Article

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The Regulatory Effect of Remifentanil on JNK Signaling during Remission of Flap Ischemia-Reperfusion Injury

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Background: The impact of remifentanil on hypogastric flap function following ischemia-reperfusion (I/R) injury remains largely unknown, limiting its potential clinical application in flap surgery. This study investigated the therapeutic effects of remifentanil on hypogastric flap I/R injury.

Methods: Aortic endothelial cells were extracted from the hypogastric flap I/R injury models established in-house using Sprague-Dawley rats, and were treated under hypoxic conditions. The cells were treated with 0.1, 1, 10 and 100 ng/mL remifentanil and 10 ng/mL anisomycin (the activator of c-Jun N-terminal kinase [JNK]). Histopathological changes and tumor necrosis factor alpha (TNF-α) content of the flaps were observed after hematoxylin-eosin staining and immunohistochemistry. Immunofluorescence, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and flow cytometry were employed for apoptosis evaluation. Western blotting, quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were utilized to assess the protein and gene expression levels of TNF receptor 1 (*TNFR1*), *JNK1*, phosphorylated (p)-JNK1, malondialdehyde (MDA), superoxide dismutase (SOD), nitric oxide (NO) and *TNF*-α in the flaps and cells.

Results: The endothelial necrosis and cell apoptosis of rat flaps induced by I/R injury were ameliorated by remifentanil, and declining aortic endothelial cell viability and augmented apoptosis induced by hypoxia were reversed by remifentanil (10, 100 ng/mL) ($p < 0.05$). Remifentanil reversed the increased expressions of *TNFR1*, *JNK1*, p-JNK1, MDA and *TNF*-α induced by I/R injury or hypoxia in the flaps and cells ($p < 0.05$), and counteracted the decreased levels of NO and SOD induced by I/R injury in the flaps ($p < 0.05$). Anisomycin reversed the effects of remifentanil on suppressing *TNFR1*, *JNK1* and p-JNK1 levels and apoptosis in the cells ($p < 0.05$).

Conclusion: Remifentanil ameliorates cell apoptosis and vascular endothelial necrosis induced by I/R injury in the hypogastric flap, likely by downregulating the TNF-α/TNFR1 pathway and JNK1 signaling. These findings suggest that remifentanil may be a promising therapeutic agent for improving hypogastric flap survival in clinical settings.

Keywords: ischemia-reperfusion injury; remifentanil; skin flap; apoptosis; tumor necrosis factor receptor 1 pathway; c-Jun N-terminal kinase 1 pathway

Introduction

Skin flap grafting, as a common method widely used in the transplantation of tissues, is performed in the surgery of organ reconstruction and wound repair [1]. The complexity of the biological process of wound healing, decreased activity or deficiency in the number of local growth factors are the inherent factors underlying the challenges for wound healing, thus restricting skin flap application in plastic surgery [2]. In addition to this, the frequency of flap necrosis occurrence increases after clinical operation [3]. Therefore, to ensure a better prognosis after flap grafting, it is necessary to improve grafted flap survival [2]. As a primary factor causing grafted flap necrosis and even influencing its survival [4], ischemia-reperfusion (I/R) injury is a major impediment that should be addressed during skin flap grafting.

Remifentanil is a type of synthetic opioid that can be used to control the pernicious stimuli in the process of operation and promote a quick recovery from the anesthesia [5]. It has been widely used as an anesthetic and pain-relief medication owing to its pharmacological characteristics [6]. Previous research regarding the effect of remifentanil on I/R injury. For instance, preconditioning with remifentanil has been shown to reduce I/R injury of the liver in mice models [7]. Nevertheless, studies regarding the effects of remifentanil on I/R injury-induced abdomen flap remain scarce.

According to a previous study, remifentanil can mitigate hepatic I/R injury and decrease the phosphorylation of c-Jun N-terminal kinase (JNK) in macrophages [8]. Reportedly, G protein-coupled receptor 124 (GPR124) regulates trophoblast proliferation, invasion, migration, apoptosis, and inflammation via the JNK pathways [9]. Previous research have shown that the JNK pathway is suppressed

during the mitigation of cerebral I/R injury [10]. Besides, the inhibition of I/R injury-induced cell death is accompanied by a reduction in the level of tumor necrosis factor alpha (TNF-α) [11]. Moreover, the TNF pathway is promoted during myocardial I/R injury [12]. It is worthy noting that remifentanil can facilitate the inhibition of the brain I/R injury and the JNK and TNF-α/TNF receptor 1 (TNFR1) pathways [13]. However, the effects of remifentanil on I/R-injured hypogastric flap, JNK pathway and TNF-α/TNFR1 pathway still remain obscure.

Therefore, in the present study, rat models of hypogastric flap I/R injury were established by means of surgical methods. According to the findings obtained from the experiments of the flap cut and cell isolated from the rats after surgery, the effects of remifentanil on the hypogastric flap, TNF-α/TNFR1 pathway and JNK pathway were discussed.

Materials and Methods

Animals and Ethics Statement

In this study, Sprague-Dawley rats (male, n = 60, 200–220 g), which were procured from Hangzhou Medical College (Hangzhou, China), were utilized. The rats were maintained in the specific pathogen-free room operated with a 12-hour light-dark cycle at 18–25 °C with 47% humidity. The rats were given access to water and standard rodent food *ad libitum*. All operations and treatments in this study on animals were conducted in line with the guidance of China Council on Animal Care and Use, and were approved by The Ethics Committee for Laboratory Animal Welfare in Zhejiang Experimental Animal Centre (Ethics Approval No. ZJCLA-IACUC-20020163).

Experimental Groups

The rats were categorized into six groups (n = 10 per group) according to the experimental design utilized in a previous study [14]: (group 1) sham group: rats were subjected to surgery to cut the flaps and isolate the vessel of flap pedicle without inducing ischemic injury; (group 2) remifentanil-high concentration (H) group: rats received 10 µg/kg remifentanil (R-024-1ML; Sigma-Aldrich, St. Louis, MO, USA) via intravenous injection in the tail; (group 3) I/R group: rats were induced with I/R as described in the next section; (groups 4–6) I/R+remifentanil-low concentration (L), -medium concentration (M) and -H groups: rats received intravenous injection of remifentanil at the doses of 0.4, 2 and 10 µg/kg for 15 min in the tail [14], respectively, and were also induced with I/R at 10 min after the remifentanil injection.

Establishment of Model of Flap Ischemia-Reperfusion Injury

The I/R injury induction was performed as per the procedures stipulated in a previous study [15]: briefly, after being anesthetized with 2% pentobarbital sodium salt (40 mg/kg; 4579, Tocris Bioscience, Bristol, UK) via intraperitoneal injection, the Sprague-Dawley rats were subjected to the operation for inducing flap I/R injury. The skin flaps of the rats were cut along the line, encompassing a 3 cm × 6 cm area on the lower left region of the abdomen. Then, the perforator vessels were ligated and the superficial hypogastric artery was clamped to induce ischemia for 8 h. In the sham group and remifentanil-H group, ischemia was not induced in the superficial hypogastric artery. Then, under an optical microscope (OCM-1; KERN & Sohn, Balingen, Germany), the skin flaps were sutured after the blood flow was blocked. While blocking the blood flow, 2% pentobarbital sodium salt was injected into each rat with a dose of 20 mg/kg every 3 h to maintain the anesthetic effect. After 6 h of blood flow blocking, the suture was removed and the superficial hypogastric artery was clamped. Observed with an optical microscope (CKX53, Olympus, Japan), the blood flow was confirmed to be restored and circulated smoothly in the flap, an indication of a successful reperfusion. Later, at 8 h after the surgery, the awakened rats were given free access to food, and their condition, especially the blood circulation of the flaps, was observed and photographed every day. They were anesthetized with 2% pentobarbital sodium salt (35–40 mg/kg) at 7-day after the surgery, after which the flaps and the serum from the superficial vein in the pedicle of hypogastric flaps were harvested after photography and disinfection, and the full-thickness flap tissues were cut from the edge of the middle part of the flaps. After the flaps and serum were harvested, all these rats were euthanized through cervical dislocation.

Skin Flap Survival Evaluation and Hematoxylin-Eosin Staining

The condition of skin flaps in rats was observed and evaluated based on the photograph before cutting. The tissues from the middle part of the flaps were subjected to hematoxylin-eosin (H&E) staining using H&E staining kit (G1120, Solarbio, Beijing, China). In brief, paraffin-embedded 4-µm-thick sections were made, stained with hematoxylin and eosin dyes, and the cell morphology was observed and photographed using an optical microscope (CKX53, Olympus, Japan). The tissue sections were soaked in 0.1% cresyl violet at 37 °C for 20 min, rinsed and dehydrated. The condition of flaps was observed under an optical microscope (CKX53, Olympus, Japan) at a 100× magnification. The morphology of neutrophils, the structure of muscle fibers, and the condition of inflammatory edema in the flap tissue were observed and recorded. For each criterion, the histological scores were calculated based on a four-point scale [16]: 0, none; 1, mild; 2, moderate; and 3, severe. At least three microscopic areas were examined in the scoring process.

Colorimetry

The skin flap tissues were treated with phosphate-buffered saline (PBS; ST476; Beyotime, Shanghai, China) and then homogenized using High speed homogenizer

(E1648, Beyotime, Shanghai, China). The tissue suspension was then centrifuged at 450 $\times g$ for 15 min. Afterward, the supernatant was removed for colorimetric analysis. The levels of nitric oxide (NO), malondialdehyde (MDA) and superoxide dismutase (SOD) were quantified using NO, MDA, and SOD kits (ab65328, ab118970, and ab65354, respectively; Abcam, Cambridge, UK) according to a previous study [17]. The experiment and quantification were conducted in accordance with the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay

The TNF-α level was determined using a rat enzyme-linked immunosorbent assay (ELISA) kit (ab236712, Abcam, Cambridge, UK) according to the manufacturer's protocol. The absorbance was measured using a multimode microplate reader (iMark, Bio-Rad, Hercules, CA, USA) at the wavelength of 450 nm. The content of the substance to be measured in a sample was determined by using an equation generated based on the standard curve plotted.

Immunohistochemistry

Immunohistochemical (IHC) assay was also used to detect TNF-α levels. The sliced flap tissues for each group were dewaxed and sectioned before the antigen was extracted. Paraffin sections were incubated in 5% goat serum for 15–20 min and then incubated in anti-TNF-α antibody (1:1000; ab220210, Abcam, Cambridge, UK) for 2 h at 37 °C. Later, the tissues were immersed with goat anti-mouse antibody (1:1000; ab6708, Abcam, Cambridge, UK). Finally, diaminobenzidine (D106468; Aladdin, Seattle, WA, USA) was added to the tissues, which were then inspected under a fluorescence microscope (100× magnification; DM6FS, Leica, Wetzlar, Germany). The area of positively stained cells was analyzed by using the ImageJ software (vesion5.0; Bio-Rad, Hercules, CA, USA).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining and Immunofluorescence for in Vivo Apoptosis Detection

After a 3% hydrogen peroxide treatment, the sliced flap tissues were immersed in 10 µmol citrate buffer (C2488, Sigma-Aldrich, St. Louis, MO, USA) for 3 min under high pressure. Then, the tissues were immersed in 10% goat serum, followed by incubation with anti-cluster of differentiation (CD)31 antibody (1:2000; ab182981; Abcam, Cambridge, UK) was conducted for 12 h at 4 °C. Later, the tissues were rinsed with PBS and incubated with donkey anti-rabbit antibody (1:200; ab150075, Abcam, Cambridge, UK) for 1 h in the dark. Subsequently, the tissues were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining after being washed with PBS thrice. For the staining, TUNEL assay kits (ab66108, Abcam, Cambridge, UK) were used according to the manufacturer's instructions. Then, the tissues were incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (C0065, Solarbio, Beijing, China) for 30 min for labeling the nuclei. A fluorescence microscope (A7-345, Leica, Wetzlar, Germany) (200× magnification) was utilized to observe the red fluorescence indicative of vascular endothelial cells, the blue fluorescence indicative of cell nuclei, and the green fluorescence indicative of TUNEL-positive cell nuclei.

Extraction of Aortic Endothelial Cells

Sprague-Dawley rats (n = 20), each weighing 200–220 g, were subjected to the extraction of aortic endothelial cells in the hypogastric flap. The isolation of the endothelial cell in the abdominal aorta was performed as per the procedures detailed in previous study [18]. After these rats were anesthetized with the 2% pentobarbital sodium salt (2 mL/kg), the abdominal aorta was incised along the abdominal incision line and the left ventricle was perfused with the 1 mL fetal bovine serum (FBS; 12103C, Sigma-Aldrich, St. Louis, MO, USA) containing heparin (1000 U/mL; YZM002106, BIOFOUNT, Beijing, China). After being removed from the rat, the aorta was incubated in the Dulbecco's modified Eagle medium (DMEM; 31870082, Gibco, Brownsville, TX, USA) supplemented with 20% fetal bovine serum (FBS) containing heparin (1000 U/mL). After the proximal part of the aorta was ligated, the ligated sections were rinsed with DMEM without serum and the other side was incubated with collagenase II (MX1002; Maokangbio, Shanghai, China) for 45 min at 37 °C. Then, the endothelial cells were taken from the aorta. After being centrifuged for 5 min at 1200 rpm, the endothelial cells were resuspended in DMEM supplemented with 20% FBS. Finally, the cell morphology was observed under an inverted microscope (Zeiss, Oberkochen, Germany).

Immunofluorescence Assay for Cell Identification

The marker of endothelial cell, CD31, in the aortic endothelial cells was identified by the immunofluorescence assay. After fixation and washing, the endothelial cells were added with PBS supplemented with 5% FBS and incubated for 0.5 h. Next, the endothelial cells were soaked with anti-CD31 antibody (ab222783, Abcam, Cambridge, UK) overnight at 4 °C. After that, the cells were further immersed with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibody (1:2000; ab6717, Abcam, Cambridge, UK) at 25 °C for 1 h in the dark. After washing with PBS and incubation with DAPI (D8417, Sigma-Aldrich, St. Louis, MO, USA) in the dark at 25 °C for 10 min, the coverslips containing the cells were rinsed with PBS and examined under a fluorescence microscope (A7-345, Wetzlar, Germany) at 400× magnification.

Cell Treatment

The hypoxia-induced aortic endothelial cells-based I/R injury models were established in adherence to a reported protocol [19], and the aortic endothelial cells were divided into several groups: (group 1) control group: the

cells were cultured with DMEM supplemented with 15% FBS at 37 °C; (group 2) hypoxia group: the cells were cultured with DMEM at 5% CO_2, 3% O_2 and 92% N_2 at 37 °C (a simulated hypoxic condition) for 12 h and then cultured with serum-free DMEM with 5% CO_2 and 95% O_2 at 37 °C for 4 h; (groups 3–6) hypoxia+remifentanil: the cells were treated with medium containing 0.1, 1, 10 or 100 ng/mL remifentanil prior to exposure to hypoxic condition; (group 7) hypoxia+remifentanil+anisomycin group: the cells were treated with medium containing 10 ng/mL anisomycin (the activator of JNK; A102397, Aladdin, Shanghai, China) and 1 ng/mL remifentanil prior to exposure to hypoxic condition.

MTT Assay

After treatment with remifentanil and/or hypoxia, methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was utilized to measure the viability of aortic endothelial cells. The cells (1×10^4 cells/well) were inoculated into 96-well plates and cultured overnight. Next, each well was added with 10 µL MTT reagent (C0009, Beyotime, Shanghai, China) and continually incubated for 4 h at 37 °C. Later, 100 µL dimethyl sulfoxide (D119415, Aladdin, Shanghai, China) was added and the absorbance at the wavelength of 570 nm was detected using a microplate reader (iMark, Bio-Rad, Hercules, CA, USA). Cell viability was determined as follows:

Cell viability (%) = $(OD_{Experimental} - OD_{blank})/(OD_{Control} - OD_{blank}) \times 100$

Flow Cytometry

Annexin V-FITC/propidium iodide (PI) cell apoptosis kit (V13242, Invitrogen, Carlsbad, CA, USA) was employed to determine the rate of apoptosis. After being plated in a 6-well plate, cells (1×10^6) were treated with remifentanil, anisomycin and hypoxia. Then, cell suspension (1×10^5 cells in 100 µL DMEM) was obtained by resuspending the cells. Next, the cell suspension was added with FITC Annexin V and PI working solution (5 µL for each), followed by incubation in the dark at 37 °C for 15 min. A flow cytometer (A24858, Attune NxT, Invitrogen, Carlsbad, CA, USA) was utilized to detect cells undergoing apoptosis. The analysis of related data was accomplished by utilizing Attune NxT (version 2.5; Invitrogen, Carlsbad, CA, USA). The rate of cell apoptosis was calculated using the formula given below:

Apoptosis rate (%) = Q2-apoptosis rate + Q3-apoptosis rate

Q2 is the upper right corner region, designated as a late apoptotic cell. Q3 is the lower right corner region, designated as an early apoptotic cell.

Quantitative Real-Time Polymerase Chain Reaction

After being extracted from rat tissue and endothelial cells, total RNA was converted into cDNA using cDNA (AW311-02, Transgenic, Beijing, China). PCR reaction was conducted with Green qPCR SuperMix (AQ132-11; Transgen, Beijing, China) in a Real-Time PCR System (4485701; Thermo Fisher, Waltham, MA, USA). The primers' sequences are listed in Table 1. The housekeeping gene used in this study was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The PCR reaction conditions are as follows: 95 °C, 10 min; 40 cycles of 94 °C, 15 sec each; and 60 °C, 1 min. The quantification of the relative gene expressions was analyzed by $2^{-\Delta\Delta CT}$ method [20].

Western Blotting

Radio immunoprecipitation assay (RIPA) lysis buffer (R0010, Solarbio, Beijing, China) was used to lyse the cells, which were then centrifuged at 12000 $\times g$ for 5 min to obtain total protein. Then, the bicinchoninic acid (BCA) kit (BCA1, Sigma-Aldrich, St. Louis, MO, USA) was utilized to detect the concentration of the protein. The protein was separated by gel reagent (P0690, Beyotime, Shanghai, China), and the separated proteins on the gel were then transferred to polyvinylidene fluoride (PVDF) membrane (FFP33, Beyotime, Shanghai, China). Skim milk (5%) was used to block the membranes at 25 °C for 2 h, followed by an overnight incubation at 4 °C, separately, with primary anti-JNK1 antibody (1:1000; 48 kDa, ab110724), anti-p-JNK1 antibody (1:1000; 50 kDa, ab47337), anti-TNFR1 antibody (1:1000; 51 kDa, ab90463), and anti-GAPDH antibody (1:1000; 36 kDa, ab8245), which were all acquired from Abcam, Cambridge, UK. The internal control employed in the Western blotting experiment was GAPDH. After incubation for 24 h, these membranes were immersed with goat anti-rabbit secondary antibody (1:2000; ab205718, Abcam, Cambridge, UK) and goat anti-mouse secondary antibody (1:2000; ab205719, Abcam, Cambridge, UK) at 25 °C for 1 h. After being washed by Tris-buffered saline Tween (ab64204; Abcam, Cambridge, UK), the membranes were processed using the enhanced chemiluminescence (ECL) kit (P0018AS, Beyotime, Shanghai, China) for chemiluminescence development, and the blots were then observed using iBright™ CL1500 Imaging System (A44240; Invitrogen, Carlsbad, CA, USA). ImageJ (vesion5.0; Bio-Rad, Hercules, CA, USA) was employed to calculate the gray values.

Statistical Analysis

In this paper, all experiment data are expressed as mean ± standard deviation. All experiments of this study were executed in triplicate. SPSS 22.0 (IBM Cor., Armonk, NY, USA) was utilized to conduct statistical analysis. Multi-group comparisons were conducted by utilizing a one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Results with $p < 0.05$ were considered statistically significant.

Table 1. Primers' sequences for qRT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
JNK1 (rat)	CCTTCTACGACGATGCCCTC	GGGTCGGTGTAGTGGTGATGT
*TNFR*1 (rat)	GAACACCGTGTGTAACTGCC	ATTCCTTCACCCTCCACCTC
GAPDH (rat)	AGGGATCCTGAGGCATCTGT	TCCGGCTCCTGGGAATCTAA

JNK, c-Jun N-terminal kinase; *TNFR1*, tumor necrosis factor receptor 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction.

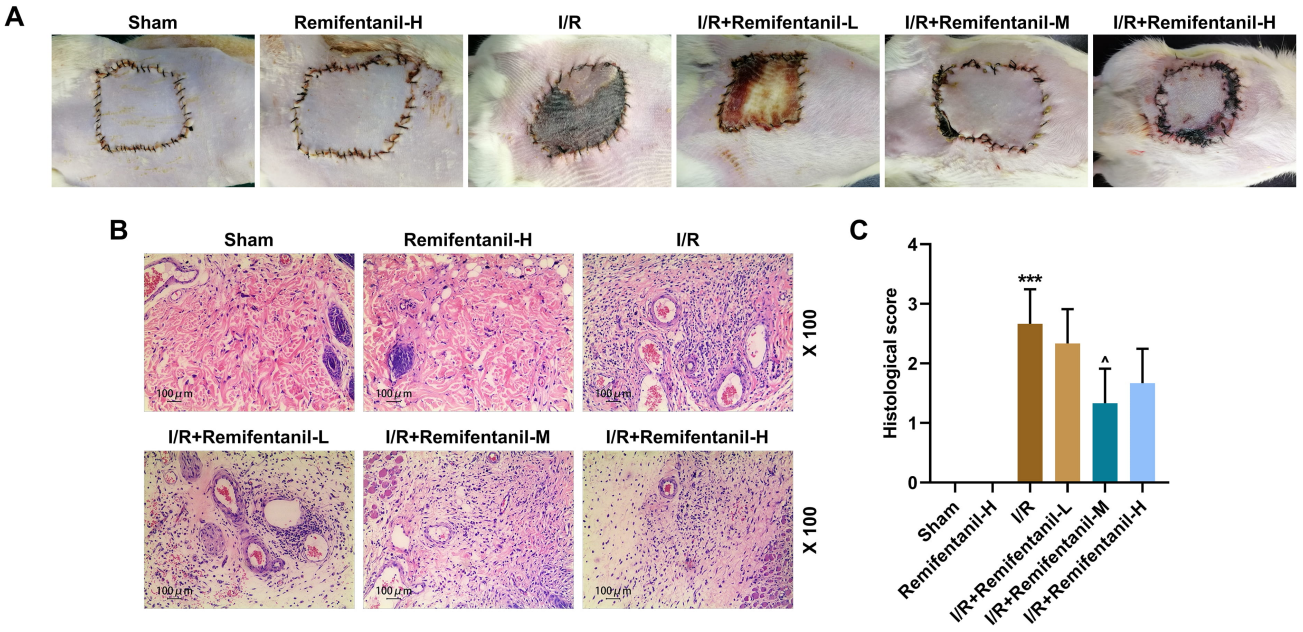

Fig. 1. Remifentanil alleviated I/R injury. (A) The conditions of the flaps were directly observed from the photographs. (B,C) The inflammatory condition of the flap was detected with the aid of H&E staining (100× magnification) and histological scores of all groups. Experimental data are expressed as mean ± standard deviation (SD). ***$p < 0.001$, $^{\wedge}p < 0.05$; * vs. sham group; $^{\wedge}$ vs. I/R group. n = 3 in each group. Abbreviations: I/R, ischemia-reperfusion; H&E, hematoxylin-eosin; remifentanil-H, remifentanil-high concentration; remifentanil-M, remifentanil-medium concentration; remifentanil-L, remifentanil-low concentration.

Results

Remifentanil Alleviated the I/R Injury of Rat Hypogastric Flaps

At 7 days after the surgery, the flaps on the lower left abdomen of rats were photographed and then cut after disinfection. As shown in Fig. 1A, in the sham group and remifentanil-H groups, the majority of the flaps survived without necrosis and atrophy, had good elasticity, and manifested a pink color. The majority of flaps in the I/R group became necrotic, with their color turning dark or light dark and some of themselves becoming crusted and atrophied without flexibility. Moreover, the minute amount of inflammatory secretions and compromised blood flowing could be observed when the flap was lifted up; necrosis appeared in the distal flap of the I/R+remifentanil-L group, but the necrotic area was smaller compared to the I/R group. Meanwhile, the proximal flap survived, being light red in color and soft in texture, manifesting a gradual transition into necrotic phenotype in the distal flap, with a transition of the color from a light red to a light black and eventually into a black brown in the distal flap. The abnormalities of the flap were alleviated in the I/R+remifentanil-M group, compared to the I/R group, with an effect much stronger relative to the I/R+remifentanil-L group; besides, the I/R+remifentanil-H group showed mitigation of the flap abnormalities in contrast to the I/R group, which was also being more pronounced as compared to the I/R+remifentanil-L group.

The inflammatory condition of the flap was evaluated with the aid of H&E staining. Fig. 1B,C display that, in the sham group and remifentanil-H group, each layer of the flap showed a well-organized tissue structure, deeply dyed nucleus and no obvious inflammatory edema; the vessel wall of tissues was infiltrated by copious neutrophils, and obscure layers and necrosis of some myofibers in the flap of the I/R group were detected; the tissues in the I/R+remifentanil-L group were in a histopathologically better condition as compared with those in the I/R group but were still fraught with extensive infiltration of inflamma-

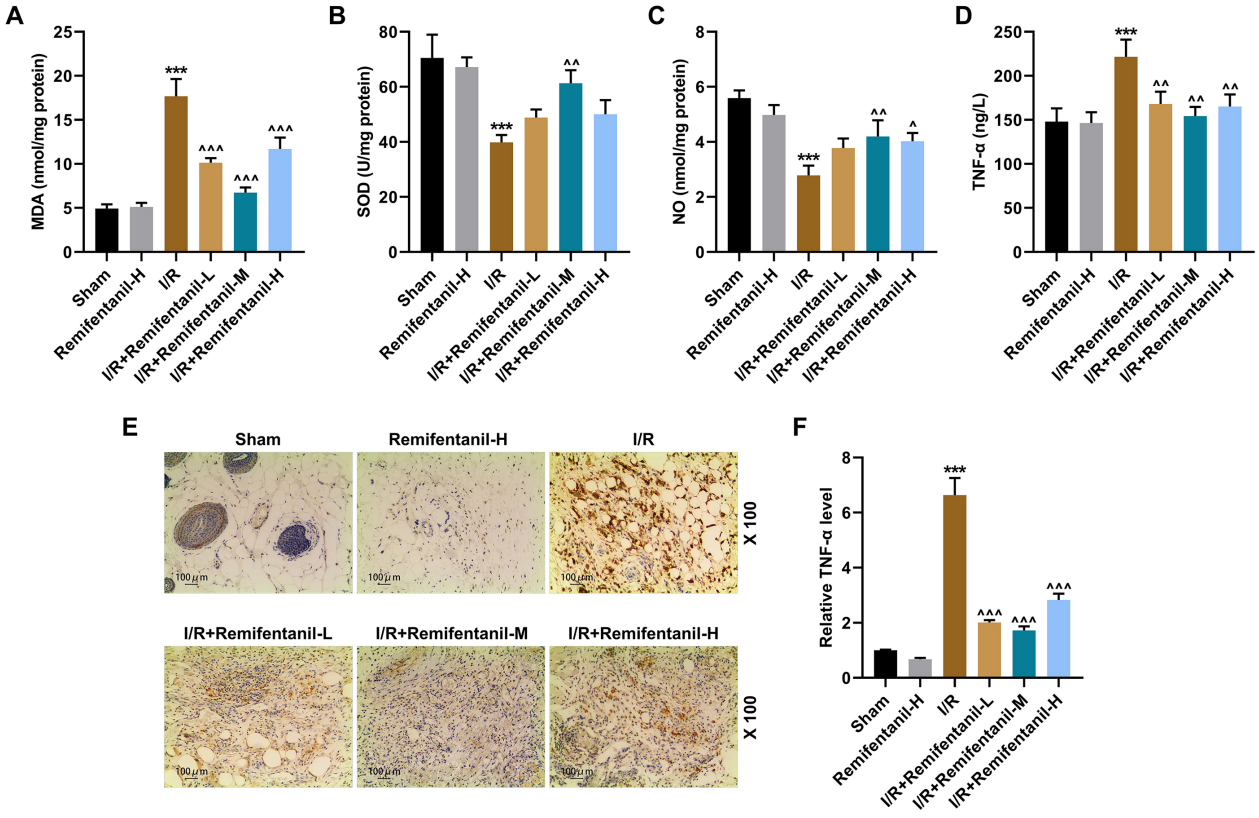

Fig. 2. Remifentanil downregulated the levels of MDA and TNF-α and upregulated those of SOD and NO in the rat hypogastric flap with I/R injury. (A–C) The levels of MDA, SOD and NO in the hypogastric flap tissues were detected colorimetrically using MDA, SOD and NO kits. (D) The level of TNF-α in the hypogastric flap tissues was detected by ELISA. (E,F) The expression of TNF-α in the hypogastric flap tissues was detected by means of immunohistochemistry (100$\times$ magnification). The experimental data are expressed as mean $\pm$ standard deviation (SD). ***$p < 0.001$, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$; * vs. sham group; $^{\wedge}$ vs. I/R group. n = 3 in each group. Abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; NO, nitric oxide; TNF-α, tumor necrosis factor alpha; ELISA, enzyme-linked immunosorbent assay.

tory cells; the tissues in the I/R+remifentanil-M group were also in a better histopathologically condition as compared with those in the I/R+remifentanil-L group, demonstrating mitigated inflammation; moreover, the condition of flap in the I/R+remifentanil-H group was similar to that in the I/R+remifentanil-L group. Overall, these results recounted above illustrated that the remifentanil could alleviate the I/R injury of the flaps.

Remifentanil Downregulated the Levels of TNF-α and MDA and Upregulated the Levels of NO and SOD in Rat Hypogastric Flap with I/R Injury

The levels of MDA, SOD and NO were quantified colorimetrically and the TNF-α level was detected by means of ELISA and IHC. Fig. 2A,D show that compared to the sham group, the levels of TNF-α and MDA were upregulated in the I/R group ($p < 0.001$), and the levels of these two indices decreased in the I/R+remifentanil-L, -M and -H groups relative to the I/R group (Fig. 2A,D; $p < 0.01$); and compared to the sham group, the levels of SOD and NO were downregulated in the I/R group (Fig. 2B,C; $p < 0.001$), and the levels of these indices were increased in the I/R+remifentanil-L, -M and -H groups in contrast to the I/R group (Fig. 2B,C; $p < 0.05$). Based on the IHC results, the TNF-α content was increased in the I/R group in contrast to the sham group. In addition, compared to the I/R group, the TNF-α content was lower in the I/R+remifentanil-L, -M and -H groups (Fig. 2E,F; 100$\times$ magnification). Taken together, these results indicate that remifentanil exerts a multi-faceted protective effect against I/R injury in the rat hypogastric flap, reducing markers of inflammation (TNF-α) and oxidative damage (MDA) while increasing the expression of antioxidants (SOD and NO).

Remifentanil Suppressed the Levels of TNFR1 and JNK1 and Cell Apoptosis in Rat Hypogastric Flap with I/R Injury

The quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were exploited to determine the gene and protein expression levels of JNK1, phosphorylated (p)-JNK1, TNFR1 and p-JNK1/JNK1 in the flap with I/R injury. Fig. 3A–D displays that compared

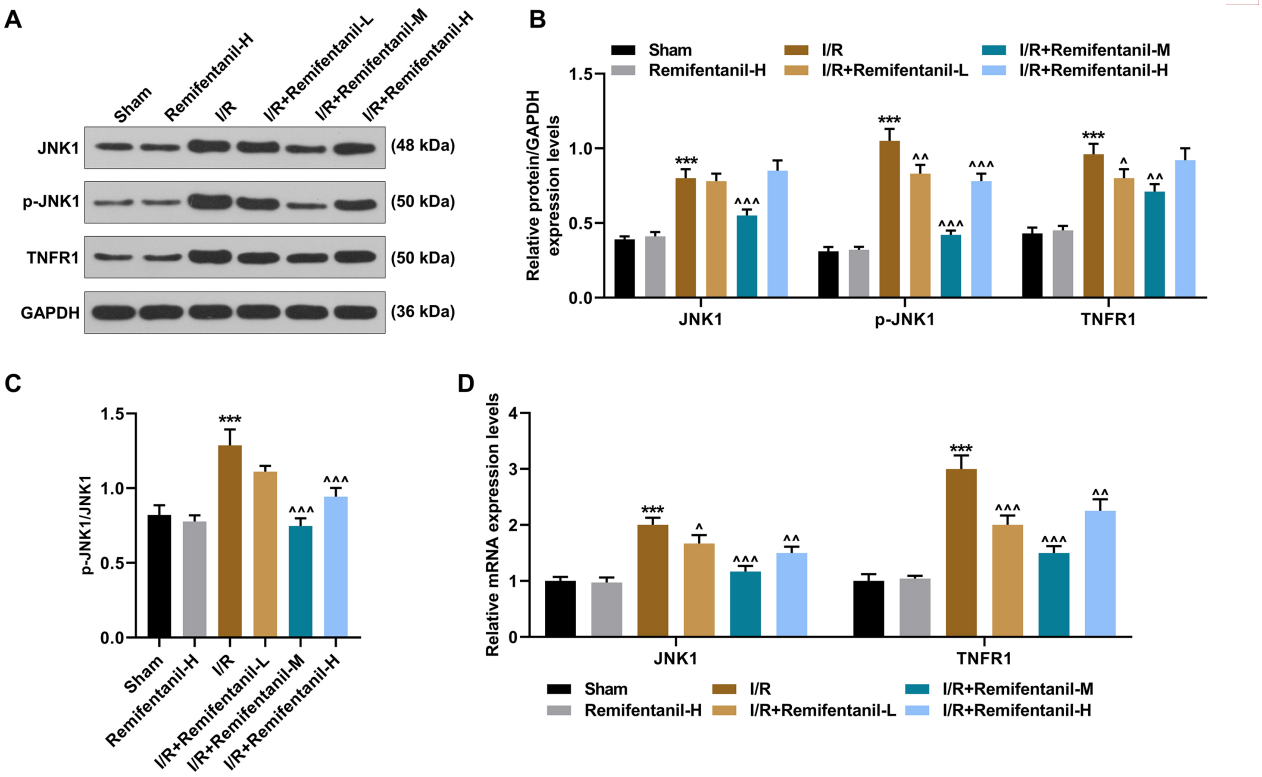

Fig. 3. Remifentanil downregulated the expression levels of JNK1, p-JNK1, TNFR1 and p-JNK1/JNK1 in the rat hypogastric flap with I/R injury. (A,B) The protein expression levels of JNK1, p-JNK1, and TNFR1 in the flap with I/R injury were detected by means of Western blotting, in which we employed GAPDH as the internal reference. (C) The p-JNK1/JNK1 ratio was analyzed according to the protein expression results derived from the Western blotting. (D) The mRNA expression levels of *JNK1* and *TNFR1* in the flap with I/R injury were detected using qRT-PCR, in which *GAPDH* was utilized as the housekeeping gene. Experimental data are expressed as mean ± standard deviation. ***$p < 0.001$, ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$; * vs. sham group; ^ vs. I/R group. n = 3 in each group. Abbreviations: p-JNK1, phosphorylated JNK1.

to the sham group, the I/R group had upregulated levels of JNK1, p-JNK1, TNFR1 and p-JNK1/JNK1 ($p < 0.001$), and the expressions of these factors were suppressed in the I/R+remifentanil-L, -M and -H groups relative to the I/R group ($p < 0.05$), with the most pronounced expression changes noted in the I/R+remifentanil-M group. CD31 is a surface marker of aortic endothelial cells. Compared to the sham group, the apoptosis of endothelial cells increased significantly after I/R injury, and decreased after remifentanil treatment. (Fig. 4; 200×). Our experimental results suggested that remifentanil suppressed the vascular endothelial cell apoptosis, the levels of JNK1 and TNFR1 in the flap with I/R injury.

Remifentanil Promoted the Viability of Aortic Endothelial Cells under the Hypoxic Condition

Endothelial cells were successfully isolated, and as shown in Fig. 5A,B, the morphology and immunofluorescence staining showed that the CD31-positive cells were aortic endothelial cells. Compared to the control group, the hypoxia group displayed suppression of the aortic endothelial cell viability ($p < 0.001$; Fig. 5C), and the cell viability was promoted in the remifentanil (10, 100 ng/mL)+hypoxia group compared to the hypoxia group ($p < 0.01$). Our results indicated that remifentanil promoted aortic endothelial cell viability under hypoxic conditions. We selected 10 ng/mL remifentanil as the subsequent treatment dose since the viability of cells treated with this dose was the highest among all the other remifentanil-treated groups.

Anisomycin Reversed the Effect of Remifentanil on Inhibiting the Apoptosis of Aortic Endothelial Cells under Hypoxic Condition

Fig. 5D,E shows that compared to the control group, the hypoxia group had an increased apoptosis rate of aortic endothelial cells ($p < 0.001$), whereas the apoptosis rate decreased in the remifentanil+hypoxia group in contrast to the hypoxia group ($p < 0.001$). Moreover, in contrast to the hypoxia+remifentanil group, the apoptosis rate of aortic endothelial cells increased in the hypoxia+remifentanil+anisomycin group ($p < 0.001$). Our experimental outcomes demonstrated that anisomycin reversed the inhibitory effects of remifentanil on the apoptosis of aortic endothelial cells under hypoxic conditions.

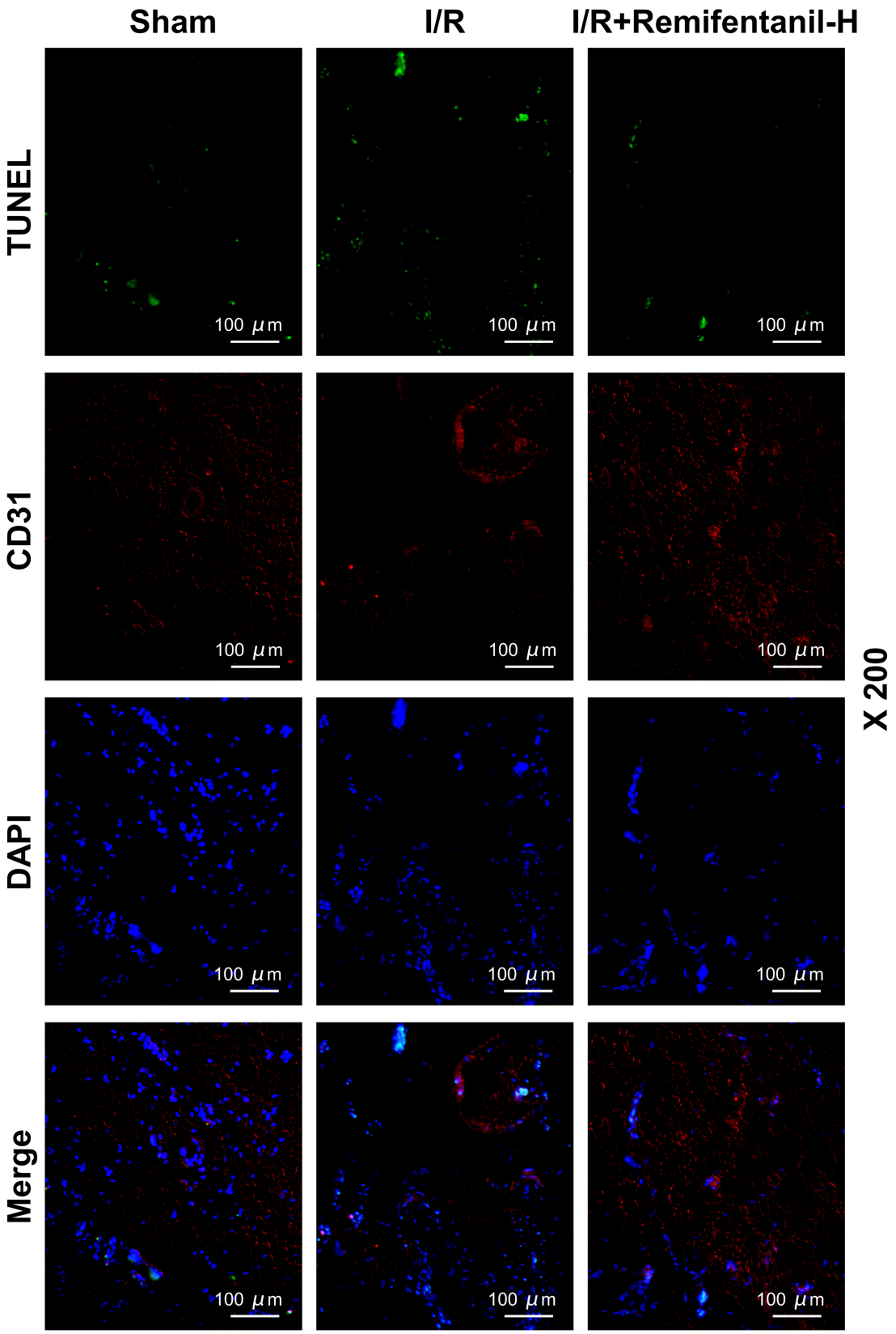

Fig. 4. Remifentanil inhibited cell apoptosis in the rat hypogastric flap with I/R injury. Vascular endothelial cell apoptosis in the flap was detected by immunofluorescence and TUNEL staining (200× magnification). Abbreviations: TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

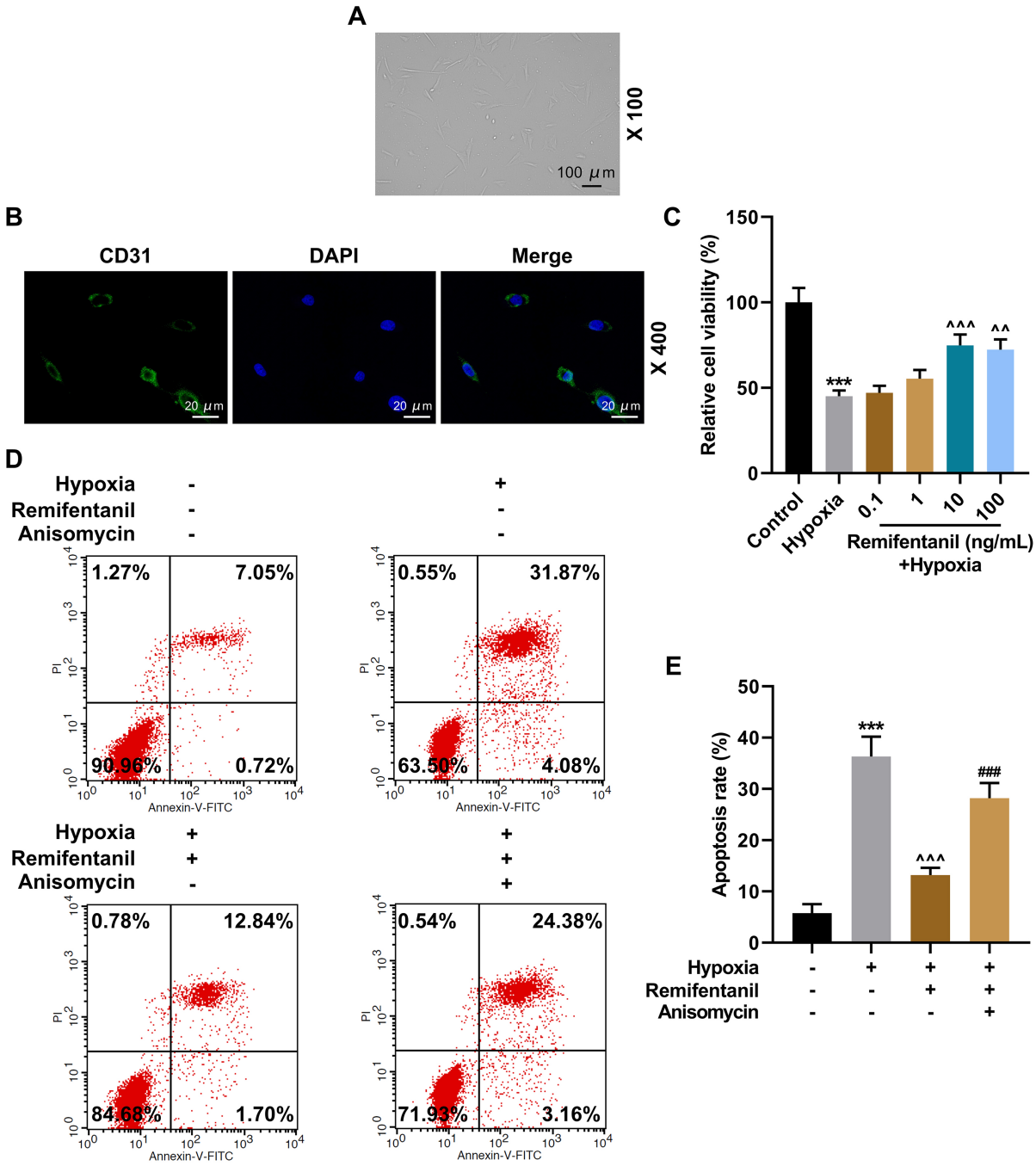

Fig. 5. Remifentanil promoted the cell viability and inhibited the apoptosis of aortic endothelial cells with I/R injury and anisomycin reversed the effects of remifentanil on apoptosis. (A) Morphology of the endothelial cells (100× magnification). (B) The endothelial cells were verified through immunofluorescence assay (400× magnification). (C) The viability of endothelial cells under hypoxic conditions was detected by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. (D,E) The apoptosis of endothelial cells under hypoxic conditions was detected by means of flow cytometry. Experimental data are expressed as mean ± standard deviation. ***$p < 0.001$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, $^{\#\#\#}p < 0.001$; * vs. control group; $^{\wedge}$ vs. hypoxia group; $^{\#}$ vs. hypoxia+remifentanil group. n = 3 in each group.

Anisomycin Reversed the Effects of Remifentanil on Downregulating the Expression Levels of JNK1, p-JNK1 and TNFR1 in the Aortic Endothelial Cells under Hypoxic Condition

The expression levels of JNK1, p-JNK1 and TNFR1 in the endothelial cells treated with remifentanil under hypoxic conditions were determined by means of qRT-PCR and Western blotting. Fig. 6A–D demonstrates that the expression levels of JNK1, p-JNK1 and TNFR1 were up-regulated in the hypoxia group compared to the control group ($p < 0.001$); although there is no significant differ-

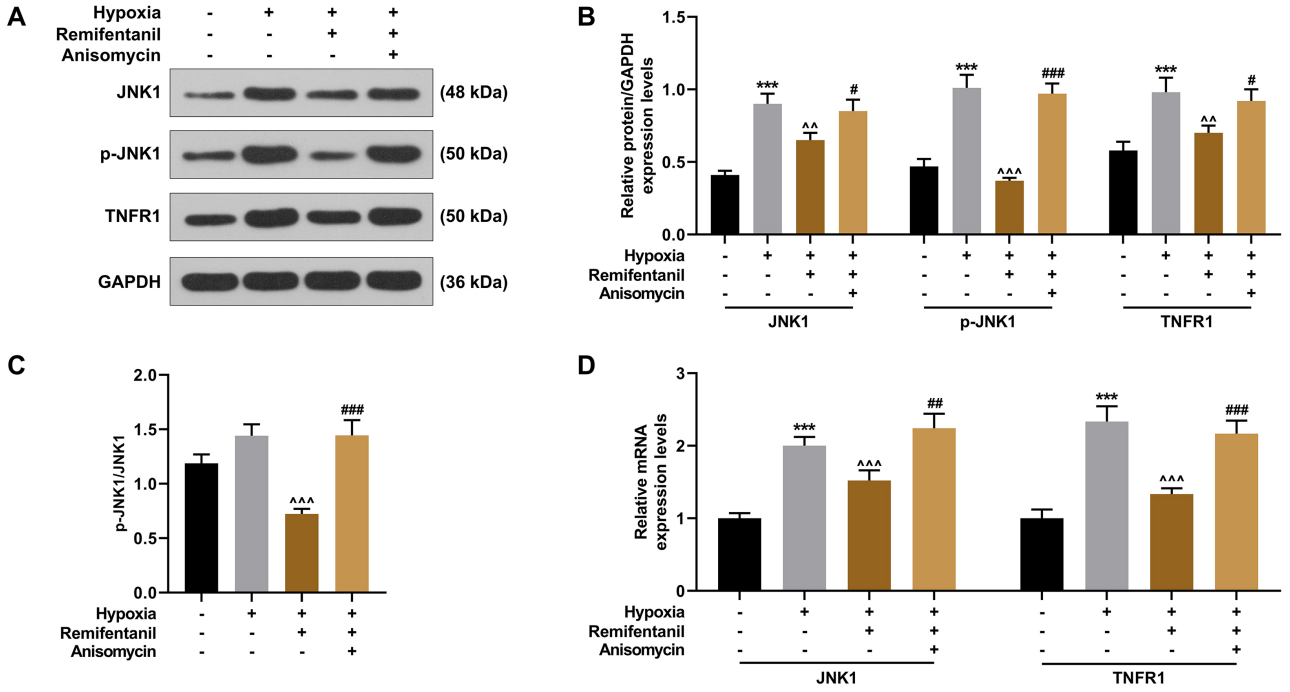

Fig. 6. Anisomycin reversed the effects of remifentanil on downregulating expression levels of JNK1, p-JNK1, TNFR1 and p-JNK1/JNK1 in the endothelial cells with I/R injury. (A,B) The protein expression levels of JNK1, p-JNK1 and TNFR1 in aortic endothelial cells under hypoxic conditions were detected by means of Western blotting, in which GAPDH was utilized as the internal reference. (C) The p-JNK1/JNK1 ratio was calculated based on the protein levels determined from the Western blotting. (D) The mRNA expression levels of *JNK1* and *TNFR1* in aortic endothelial cells under hypoxic conditions were detected using qRT-PCR, in which *GAPDH* was employed as the housekeeping gene. Experimental data are expressed as mean ± standard deviation. $^{***}p < 0.001$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; * vs. control group; $^{\wedge}$ vs. hypoxia group; $^{\#}$ vs. hypoxia+remifentanil group. n = 3 in each group.

ence in p-JNK1/JNK1, there is still an upward trend between the two groups. The expression levels of these factors were downregulated in the hypoxia+remifentanil group compared to the hypoxia group ($p < 0.01$); and the expression levels of these factors were upregulated in the hypoxia+remifentanil+anisomycin group relative to the hypoxia+remifentanil group ($p < 0.05$). These outcomes revealed that anisomycin reversed the inhibitory effects of remifentanil on JNK1, p-JNK1 and TNFR1 expression in the aortic endothelial cells under hypoxic conditions.

Discussion

Skin flap grafting is widely used in operations for wound recovery [2], in which I/R injury constitutes a major obstacle to flap survival [21]. Numerous studies have proven that remifentanil is a potent therapy for I/R injury [22–24]. Nevertheless, the effects of remifentanil on the hypogastric flap I/R injury, and the underlying mechanism, still remain obscure. Thus, the rodent I/R models were constructed in this study to explore such effects and mechanisms.

The survival of grafted flaps is important for the surgery for wound repair [2]. Unfortunately, flap necrosis often becomes more frequent after clinical surgery [3]. Flap transplant often sustains ischemia and hypoxia for a period of time, and then the reperfusion of blood can lead to inevitable damage to the flap tissue and even affect its survival [4], which is also associated with the inflammation of the damaged tissues [25]. According to the H&E staining results, the damaged tissue structure and inflammatory edema resulting from the I/R injury were alleviated by remifentanil, an indication that remifentanil could attenuate I/R injury-caused necrosis of the flap after surgery and thus improve flap survival.

These results were further verified by molecular analysis in the present study. I/R injury can result in the acute generation of reactive oxygen species (ROS), which causes the death of cells and failure of organs [26]. The function of MDA is to promote oxygen-free radical formation and that of SOD is to protect tissues and cells against acute ROS-induced oxidative stress [27,28]. SOD and MDA are regarded as the indicators of oxidative stress in tissues and cells [29]. The formation of NO was reduced in the process of I/R injury [30]. Therefore, the increases in the MDA levels and the reductions in the NO and SOD levels were exploited to reflect the damage caused by I/R injury. Besides, the receptors from the TNF-α receptor family promoted inflammation either directly by inducing inflammatory gene

expression or indirectly by triggering cell death [31]. According to a previous study, the downregulation of TNF-α can mitigate the I/R injury [12]. Moreover, remifentanil relieves the I/R injury by regulating the MDA, SOD, NO and TNF-α levels, according to previous studies [13,32,33]. As shown by the results of the current set of experiments, I/R injury led to the upregulation of TNF-α and MDA and the downregulation of SOD and NO in the flaps, which were counteracted by remifentanil—a testament to the suppressive role of remifentanil in oxidative stress and cell death of flap induced by I/R injury. In addition, our study demonstrated that among the other doses tested, 2 µg/kg per min of remifentanil exerted the most evident effect against I/R injury. However, the effects of remifentanil on the I/R injury, as well as the underlying mechanism, were not completely elucidated in the current study.

In this investigation, the expression levels of JNK1, p-JNK1 and TNFR1 were also detected. The JNK pathway is essential for the progression of I/R injury [34], cell inflammation and death [35]. TNF-α is the major proinflammatory cytokine central to the construction of an inflammatory microenvironment, acted via binding to its receptors, such as TNFR1 [36]. Elevated expression level of TNFR1 is regarded as an indicator of the activation of TNF receptor in the context of I/R injury [37]. In face of stress inflicted upon the cells or tissues, the JNK will become phosphorylated [34], and the inhibition of JNK could alleviate I/R injury [38]. According to our experimental results, I/R injury upregulated the expression levels of JNK1, p-JNK1, TNF-α and TNFR1 and raised the JNK1/p-JNK1 ratio, while these effects could be reversed by remifentanil, suggesting that the potential links among TNF-α/TNFR1 pathway, JNK1 signaling and hypogastric flap I/R injury, which could be alleviated by remifentanil. However, more experiments are warranted to confirm the roles of TNF-α/TNFR1 pathway and JNK1 signaling pathway in the remifentanil-mediated mitigation of hypogastric flap I/R injury.

Apart from the tissue experiments, the effects of remifentanil on rat aortic endothelial cells were also studied in the present study. The vascular endothelial cells are vital in the flap and the proper endothelial cell growth is critical to the skin flap survival [39]. Moreover, the apoptosis of vascular endothelial cells represents another major factor contributing to the progression of I/R injury [40]. Therefore, the viability and apoptosis of the vascular endothelial cells treated with remifentanil and put through the hypoxic condition were also quantified in this study. Anisomycin, an activator of JNK, was used to further enhance the effects of the JNK pathway in the aortic endothelial cells in this study [41]. The endothelial cells under hypoxic conditions can be used as *in vitro* I/R injury models [19], which were established using the same method in our study. Herein, we showed that the cell viability was inhibited and the apoptosis of aortic endothelial cells was promoted under hypoxic conditions, while these effects were neutralized by remifentanil. However, the inhibitory effect of remifentanil on apoptosis of aortic endothelial cells under hypoxic conditions could be counteracted by anisomycin. In addition, the expression levels of JNK1, p-JNK1 and TNFR1 in the cells were upregulated after being exposed to a hypoxic condition, alongside a rise in the ratio of JNK1/p-JNK1, and these hypoxia-induced alterations could be offset by remifentanil. These results attest to the potential involvement of TNF-α/TNFR1 pathway and JNK1 signaling pathway in the remifentanil-regulated viability and apoptosis of vascular endothelial cells exposed to hypoxic stress. Future investigations will encompass the attempts to reveal the underlying molecular mechanisms.

Several limitations of this study warrant our considerations. Firstly, only the cellular and rat models were employed in this research, yielding findings that might not be translatable to human patients. Secondly, the mechanisms behind the mitigation of hypogastric flap I/R injury, involving the TNF-α/TNFR1 pathway and JNK1 signaling pathway, remain largely unclear, necessitating further investigations in the future.

Conclusion

In conclusion, remifentanil alleviates the necrosis of I/R-injured hypogastric flap tissues and the apoptosis of aortic endothelial cells, as well as improves the viability of aortic endothelial cells in *in vitro* flap I/R injury models. Furthermore, our data indicate that remifentanil suppresses both the JNK1 signaling and TNF-α/TNFR1 pathways. The JNK activator anisomycin was able to counteract these effects, reversing the inhibitory effects of remifentanil on aortic endothelial cell apoptosis and restoring the TNF-α/TNFR1 and JNK1 signaling pathways under hypoxic conditions.

Availability of Data and Materials

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

Author Contributions

WF designed the research study. WF, XF, and SLW performed the research. WF, XF, and SLW collected and analyzed the data. All authors have been involved in drafting the manuscript and involved in revising it critically for important intellectual content. All authors give 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 animal experiments were conducted in line with the guidance of China Council on Animal Care and Use, and were approved by The Ethics Committee for Laboratory Animal Welfare in Zhejiang Experimental Animal Centre (Ethics Approval No. ZJCLA-IACUC-20020163). Incidentally, the minimization of discomfort and pain on animals was the one of the pursuits in our study.

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Conflict of Interest

The authors declare no conflict of interest.

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