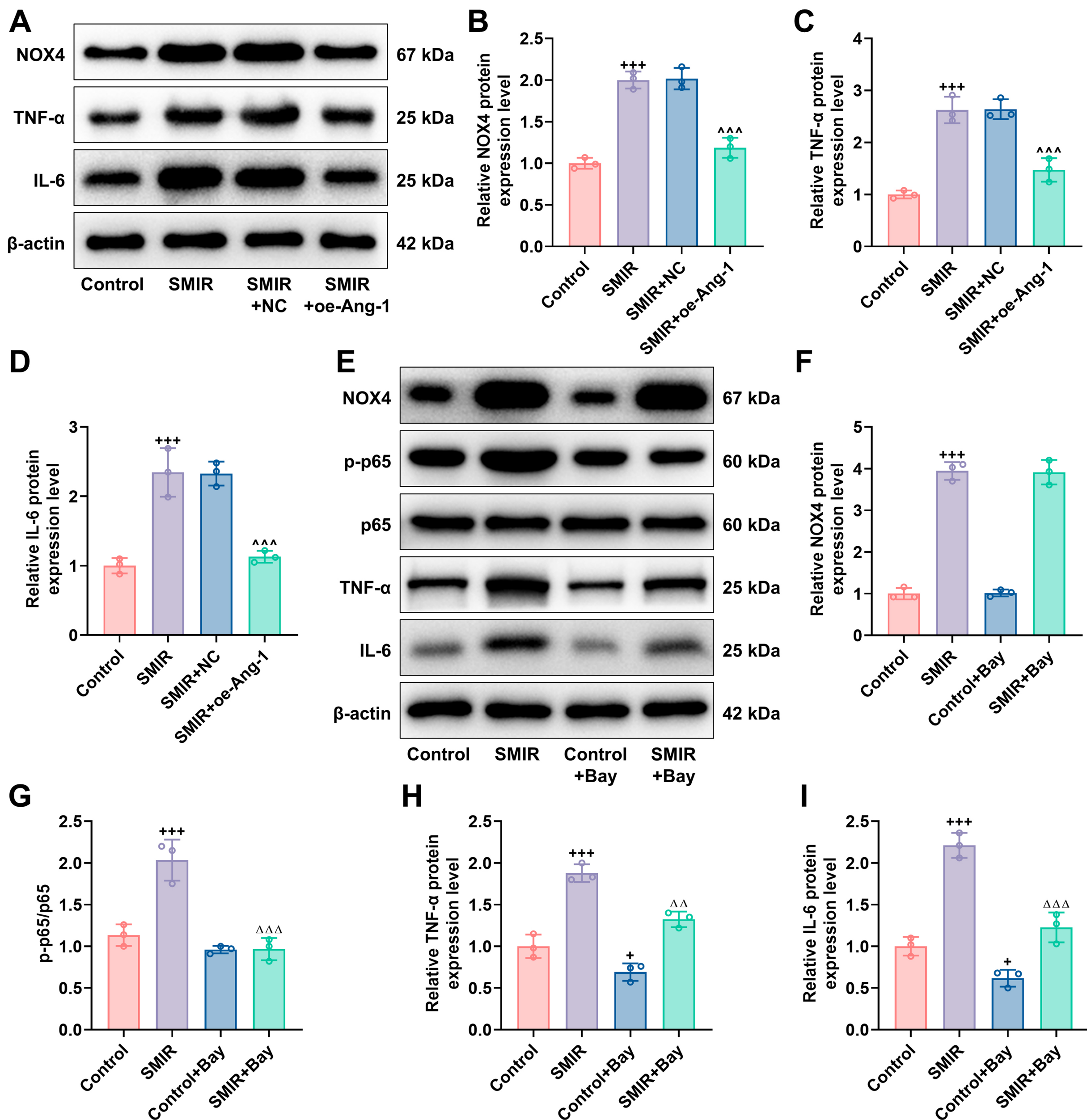

Fig. 4. Impact of Ang-1 overexpression and NF-κB inhibition on NOX4, TNF-α, IL-6 protein levels in DRG neurons. (A–D) Representative Western blot bands (A) and quantitative analysis of NOX4 (B), TNF-α (C), and IL-6 (D) protein levels in DRG neurons (Western blotting, β-actin as an internal control). (E–I) Representative Western blot bands (E) and quantitative analysis of NOX4 (F), phosphorylated p65 (p-p65), p65 (G), TNF-α (H), and IL-6 (I) protein levels in DRG neurons (Western blotting, β-actin as an internal control). Each experiment was performed independently three times. $^{+}p < 0.05$, $^{+++}p < 0.001$ compared to Control group. $^{\wedge\wedge\wedge}p < 0.001$ compared to SMIR+NC group. $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared to SMIR group.

pro-inflammatory cytokine production in DRG neurons, thereby aggravating pain perception [38]. Our findings demonstrated that SMIR treatment increased NOX4 expression in DRG neurons, whereas Ang-1 overexpression effectively suppressed this upregulation. These results indicate that Ang-1 may relieve post-surgical pain by downregulating NOX4, reducing oxidative stress, and suppressing inflammatory responses.

NF-κB, a pivotal transcription factor family comprising p65 (RelA), p50, p52, c-Rel, and RelB, regulates inflammation, immune responses, proliferation, and apoptosis [39,40]. It is critical in pain-related inflammation by upregulating the expression of inflammatory mediators, thereby enhancing nociceptive signaling [41]. In models of sciatic nerve injury and diabetic neuropathic pain, NF-κB activation increases the expression of pain-related genes

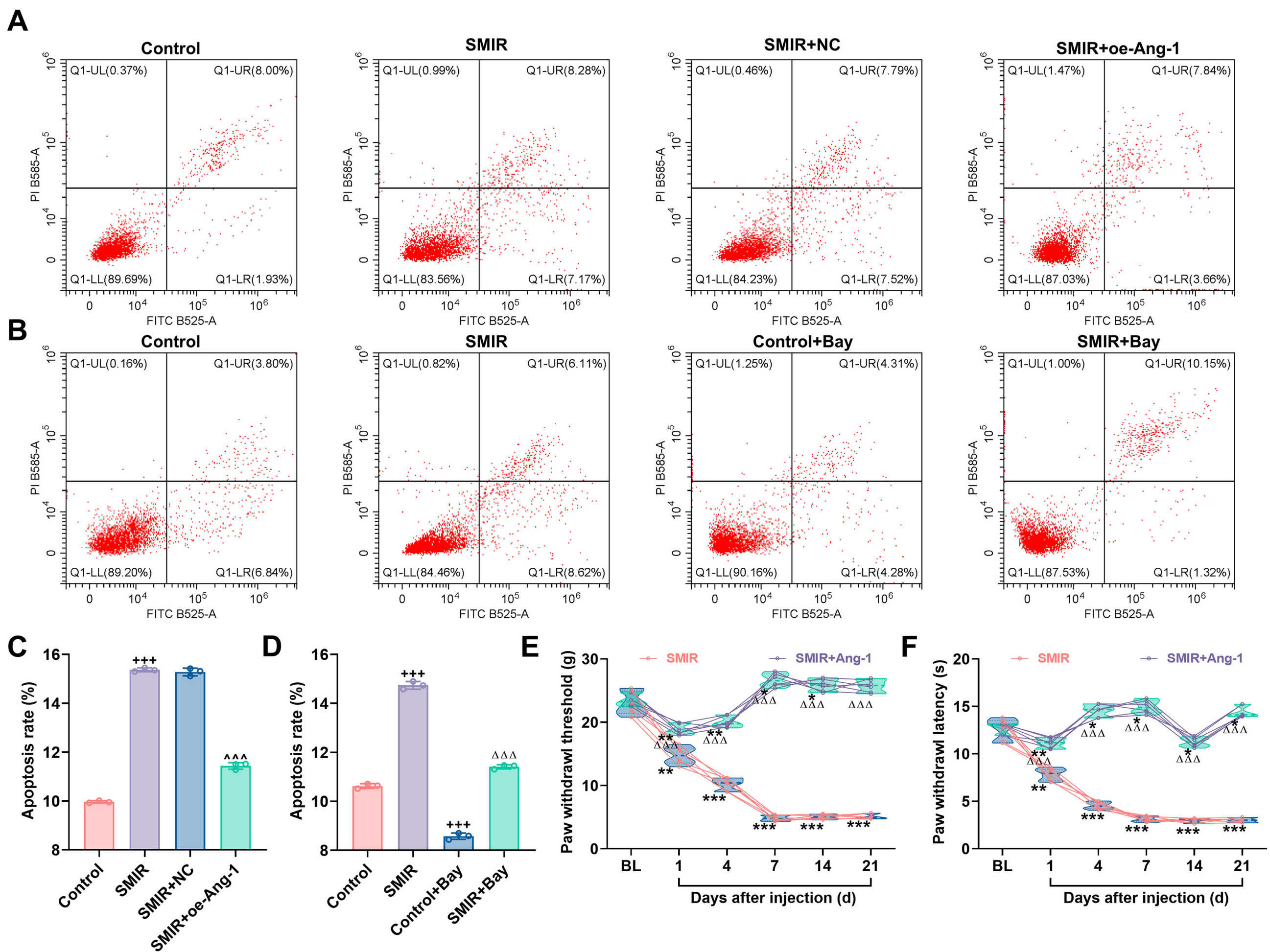

Fig. 5. Effects of Ang-1 overexpression and NF-κB inhibition on apoptosis and pain behavior post-SMIR. (A,C) Apoptosis in DRG neurons (Flow cytometry). (B,D) Apoptosis in DRG neurons (Flow cytometry). (E,F) PWT and PWL in rats from the SMIR and SMIR+Ang-1 groups at BL and on days 1, 4, 7, 14, and 21. Each experiment was performed independently three times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to BL group. $^{+++}p < 0.001$ compared to Control group. $^{\wedge\wedge\wedge}p < 0.001$ compared to SMIR+NC group. $^{\Delta\Delta\Delta}p < 0.001$ compared to SMIR group.

and pain sensitivity [42]. Consistent with these findings, SMIR treatment activated NF-κB in DRG neurons, an effect suppressed by Ang-1. Moreover, NF-κB inhibition significantly reduced SMIR-induced pain, supporting the notion that Ang-1 alleviates post-surgical pain, at least in part, by downregulating NF-κB signaling and modulating inflammation [43].

There are several limitations. First, behavioral assessments were performed only for 21 days, which may not completely capture the long-term effects of Ang-1 treatment on post-surgical pain. Second, although the study demonstrates that Ang-1 modulates the NOX4/ROS/NF-κB pathway, the precise molecular mechanisms governing this regulation remain unclear. Further studies are needed to elucidate the detailed intracellular signaling networks through which Ang-1 influences oxidative stress, inflammation, and neuronal apoptosis. Finally, the use of primary cultured DRG neurons does not reflect the full heterogeneity of the intact DRG tissue. In future research, we will perform immunofluorescence assays on intact DRG tissue samples to verify the spatiotemporal expression of key molecules (e.g., Ang-1, NOX4, NF-κB), thereby enhancing the translational value of our findings.

Conclusion

In summary, using rat models of post-surgical pain, we demonstrate Ang-1 overexpression alleviates pain hypersensitivity by reducing ROS production, inflammatory mediator release, and neuronal apoptosis. SMIR-induced post-surgical pain is pertinent to activation of the NOX4/ROS/NF-κB pathway, which is effectively mitigated by Ang-1. These findings highlight the therapeutic potential of Ang-1 for managing post-surgical pain and provide a foundation for developing novel analgesic strategies.

DM
DISCOVERY MEDICINE
2001

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author Contributions

BH and FFM designed the research study; LLL, XNS and JHH performed the research; XFW and MML collected and analyzed the data. JHH has been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

All procedures were approved by the Zhejiang Experimental Animal Centre (No. ZJCLA-IACUC-20040181).

Acknowledgment

Not applicable.

Funding

This research was funded by Science and Technology Development Plan Project of Jinan Municipal Health Commission (grant number: 2025302009).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Treede RD, Rief W, Barke A, Aziz Q, Bennett MI, Benoliel R, *et al*. Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). Pain. 2019; 160: 19–27. https://doi.org/10.1097/j.pain.0000000000001384.

[2] Mayoral Rojals V, Charaja M, De Leon Casasola O, Montero A, Narvaez Tamayo MA, Varrassi G. New Insights Into the Pharmacological Management of Postoperative Pain: A Narrative Review. Cureus. 2022; 14: e23037. https://doi.org/10.7759/cureus.23037.

[3] Keast M, Hutchinson AF, Khaw D, McDonall J. Impact of Pain on Postoperative Recovery and Participation in Care Following Knee Arthroplasty Surgery: A Qualitative Descriptive Study. Pain Management Nursing: Official Journal of the American Society of Pain Management Nurses. 2022; 23: 541–547. https://doi.org/10.1016/j.pmn.2021.11.011.

[4] Yeung SC, Irwin MG, Cheung CW. Environmental Enrichment in Postoperative Pain and Surgical Care: Potential Synergism With the Enhanced Recovery After Surgery Pathway. Annals of Surgery. 2021; 273: 86–95. https://doi.org/10.1097/SLA.0000000000003878.

[5] Geoffrion R, Koenig NA, Zheng M, Sinclair N, Brotto LA, Lee T, *et al*. Preoperative Depression and Anxiety Impact on Inpatient Surgery Outcomes: A Prospective Cohort Study. Annals of Surgery Open: Perspectives of Surgical History, Education, and Clinical Approaches. 2021; 2: e049. https://doi.org/10.1097/AS9.0000000000000049.

[6] Di Maio G, Villano I, Ilardi CR, Messina A, Monda V, Iodice AC, *et al*. Mechanisms of Transmission and Processing of Pain: A Narrative Review. International Journal of Environmental Research and Public Health. 2023; 20: 3064. https://doi.org/10.3390/ijerph20043064.

[7] Jin Z, Lee C, Zhang K, Gan TJ, Bergese SD. Safety of treatment options available for postoperative pain. Expert Opinion on Drug Safety. 2021; 20: 549–559. https://doi.org/10.1080/14740338.2021.1898583.

[8] Peng J, Zhang P, Zheng H, Ren YQ, Yan H. Dexmedetomidine reduces hippocampal microglia inflammatory response induced by surgical injury through inhibiting NLRP3. Chinese Journal of Traumatology = Zhonghua Chuang Shang Za Zhi. 2019; 22: 161–165. https://doi.org/10.1016/j.cjtee.2019.03.002.

[9] Huang W, Li J, Geng X, Li S, Zou Y, Li Y, *et al*. The Reactive Astrocytes After Surgical Brain Injury Potentiates the Migration, Invasion, and Angiogenesis of C6 Glioma. World Neurosurgery. 2022; 168: e595–e606. https://doi.org/10.1016/j.wneu.2022.10.064.

[10] Heidary D, Bahremand M, Mehdipour A, Zandieh Z. The Impact of Different Types of Exercise Training on the Angiopoietin Family. New Approaches in Exercise Physiology. 2022; 4: 57–76.

[11] Joussen AM, Ricci F, Paris LP, Korn C, Quezada-Ruiz C, Zarbin M. Angiopoietin/Tie2 signalling and its role in retinal and choroidal vascular diseases: a review of preclinical data. Eye (London, England). 2021; 35: 1305–1316. https://doi.org/10.1038/s41433-020-01377-x.

[12] Ha JM, Jin SY, Lee HS, Kum HJ, Vafaeinik F, Ha HK, *et al*. Akt1-dependent expression of angiopoietin 1 and 2 in vascular smooth muscle cells leads to vascular stabilization. Experimental & Molecular Medicine. 2022; 54: 1133–1145. https://doi.org/10.1038/s12276-022-00819-8.

[13] Sharma A, Behl T, Sharma L, Shah OP, Yadav S, Sachdeva M, *et al*. Exploring the molecular pathways and therapeutic implications of angiogenesis in neuropathic pain. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie. 2023; 162: 114693. https://doi.org/10.1016/j.biopha.2023.114693.

[14] Azouz AA, Abdel-Rahman DM, Messiha BAS. Balancing renal Ang-II/Ang-(1-7) by xanthenone; an ACE2 activator; contributes to the attenuation of Ang-II/p38 MAPK/NF-κB p65 and Bax/caspase-3 pathways in amphotericin B-induced nephrotoxicity in rats. Toxicology Mechanisms and Methods. 2023; 33: 452–462. https://doi.org/10.1080/15376516.2023.2177218.

[15] Chen YYK, Boden KA, Schreiber KL. The role of regional anaesthesia and multimodal analgesia in the prevention of chronic postoperative pain: a narrative review. Anaesthesia. 2021; 76 Suppl 1: 8–17. https://doi.org/10.1111/anae.15256.

[16] Ma R, Xie Q, Li H, Guo X, Wang J, Li Y, *et al*. *l*-Borneol Exerted the Neuroprotective Effect by Promoting Angiogenesis Coupled With Neurogenesis via Ang1-VEGF-BDNF Pathway. Frontiers in Pharmacology. 2021; 12: 641894. https://doi.org/10.3389/fphar.2021.641894.

[17] Li T, Liu T, Chen X, Li L, Feng M, Zhang Y, *et al*. Microglia induce the transformation of A1/A2 reactive astrocytes via the CXCR7/PI3K/Akt pathway in chronic post-surgical pain. Journal of Neuroinflammation. 2020; 17: 211. https://doi.org/10.1186/s12974-020-01891-5.

[18] Cai HY, Tian KW, Zhang YY, Jiang H, Han S. Angiopoietin-1 and $\alpha v\beta 3$ integrin peptide promote the therapeutic effects of

L-serine in an amyotrophic lateral sclerosis/Parkinsonism dementia complex model. Aging. 2018; 10: 3507–3527. https://doi.org/10.18632/aging.101661.

[19] de Geus TJ, Franken G, Joosten EAJ. Spinal Cord Stimulation Paradigms and Alleviation of Neuropathic Pain Behavior in Experimental Painful Diabetic Polyneuropathy. Neuromodulation: Journal of the International Neuromodulation Society. 2024; 27: 1330–1337. https://doi.org/10.1016/j.neurom.2024.06.007.

[20] Panušková K, Voděrová L, Vaculín Š. Methylphenidate attenuates signs of evoked neuropathic pain in animal model. Physiological Research. 2023; 72: S551–S558. https://doi.org/10.33549/physiolres.935215.

[21] Qian J, Lin X, Zhou Z. Skin/muscle incision and retraction regulates the persistent postoperative pain in rats by the Epac1/PKC-βII pathway. BMC Anesthesiology. 2022; 22: 230. https://doi.org/10.1186/s12871-022-01771-w.

[22] Xu H, Zeng Q, Zou K, Huang H, Chen J, Wang P, *et al*. Glucocorticoid-induced activation of NOX/ROS/NF-κB signaling in MSCs contributes to the development of GONFH. Apoptosis: an International Journal on Programmed Cell Death. 2023; 28: 1332–1345. https://doi.org/10.1007/s10495-023-01860-2.

[23] Paul D, Das DN, Mondal S, Gnanasekaran S, Mor A, Subramanya K. Optimization of β-actin gene as a housekeeping gene for quantification of mRNA levels of target genes in buffalo endometrium. Indian Journal of Physiology and Allied Sciences. 2022; 74: 7–11. https://doi.org/10.55184/ijpas.v74i04.65.

[24] Rotariu D, Babes EE, Tit DM, Moisi M, Bustea C, Stoicescu M, *et al*. Oxidative stress - Complex pathological issues concerning the hallmark of cardiovascular and metabolic disorders. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie. 2022; 152: 113238. https://doi.org/10.1016/j.biopha.2022.113238.

[25] Silva Santos Ribeiro P, Willemen HLDM, Eijkelkamp N. Mitochondria and sensory processing in inflammatory and neuropathic pain. Frontiers in Pain Research (Lausanne, Switzerland). 2022; 3: 1013577. https://doi.org/10.3389/fpain.2022.1013577.

[26] Xu J, Wu S, Wang J, Wang J, Yan Y, Zhu M, *et al*. Oxidative stress induced by NOX2 contributes to neuropathic pain via plasma membrane translocation of PKCε in rat dorsal root ganglion neurons. Journal of Neuroinflammation. 2021; 18: 106. https://doi.org/10.1186/s12974-021-02155-6.

[27] Shi Y, Yuan S, Tang SJ. Reactive Oxygen Species (ROS) are Critical for Morphine Exacerbation of HIV-1 gp120-Induced Pain. Journal of Neuroimmune Pharmacology: the Official Journal of the Society on NeuroImmune Pharmacology. 2021; 16: 581–591. https://doi.org/10.1007/s11481-020-09951-6.

[28] Fakhri S, Sabouri S, Kiani A, Farzaei MH, Rashidi K, Mohammadi-Farani A, *et al*. Intrathecal administration of naringenin improves motor dysfunction and neuropathic pain following compression spinal cord injury in rats: relevance to its antioxidant and anti-inflammatory activities. The Korean Journal of Pain. 2022; 35: 291–302. https://doi.org/10.3344/kjp.2022.35.3.291.

[29] Harvanová G, Duranková S, Bernasovská J. The role of cytokines and chemokines in the inflammatory response. Alergologia Polska-Polish Journal of Allergology. 2023; 10: 210–219. https://doi.org/10.5114/pja.2023.131708.

[30] Aliyu M, Zohora FT, Anka AU, Ali K, Maleknia S, Saffarioun M, *et al*. Interleukin-6 cytokine: An overview of the immune regulation, immune dysregulation, and therapeutic approach. International Immunopharmacology. 2022; 111: 109130. https://doi.org/10.1016/j.intimp.2022.109130.

[31] Mo Q, Zhang W, Zhu A, Backman LJ, Chen J. Regulation of osteogenic differentiation by the pro-inflammatory cytokines IL-1β and TNF-α: current conclusions and controversies. Human Cell. 2022; 35: 957–971. https://doi.org/10.1007/s13577-022-00711-7.

[32] Seifert O, Baerwald C. Interaction of pain and chronic inflammation. Zeitschrift Fur Rheumatologie. 2021; 80: 205–213. https://doi.org/10.1007/s00393-020-00951-8.

[33] Chen L, Zhuang K. Kaempferol counteracts bupivacaine-induced neurotoxicity in mouse dorsal root ganglia neurons by regulating TRAF6-dependent NF-κB signaling. The Kaohsiung Journal of Medical Sciences. 2023; 39: 710–717. https://doi.org/10.1002/kjm2.12682.

[34] Jeon BH, Khanday F, Deshpande S, Haile A, Ozaki M, Irani K. Tie-ing the antiinflammatory effect of angiopoietin-1 to inhibition of NF-kappaB. Circulation Research. 2003; 92: 586–588. https://doi.org/10.1161/01.RES.0000066881.04116.45.

[35] Szanto I. NADPH Oxidase 4 (NOX4) in Cancer: Linking Redox Signals to Oncogenic Metabolic Adaptation. International Journal of Molecular Sciences. 2022; 23: 2702. https://doi.org/10.3390/ijms23052702.

[36] Dzubanova M, Bond JM, Craige SM, Tencerova M. NOX4-reactive oxygen species axis: critical regulators of bone health and metabolism. Frontiers in Cell and Developmental Biology. 2024; 12: 1432668. https://doi.org/10.3389/fcell.2024.1432668.

[37] Li ZM, Xu SY, Feng YZ, Cheng YR, Xiong JB, Zhou Y, *et al*. The role of NOX4 in pulmonary diseases. Journal of Cellular Physiology. 2021; 236: 1628–1637. https://doi.org/10.1002/jcp.30005.

[38] Liu Z, Liu S, Zhao Y, Wang Q. Biological Mediators and Partial Regulatory Mechanisms on Neuropathic Pain Associated With Chemotherapeutic Agents. Physiological Research. 2024; 73: 333–341. https://doi.org/10.33549/physiolres.935162.

[39] Ghosh G, Wang VYF. Origin of the Functional Distinctiveness of NF-κB/p52. Frontiers in Cell and Developmental Biology. 2021; 9: 764164. https://doi.org/10.3389/fcell.2021.764164.

[40] Zhang T, Ma C, Zhang Z, Zhang H, Hu H. NF-κB signaling in inflammation and cancer. MedComm. 2021; 2: 618–653. https://doi.org/10.1002/mco2.104.

[41] Duan YW, Chen SX, Li QY, Zang Y. Neuroimmune Mechanisms Underlying Neuropathic Pain: The Potential Role of TNF-α-Necroptosis Pathway. International Journal of Molecular Sciences. 2022; 23: 7191. https://doi.org/10.3390/ijms23137191.

[42] Guo HM, Zhang Y, Zhang Y, Jiao PF, Fan XC, Kong CL, *et al*. Spinal Ninjurin2 contributes to the neuropathic pain via NF-κB-mediated neuroinflammation in the spared sciatic nerve injury rats. International Immunopharmacology. 2021; 99: 107918. https://doi.org/10.1016/j.intimp.2021.107918.

[43] Zhao L, Tao X, Song T. Astaxanthin alleviates neuropathic pain by inhibiting the MAPKs and NF-κB pathways. European Journal of Pharmacology. 2021; 912: 174575. https://doi.org/10.1016/j.ejphar.2021.174575.